Female and Male Fertility Preservation

Michael Grynberg
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Editors

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Preface

The field of reproductive medicine has fully embraced the new branch of fertility preservation and is now poised to offer a full range of options to both male and female patients at risk of losing their future reproductive potential.

Many advances in fertility preservation have been developed over the course of the last decade and many more are in the pipeline. To offer guidance and to overcome challenges for reproductive medicine practitioners at all stages in their careers, we edited this comprehensive volume with the collaboration of leaders in the field discussing state-of-the-art technologies with the aim to illustrate and reinforce appropriate treatment strategies not only for patients stricken by cancer but also for those impacted by medical, non-oncological, conditions posing future reproduction at risk. The common theme of the book has been to include the whole spectrum of medical issues requiring fertility preservation, emphasizing integrative thinking, teamwork with oncologists and ultimately to improve skills for health care professionals.

We strongly believe that this book provides a useful and excellent resource also for teaching nursing staff, medical students, residents and fellows and for continuing individual professional development.

Clamart, France — Michael Grynberg
New Haven, CT, USA — Pasquale Patrizio

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Part I

Female Fertility Preservation

Normal Ovarian Ageing

Frank J. Broekmans and Annelien C. de Kat

Clinical Cases

- A 37-year-old woman has experienced a spontaneous miscarriage. What can you tell her about the potential causes?
- A 45-year-old woman with previously an average cycle duration of 28 days presents with cycle durations over the past 3 months of 20, 15, and 26 days. Which condition could be present here?
- A 30-year-old woman has measured her AMH level with a commercial test and has a value on the tenth percentile. What does this mean with regards to her near-future fecundability?

Introduction

It has been long known from both natural population studies and from assisted reproduction studies that with increasing age female fecundity – the ability to produce offspring – decreases [1, 2]. This age-related decrease in female fecundity has distinct implications for the current trend to postpone childbearing in Western societies, as well as for recent developments regarding fertility preservation and pre-implantation genetic testing for hereditary disease.

By postponing childbearing, a growing proportion of women attempting to conceive will fail in achieving this goal within a time frame of 12 months, a condition referred to as female infertility [3]. An increasing proportion of couples will therefore depend on assisted reproduction technology (ART) in order to achieve a pregnancy. However, ART will only to a limited extent be able to compensate for the decreased natural fertility [4, 5], leaving many couples permanently childless after prolonged and demanding infertility therapies.

Fertility preservation strategies in the female have become increasingly available for both benign and malignant disease indications, as well as for non-medical indications, the latter highly related to the current trends in postponing childbearing and family building. For all options of preservation, reproductive ageing may affect future prospects for success from the use of the preserved gametes, embryos, or reproductive tissues. Knowledge on the mechanisms steering this ageing process in the female may therefore be very relevant.

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Today, assisted reproduction technology is more and more frequently applied for the purpose of embryo testing on the presence of genetic, hereditary conditions such as Huntington's disease, cystic fibrosis, or dystrophia myotonica, of unbalanced translocations as in couples with recurrent miscarriage, and of susceptibility genes such as the BRCA (breast cancer)gene-1 and -2 mutations. In this patient group, information derived from reproductive ageing studies will help in assessing the potential of the couple in obtaining sufficient numbers of embryos, with a sufficient level of implantation capacity, in order to provide reliable expectations of the prognosis for achieving the birth of a healthy child where the disease state of interest is ruled out.

Female reproductive ageing is based on age-related changes in the ovarian functional status, with focus mostly on changes in follicle numbers and oocyte quality. Between individual females, the age-related decline in numbers and quality may vary to a great extent, so that age alone may not correctly mark the actual state of this ageing process. Decreasing numbers of follicles and diminishing levels of oocyte quality are expressed by the gradual changes in menstrual cycle regularity and monthly fecundity. The mechanisms steering the gradual decline of the follicle pool size and the quality of oocytes are far from understood. The forthcoming will summarize the current state of knowledge concerning reproductive ageing and discuss the current methods for assessing a woman's individual reproductive age status.

Ovarian Ageing: Clinical Features

The first noticeable clinical sign of advancement in the reproductive ageing process is a slight shortening of the length of the menstrual cycle [6, 7], while regularity remains unaffected. This shortening of the cycle duration results from reduced numbers of antral follicles present, and thereby reduced ovarian-pituitary feedback, creating an increased driving force from follicle-stimulating hormone (FSH) in the late luteal phase, resulting in advanced dominant follicle selection and ovulation in the subsequent cycle [8, 9]. It is only at the time when cycles become overtly irregular, and shortened cycles are alternated with skipped cycles, that women first notice the signs of the reduction in follicle numbers. This period with overt cycle irregularity is referred to as the menopausal transition, and its onset occurs on average at the age of 46 years, some 5 years before the average occurrence of menopause [7, 10]. The final menstrual period (menopause), an event that can only be recognized in retrospect, eventually represents an almost exhausted follicle pool [11, 12] and occurs at a mean age of 51 years [13].

Even with regular menstrual cycles maintained over a period of many years, monthly fecundity dramatically decreases from the mean age of 31 years onwards [14], with the end of natural fertility occurring at the mean age of 41 years [15, 16]. Compromised follicle/oocyte and embryo quality is mainly held responsible for the increased time to pregnancy, increased rates of miscarriage, and increased rates of infertility with increasing female age. Although chromosomal aneuploidy likely constitutes a large deal of this quality loss, other early embryonic dysfunctions may also play a role [17]. Ultimately, a potential role at the level of the endometrium is more and more recognized and research today may be much propelled by the development of endometrial organoids that allow for in-depth research into embryo–endometrium interaction and the effects of ageing [18, 19].

Between the various reproductive events like onset of decreasing fecundity, loss of natural fertility, start of cycle irregularity, and the occurrence of menopause, a fixed temporal relationship is believed to be present [20]. Evidence for this mainly stems from cross-sectional observations, while longitudinal data establishing such relationship for individuals are scarce [21]. The variation distributions across individuals for age at loss of natural fertility and age at menopause tend to be remarkably similar [20] (Fig. 1). The demonstration of a relation between age at onset of cycle irregularity and subsequent age at

menopause has further corroborated the 'fixed interval' hypothesis. Still, recent studies in women with very early menopause have made clear that pregnancies leading to live birth may arise very close to the moment of final cycle arrest, indicating that the time interval between loss of fecundity and menopause may be less fixed than assumed [22, 23].

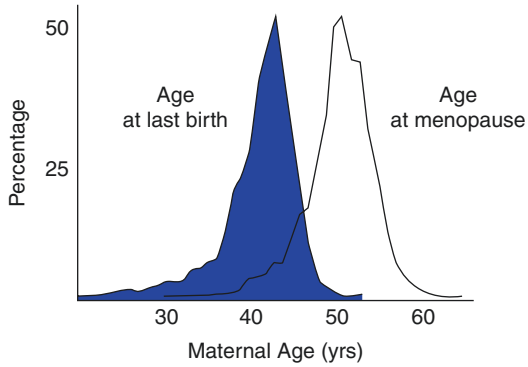

Fig. 1 Distribution for ages at menopause and age at last childbirth, demonstrating a high degree or parallelism. Data are based on studies in historical populations [68] and a contemporary population [69])

Ovarian Ageing Mechanisms: Quantity

The ovarian primordial follicle pool is established during female development in utero and reaches its peak in early-mid-pregnancy [24]. An ovarian follicle comprises an oocyte surrounded by granulosa and theca cells. A woman is born with her follicle pool at its peak of her lifetime. Pool size will steadily diminish with increasing age during the continuous recruitment and subsequent follicle apoptosis, and, to a far lesser degree, cyclic follicle recruitment towards ovulation.

Primordial follicles remain quiescent awaiting recruitment from their resting phase into the growing follicle pool of preantral and antral follicles. This process is called initial or continuous recruitment [25] and will lead these follicles to enter atresia at some stage during this maturation process [26, 27] (Fig. 2).

Cyclic recruitment of antral follicles is initiated with the advent of menstrual cycles after puberty. The gonadotropin-releasing hormone (GnRH) pulse generator in the hypothalamus has been set into motion to stimulate the pituitary to release

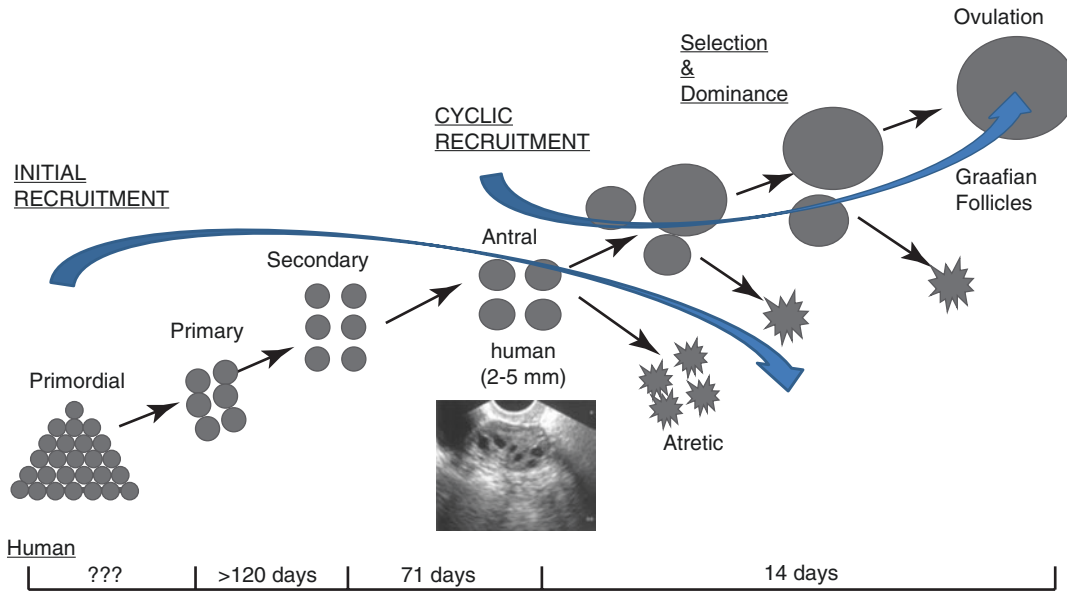

Fig. 2 Schematic representation of folliculogenesis in the ovary, demonstrating initial continuous and cyclic intermittent recruitment of primordial and antral follicles

follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Under the influence of FSH, a part of the antral follicle cohort is selected for further development. One of these follicles is able to grow faster than the rest of the cohort, potentially due to a differing degree of FSH receptor expression or under the influence of local growth factors. The increased levels of oestrogen and inhibins produced by the granulosa cells of this dominant follicle suppress circulating FSH levels, impeding the further growth of the smaller, less sensitive follicles, which will then go into atresia. The dominant follicle is henceforth singly able to reach the preovulatory phase and subsequently release an oocyte for ovulation [26].

From this knowledge it can be derived that all follicles built into the ovaries in the foetal life period are destined to be discarded, while only very few will potentially contribute to reproduction. Due to the continuously occurring follicular atresia, the total number of follicles in the ovaries decreases with increasing age. At the fourth month of foetal development the ovaries contain some 6–7 million oocytes surrounded by a layer of flat granulosa cells to form primordial follicles [24]. Due to a rapid loss via apoptosis in the second half of the foetal life period, at birth only 1–2 million primordial follicles remain [28]. After birth, this high rate of follicle loss slows down so that at menarche approximately 300,000–400,000 follicles remain [20]. During the reproductive years, the further decline in the number of primordial follicles remains steady at some 1.000 follicles per month and accelerates after the age of 37 years. At the time of menopause, the number of remaining follicles has dropped clearly below 1.000 [12, 29]. Variation within this average decline pattern is thought to be the basis for the variation in timing of menopause between individual women. Evidence for this may stem from studies where markers for the remaining pool are followed over the years and related to the final menstrual period.

Ovarian Ageing Mechanisms: Quality

The human species can be considered as relatively subfertile compared to other mammalian animals. The average monthly fecundability (the chance of reproducing within certain time frames) of about 20% implies that among human couples trying to conceive, many months of exposure may be needed to achieve their goal [3]. Female fecundability is shown to decrease after the age of 31 years, a decrease that may accelerate after age 37, leading to sterility (fecundability has become zero) at a mean age of 41 [15]. The degree of fertility (i.e. fecundability) in the human and the rate of decline in fertility degree may vary considerably between women of the same age. A nineteenth-century natural fertility population study revealed that a reduced birth rate in the early stages of marriage (20–30 years) was associated with an early age at last childbirth (approximately 35 years). This suggests that early loss of natural fertility is preceded by reduced fecundity already before the age of 30 years [30]. Thus, for a woman at the age of 35 years fecundability may vary between being close to natural sterility having a degree of fertility comparable to a 25 year old. The level and decrease of female fertility degree are believed to exhibit the same range of variation as for the occurrence of menopause, as indicated above [20]. Based on the presumptive fixed time period of 10 years between age at menopause and age at loss of natural fertility (sterility), it is believed that the correct prediction of menopause in an individual woman could therefore potentially provide valuable information regarding her fertile lifespan (Fig. 1).

This putative relation between quantity of follicles and quality at the oocyte level may well be much more complicated. The variation in fecundability within female age groups is notable [20, 31], while within quantity groups, defined according to markers such as the antral follicle count (AFC) or anti-Müllerian hormone (AMH) level, fertility level is highly influenced by the age of the female. Unfortunately, studies that address the variation of female fertility depending on both age and quantitative ovarian reserve status are lacking due to the fact that simple tests for qualitative ovarian reserve, that is, oocyte and embryo quality, are not present at the current time [32, 33].

As indicated previously, fertility decline with age, but putatively also variation of fecundability within female age classes is believed to stem

mainly from loss of or variation in the competence of the oocyte to produce a viable embryo, leading to implantation and a subsequent live birth. Much of this low competence is based on failures in the process of meiosis. During early foetal development, human oocytes are formed and already then initiate meiosis, which becomes arrested at the meiosis I stage (MI) [34]. The meiotic division of an oocyte is only completed (meiosis MII) at the end of the long trajectory of follicle development around the ovulation, induced by the mid-cycle LH surge.

Meiosis thus comprises two subsequent cell divisions without DNA replication, resulting in halving the chromosome content. It remains poorly understood how these two sequential steps of replication and segregation are controlled [35]. Cell cycle checkpoints involve DNA replication, spindle formation, and chromatid segregation [34, 36] (Fig. 3). Especially the segregation pro-

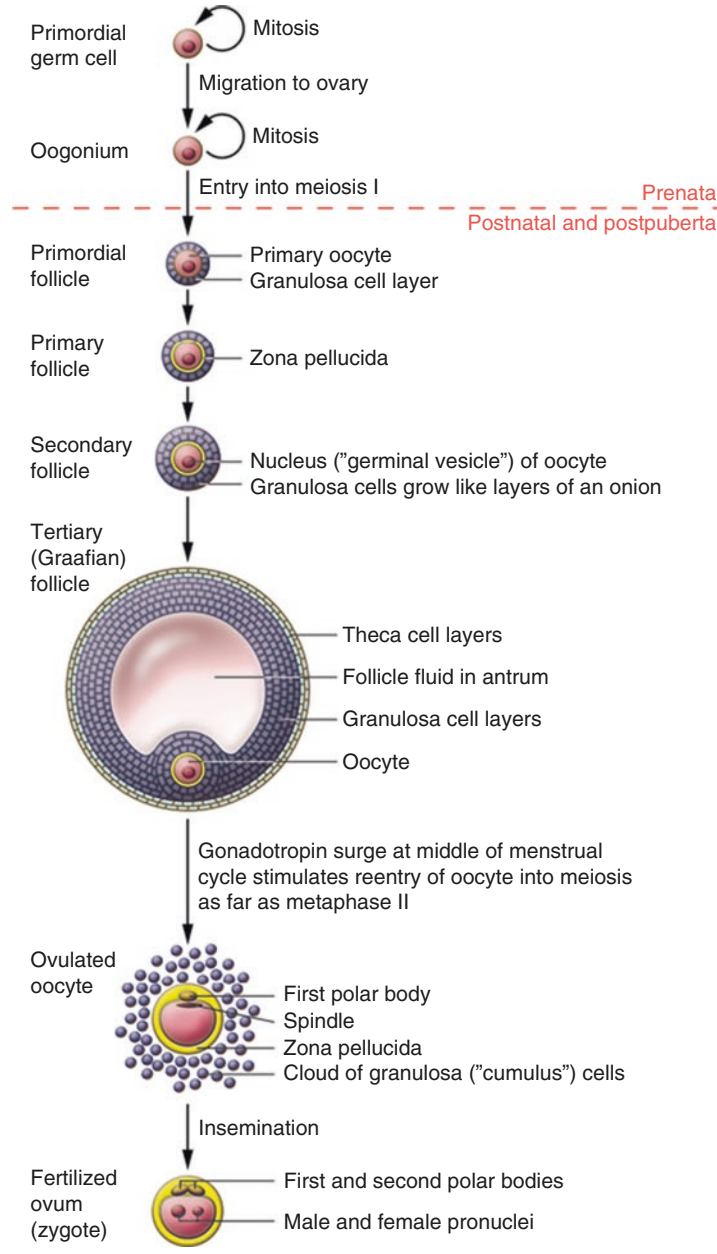

Fig. 3 Follicle/Oocyte maturation scheme, showing the interrelation between morphological development and meiotic events (ref Gosden [70])

cess in oocytes is error prone, with human oocytes being extraordinarily 'meiotically vulnerable'. The high rate of aneuploid oocytes in the human (up to 25%) will give rise to aneuploid embryos when fertilized. In humans, most aneuploidies are lethal and result in spontaneous miscarriages. As error rates go up with increasing female age, miscarriage rates and the rates of birth of Down syndrome children increase rapidly, especially above the age of 40 years [36, 37].

Changes for meiotic errors augment exponentially with increasing female age. It remains highly speculative how age influences oocyte meiotic maturation [38]. However, a close relationship between female age and aneuploidy of oocytes [39] and embryos in women undergoing IVF has been established convincingly [40, 41]. Studies involving human oocytes could indeed confirm age-related changes in spindle formation and chromosome alignment [42]. Other suggested mechanisms for the age-related increase in oocyte aneuploidy involve the different stages of foetal development where oocyte meiotic arrest is completed, the depletion of the follicle pool throughout life, the number of years of primordial follicle arrest, direct effects of increasing FSH concentrations with age, and possibly many others.

Markers for Quantity and Quality Aspects of Ovarian Ageing

For various clinical conditions, such as infertility treatments, ovarian surgery and preservation, and the diagnosis of ovulatory dysfunction, it can be useful to determine the size of the remaining follicle pool (or 'ovarian reserve') at any given time. Ideally, this would constitute the measurement of all follicles including the dormant primordial follicles, which make up >99% of the entire follicle pool. However, primordial follicles are only distinguishable by microscope with histological tissue analysis. The quantification of ovarian reserve therefore requires the use of proxy measures, such as the antral follicle count (AFC), anti-Müllerian hormone (AMH), and serum FSH. These markers of ovarian reserve are discussed below, as are the potential future options for genetic testing.

As follicles are recruited from the primordial follicle pool to become preantral follicles, they increase in size, after which a small proportion is selected to develop further into antral follicles. Preantral follicles can range in size between 0.05 and 2 mm and are therefore too small to be detected by clinical imaging [26, 43]. Antral follicles can range in size from 2 to 10 mm due to an increasing amount of fluid volume within the follicles as they mature, rendering them accessible to measure with transvaginal ultrasound. The associated inter- and intra-observer reproducibility for AFC assessment is high [44]. However, the presence of large antral and preovulatory follicles is by definition dependent on the timing of the menstrual cycle. This intra-cycle variation of AFC may be less pronounced for small antral follicles; in a prospective analysis the number of follicles <6.0 mm remained stable throughout the menstrual cycle [45]. In clinical practice, AFC is currently most commonly used in fertility treatments as a measure to predict ovarian response to gonadotrophins or oocyte yield for in vitro fertilization (IVF). There is a linear, though imprecise, relationship of AFC with oocyte yield [46] and a high correlation with the prediction of low, normal, and high response to ovarian stimulation [47].

AMH is produced by the granulosa cells of preantral and small antral follicles [48, 49]. In the ovary, AMH acts as a local paracrine inhibitory agent on the regulatory process of follicle recruitment and possibly also for the selection of the dominant follicle [50, 51]. The production of AMH ceases when the follicles reach the phase of FSH sensitivity at a size of 2–6 mm. Outside the ovary, circulating AMH levels can be detected in the peripheral blood stream. In ovarian hyperstimulation, the development of dominant follicles is accompanied by a tremendous, though temporary, drop in AMH levels. In the normal cycle, circulating AMH levels remain relatively stable independently of timing of the menstrual cycle and seem to vary slightly along with the variation in the composition of the antral follicle cohort [52]. Animal studies have shown that the concentration of circulating AMH is highly cor-

related to the size of the primordial follicle pool [53]. A positive correlation was also found in human populations, albeit with a higher degree of variability of AMH levels unexplained by true ovarian reserve as measured by histological analysis [54]. It is indeed known that the level of AMH can significantly differ in association with smoking or the use of oral contraception, for example [55]. Taking these potential variations into account, AMH is clinically used as a marker representing current ovarian reserve or expected response to ovarian stimulation in assisted reproduction techniques.

Throughout the menstrual cycle, a neuroendocrine balance between the hypothalamo-pituitary unit and the ovaries is maintained. The pituitary gland secretes LH and FSH through pulsatile GnRH stimulation from the hypothalamus. Oestrogen and inhibins produced by developing follicles temper the LH and FSH production by the pituitary. As the ovarian reserve pool diminishes with age, decreased overall concentrations of inhibins and oestrogen exert a reduced negative feedback on the pituitary. Consequently, FSH levels gradually increase over time. Increased FSH levels in the luteo-follicular transition can lead to accelerated dominant follicle selection and ovulation, resulting in shorter or irregular menstrual cycle duration [56]. Therefore, basal (assessed on days 2–3 of the spontaneous menstrual bleed) FSH levels will roughly express the number of antral follicles still contributing to the feedback system. In large studies, the precision of this tool to estimate ovarian reserve status has been shown to be less optimal than for the AFC and AMH [47].

As described above, besides the quantity of follicles remaining, oocyte quality plays an important role in the truly relevant effects of ovarian ageing, that is, the effects on fecundability. The genetics of the timing of the age at menopause are evident in the sense that heritability of the 'trait' age at menopause is quite high [57]. Candidate gene and Genome Wide Association Studies (GWAS) have put effort in identifying the relevant genetic loci. They mostly seem to be genes involved in DNA repair and immune maintenance [58]. Still, the variation within such loci together may only explain some 25% of the variation in age at menopause. It can therefore be theorized that women who are better at maintaining their 'soma' will also be more likely to experience menopause at a later age than average [59]. In concordance, there is some evidence to suggest that longer-living women are able to reproduce until a later age than average, thereby suggesting that long survival genes are also capable of creating both optimal and extended fecundability [60].

Prediction of Ovarian Ageing Milestones

As the age at which women reach menopause can vary widely, there is a vast body of research focused on the prediction of age at menopause. Several studies developing multivariable prediction models for age at the occurrence of menopause identified AMH as an important predictor [61]. This was a hopeful finding as measuring a proxy marker of current ovarian reserve could potentially give an insight into the remaining duration of the fertile lifespan of someone contemplating the postponement of pregnancy. Prospective analysis of AMH decline revealed that the speed of AMH decline differs between individuals and changes with increasing age [62]. Thus, a single measurement of AMH does not give sufficient indication of the projected trajectory of lifetime decline. The inclusion of multiple AMH measurements per individual, in an effort to circumvent this problem, did not improve the capacity of prediction models to determine who would enter menopause early [63] (Table 1). It therefore seems that the variability in age at menopause cannot be solely explained by the variability in ovarian reserve, as expressed by AMH, or the speed at which it diminishes.

When included as a predictor for ongoing pregnancy or live birth, conditions that are largely dependent on oocyte and embryo quality, AMH performs more poorly [64–66]. Although there is a hypothetical relationship between ovarian reserve quantity and quality, this remains challenging to establish as there is currently no way

Table 1 Systematic review based risk estimates of early or late menopause for age specific AMH level categories

Age specific AMH	Menopause <age 45 years (9.6) (%)	Menopause >age 55 years (11.1) (%)
p5	28.1	3.8
p25	17.7	6.5
p50	8.0	14.2
p75	2.7	30.9
p95	0.9	51.1

Data were derived from a Weibull predicted age at menopause (AMP) distribution
The left column displays age-specific AMH percentile categories
The top row displays the AMP categories and their respective incidence in the full cohort
The five bottom rows display the distribution of AMP categories per AMH percentile [71]

of directly measuring oocyte quality. In comparison to older women with the same level of quantitative ovarian reserve, younger women have better pregnancy prospects [67], emphasizing the role of female age as indicator of fecundability. It thus stands to reason that the same processes that govern the ageing of somatic cells additionally influence the quality status of gametes in the female. To date, there is still no conclusive evidence to suggest that a proxy marker for somatic cell ageing, such as telomere length, may be a beneficial predictor of the duration of the reproductive lifespan in terms of fecundity.

Summary

The ovarian ageing process can be well described as a continuous wastage of follicles and oocytes commencing already before birth and leading to the ovaries become devoid of follicles at the end of the fifth life decade. This autonomous, paracrine-steered recruitment maintains the presence of a cohort of antral follicles sized 1–8 mm in diameter that create the opportunities for cyclic events regulated by neuroendocrine control at the hypothalamo-pituitary level. Resulting in the menstrual cycle this process allows for the ovulation of a single follicle and ovulation of a fertilizable oocyte. This oocyte started her meiotic division many, many years before, at the time the ovaries were created, and at ovulation this meiotic division of the nuclear material is finished, enabling fertilization of this oocyte and subsequent embryo development. The quality of this monthly ovulated oocyte may be the key factor in the on average low level of and variation in fecundability in the human.

Current knowledge on the ovarian ageing process has enabled the use of tools like AMH and the AFC to estimate the quantitative follicle reserve status, but assessment of the average ovulated oocyte quality in individuals remains largely illusive at the current time. It is the latter that is the big avenue to open as it may revolutionize strategies on infertility prevention strategies, infertility treatments, and fertility preservation.

Practical Clinical Tips

- In cases applying for fertility preservation treatments, assessment of the ovarian reserve status by using AFC or AMH can be considered useful for monitoring the ovarian damage from gonadotoxic treatment, for anticipating response of the ovaries with the purpose of circumventing the early OHS syndrome, and for research into the role of quantitative ovarian reserve in fertility preservation management and follow up.
- In patient counselling, it must be emphasized clearly that fecundity is *mainly* dictated by the average egg quality and not by numbers of antral follicles present at some time moment in the ovary(ies).

Take Home Messages

- AMH and AFC and basal FSH are markers for the quantitative ovarian reserve. For the prediction of spontaneous pregnancies, these markers have no value. In assisted reproduction, these tests may, within age classes, refine the prospects for live birth as a higher number of follicles and oocytes will affect the chances of having normal quality embryos.

- Fecundability in the human is on average moderate and may vary among individual women. When after the age of 30 years fecundity will decline more rapidly, those women with a below average fecundity in their twenties will be most at risk of infertility in their thirties.
- AMH and AFC will have only limited capacity in predicting the long-term event 'age at menopause'.

Key Reading

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The Effect of Chemotherapy on the Ovary Clinical and Pathophysiological Review

Noam Domniz, Dror Meirow, Hila Raanani, and Hadassa Roness

Introduction

Advances in oncological therapy have resulted in an increasing population of young cancer survivors who must contend with the harmful side effects of chemotherapy on subsequent fertility.

Chemotherapy, whether for cancer or other diseases, is known to adversely affect ovarian reserve and in certain cases lead to premature ovarian insufficiency, with high levels of gonadotropins, oligo- or amenorrhea, and sterility.

The magnitude of ovarian insult depends on several factors including the chemotherapeutic regimen and the age of the patient as women above 35 years old have almost double the rate of post-chemotherapy amenorrhea when compared to women under this age. The most harmful chemotherapeutic agents are the alkylating agents and platinum derivatives. The type of cancer also affects the degree of gonadotoxicity associated with the different therapeutic protocols and chemotherapy mechanism.

Fertility preservation is an important consideration when planning the drug regimen prescribed for young cancer patients. Understanding the mechanisms and means of action by which chemotherapy affects the female reproductive system is vital in order to assess patient risk and to determine the optimal methods of fertility preservation. The purpose of this review is to summarize current knowledge regarding the impact of chemotherapy on the female reproductive tract.

Clinical Consequences of Exposure to Chemotherapy

Among pre- and peri-menopausal women, the most common cancers are breast cancer (over 40% of cases), thyroid carcinoma, melanoma, uterine and cervical tumors, and central nervous system tumors [1].

It has been reported that cancer diagnosis and treatment reduce the likelihood of achieving pregnancy by 38–50% when compared to the general population [2–4]. Reduction in fertility is most often the loss of ovarian follicle reserve after chemotherapy. Chemotherapy negatively affects the fertility potential of young patients suffering from cancer, autoimmune diseases, or hematological conditions such as thalassemia.

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The impairment of infertility varies from single figures to almost 100%, depending on the type of chemotherapeutic agent and the patient's age [5]. However, pregnancy is considered to be safe if occurring a number of years after chemotherapy.

The harmful effect of chemotherapy on the ovary was first described in the 1970s when cyclophosphamide (Cy) was shown to cause amenorrhea, a diminished follicle pool and premature ovarian insufficiency (POI) [6–8]. Other chemotherapeutic agents such as platins [9], anthracyclines [10], and taxanes [11] were also found to be ovotoxic. Currently, most chemotherapeutic regimens involve combinations of drugs; therefore in order to minimize ovarian damage, the effects of different drugs should be taken into account, and the least harmful regimens should be selected wherever possible. In cases where the patient is at risk for post-treatment premature ovarian insufficiency (POI) or significant loss of ovarian follicle reserve, an appropriate program of fertility preservation should be planned prior to chemotherapy.

The mechanisms by which chemotherapy induces ovarian damage have been investigated using various methods, including histological analysis of human ovarian segments after chemotherapy exposure (i.e., after ovarian tissue harvesting and cryopreservation for fertility preservation), animal studies, xenograft models, and tissue cultures of human ovarian cortical fragments exposed to chemotherapeutic agents *in vitro*. However, the exact mechanisms by which chemotherapeutic agents cause loss of the ovarian follicle reserve are not fully comprehended and are still actively being investigated [12, 13].

Assessment of Ovarian Damage Post-Chemotherapy

An optimal indicator for premature ovarian insufficiency (POI) has yet to be determined. Amenorrhea is a marker of POI, but amenorrhea represents the end stage of POI (Fig. 1). An earlier marker is required to show the extent of ovarian follicle loss before amenorrhea. The gold standards for ovarian reserve are anti-Mullerian hormone (AMH) level and antral follicle count (AFC), and are also used as a baseline before the commencement of chemotherapy in order to enable a comparison with postexposure levels [15, 16]. Other parameters that may be used are follicular-stimulating hormone (FSH) with estradiol (E2) levels on days 2–4 of the menstruation cycle.

AMH levels have been shown to decline rapidly in young lymphoma patients after the first exposure to the BEACOPP protocol that contains alkylating agents, and little recovery is seen 1 year after treatment (median level of 0.11 pmol/L) [10] (Fig. 2). Similar correlations have been found between AMH levels and other chemotherapy protocols and cancers [17]. Therefore, currently AMH is considered the most reliable marker for assessing follicle reserve and measuring gonadotoxicity.

Effect of Age at Treatment

The patient's age is one of the most important factors predicting the impact of chemotherapy on fertility, with the risk of subsequent POI increasing with age [19–21]. Petrek [22] reported that approximately 85% of breast cancer patients younger than 35 years of age recovered menstruation post treatment, but this rate dropped to 45–61% in women aged between 35 and 40, and the rate of recovery was even lower in women over 40, with many women remaining amenorrheic. This significant effect of age at treatment was particularly pronounced after Hodgkin's lymphoma, where patients tend to be younger than patients with other types of cancer. In Hodgkin's lymphoma, patients younger than 25 years of age have been reported to exhibit significantly less chemotherapy-induced gonadotoxicity [23].

The significant difference in fertility outcomes in younger women is due to younger women having a larger ovarian reserve, with many nongrowing primordial follicles [14]. The impact of follicle loss is therefore far more significant in older patients who have a lower initial ovarian reserve.

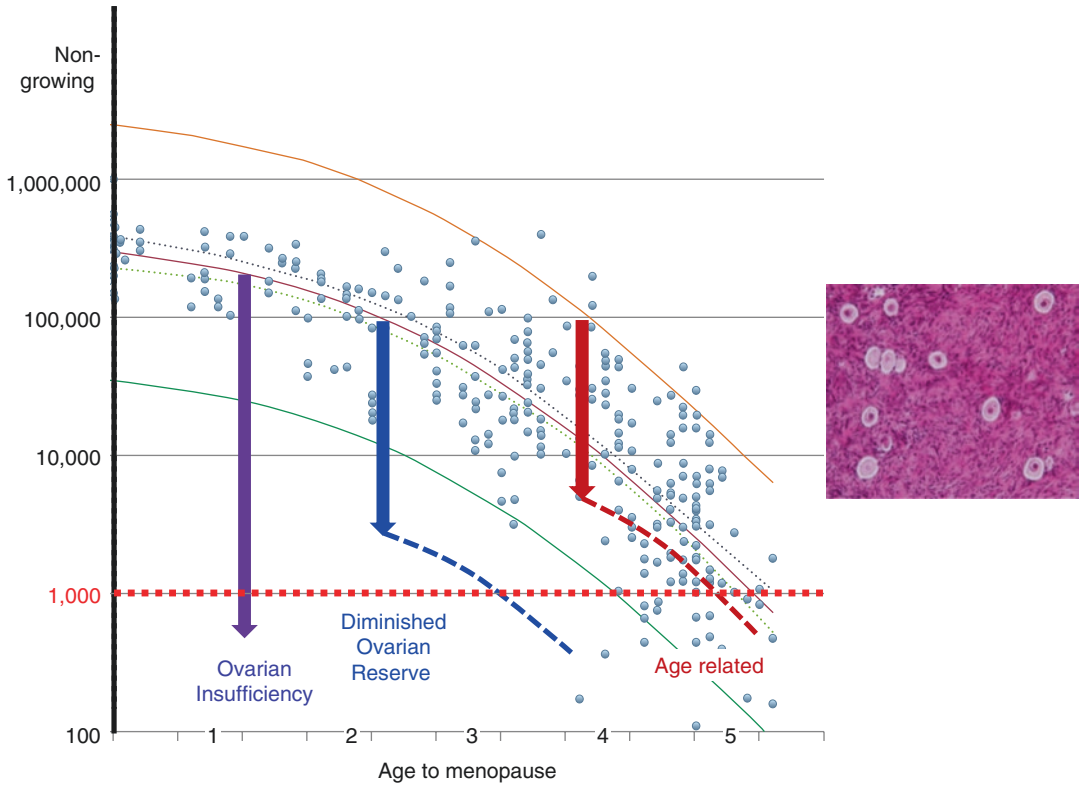

Fig. 1 Human follicle count representing the reserve of the ovary, modified from Wallace and Kelsey [14]. Menopause occurs when the number of follicles drops below 1000. Age-related menopause is a natural process while diminished ovarian reserve and subsequently premature ovarian insufficiency represent pathological processes

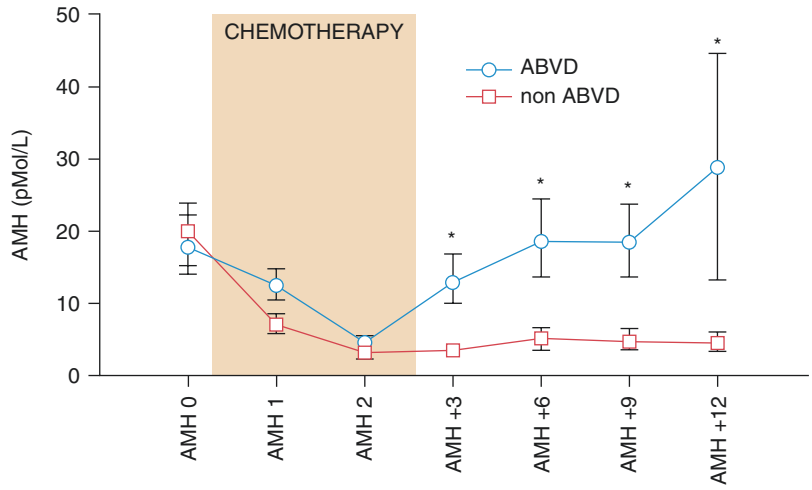

Fig. 2 AMH levels differences between low- and high-risk chemotherapy regimen, ABVD and non-ABVD protocols, respectively, modified from Decanter 2010 [18]. AMH levels remained decreased after the exposure to high-risk chemotherapy regimens while a slow and steady recovery was demonstrated within a year post exposure to low-risk protocols

Types of Chemotherapy Agents

There are six main classes of chemotherapeutic drug groups, classified according to mechanism of action. However, as regards ovotoxicity, chemotherapeutic agents are often divided into two: alkylating agents (cyclophosphamide, busulphan, etc.), which are highly ovotoxic, and nonalkylating agents, (including the other five classes of chemotherapy), which are generally less ovotoxic. The nonalkylating agents include antimetabolites (such as methotrexate, 5-fluorouracil), platinum-based agents (cisplatin and carboplatin), topoisomerase inhibitors (such as etoposide), and antibiotics [such as anthracyclanes including doxorubicin (Dox)] and plant alkaloids [Vinca alkaloids (Vincristine, etc.)] and taxanes (docetaxel, paclitaxel, etc.). As each class acts via different mechanisms, each has different effects on the ovary and follicular reserve and has different levels of risk for developing POI [24].

Alkylating Agents

Cyclophosphamide (Cy): Cy is the most commonly used alkylating agent [25]. In addition, it is used in the treatment of autoimmune diseases, such as systemic lupus erythematosis and rheumatoid arthritis.

Cy acts by inducing covalent binding of alkyl groups on DNA, thereby preventing DNA replication and consequently cell death. Cy is considered one of the most gonadotoxic chemotherapeutic agents [26, 27] and has been shown to induce primordial follicle loss [28–30]. Surviving primordial follicles are capable of developing into normal pregnancies, but growing follicles exposed to Cy have been associated with fetal abnormalities [31], a high proportion of miscarriages, and a tenfold increase in the number of malformations, [32]. Additionally, preconception exposure can also affect fetal development [33].

It is debatable whether the subsequent effects on fetal development are the result of direct damage, for example, apoptosis [34, 35], or indirect mediated by accelerated follicular activation ("burn out") [28, 36, 37], or a stromal effect such as inflammation and ischemia [38, 39], or a combination of all [40].

Busulfan: Busulfan is associated with significantly decreased pregnancy rate and has consistently been associated with a harmful effect on reproductive outcomes. Only Cy at the highest quartile dose is equivalent to busulfan regarding the damaging effect upon fertility [41].

Moreover, the combination of busulfan and cyclophosphamide, given as part of hemopoietic cell transplantation, is a recognized risk factor for POI and reduced pregnancy rates [42].

Platinum Derivatives

Platinum chemotherapeutic agents are commonly used in pediatric oncology treatment protocols for various types of cancer [43]. There are two main drug generations: cisplatin (first generation) and carboplatin (second generation) [44]. Toxicity is mainly attributed to DNA damage where the active metabolites bind to purine bases, resulting in crosslinking of the DNA molecules [43] and the creation of DNA adducts [45]. However, other molecular mechanisms have been linked to the clinical activity of these drugs, including induction of oxidative stress, modulation of calcium signaling, and activation of cellular pathways [43].

Cisplatin: Cisplatin (Cis) has been shown to be gonadotoxic for pediatric patients [41].

Cis interferes with DNA repair mechanisms, blocks cell division, causes DNA damage, and triggers apoptotic cell death [43]. Cis binds to the N-7 positions of adenine and guanine and produces inter- and intra-strand DNA adducts [46]. These DNA adducts trigger several signal transduction pathways, such as Akt, c-ABL, ATR, and MAPK/JNK/ERK [47, 48], which result in cell cycle arrest [47].

Cis has a moderate damage potential when compared to Cy, but, in studies conducted on cultured human ovarian cortical pieces or granulosa cells, exposure to Cis has resulted in a decline in follicle numbers and steroidogenic activity [49–51].

Most of the data regarding Cis-induced ovarian damage is derived from animal studies,

mainly mouse and rat models that showed diminished ovarian reserve and accelerated follicular atresia [34, 51–55]. The most sensitive cell appears to be immature oocytes [53, 55]. In addition, Cis was demonstrated to decrease AMH and inhibin A levels, reflecting a reduction in the AMH secreting follicles [56–58].

Cisplatin is also used as a traditional radiation sensitizer and radiation enhancement factor, thus diminishing the amount of radiation for local radiotherapy and therefore the effect upon fertility.

Carboplatin: Carboplatin is one of the newer generations of the platinum-derivative class of drugs that were developed with reduced toxicity and has been widely and successfully used as a replacement of cisplatin in specific cancers, such as breast and gynecological cancers [44, 59]. Pharmacologically, carboplatin is 8–45 times less potent than cisplatin and more stable, possibly due to reduced DNA-damaging effects [60, 61].

As a consequence, carboplatin requires higher doses in order to achieve the same therapeutic effect as cisplatin [43] (a dose 5–15 times higher may be necessary as compared to Cis in pediatric patients) [62, 63].

However, despite carboplatin's lower toxicity, it is as gonadotoxic as Cis, based on current classification systems [64]. Allen et al. [65] compared the gonadotoxic effect of carboplatin and cisplatin in an *in vitro* mouse model. The therapeutic dose of carboplatin has been shown to be equally gonadotoxic to Cis and caused a concomitant reduction in follicle numbers [65].

Anthracyclines

Doxorubicin (Dox)

Dox, also known as Adriamycin or Rubex, is used for the treatment of various types of cancer such as hematological malignancies [66–68] and is the most commonly used of this group of chemotherapeutic agents. Dox's mechanisms of action include topoisomerase II inhibition resulting in accumulation of DNA fragments and eventually cell death, increased production of oxygen-free radicals and other reactive oxygen species [69], induction of mitochondrial dysfunction [70] and DNA replication, and RNA and protein synthesis impairment [71–74]. Studies on human ovaries have revealed direct damage inflicted by Dox that preferentially cause damage to the granulosa cells [53] as well as vascular and stromal damage, especially to the microvasculature [56, 75]. In young patients with Hodgkin lymphoma, treatment with Dox does not seem to impair fertility [23]. It is challenging to isolate the precise effect Dox has on fertility as in most cases it is given in combination with other drugs.

Taxanes

Docetaxel is a second-generation taxane and is one of the most potent chemotherapeutic drugs in the treatment of local or metastatic breast cancer, non-small cell lung cancer, and ovarian cancer. It is extremely useful in inducing radio-sensitizing activity [76].

Taxanes lead to apoptosis, mainly by phosphorylation of bcl-2 [77].

Taxanes are usually administered in association with other drugs and adjuvants, and therefore it is challenging to evaluate the sole effect of taxanes [78, 79]. In animal models, paclitaxel (a member of the taxane family) has been associated with a loss of primordial follicles following treatment [80]. The addition of paclitaxel to the conventional treatment regimen of AC (doxorubicin/Adriamycin, cyclophosphamide) for breast cancer significantly increased ovarian toxicity [22].

However, subsequent studies showed that restoration of menstruation after treatment with taxanes was more common when compared to Cy, MTX, and 5FU [81, 82]. However, most studies, however, have used amenorrhea as a marker of POI, which does not detect partial ovarian damage.

Antimetabolites

These chemotherapeutic agents include methotrexate (MTX) and 5-fluorouracil (5FU).

Antimetabolites cause cytotoxicity in proliferating S-phase cells by inhibiting nucleotide

synthesis and incorporating the metabolites of MTX or 5FU into RNA and DNA [83].

In addition to tumors, MTX is used as treatment of ectopic pregnancies [84]. In the doses used, MTX does not seem to influence ovarian reserve nor the response to gonadotropin stimulation in artificial reproductive technology, the clinical pregnancy rate, or the live birth rate [85].

The possible ovotoxic effect of 5-fluorouracil was not evaluated until recently. However, recent *in vivo* studies in mouse model found that exposure to 5-FU caused atresia of secondary and antral follicles but did not induce apoptosis of primordial and primary follicle numbers, and therefore do not harm ovarian reserve [86].

Immunotherapy Regimens

Immunotherapy attempts to modulate the host's immune system to attack and destroy cancer cells. The main targets of immunotherapy are the checkpoint inhibitors programmed cell death-1 (PD-1) and its ligand PD-1 [87] and CTLA-4, which is upregulated in activated T cells in cancerous tumors [88]. Inhibition of PD-1, its ligand, and CTLA-4 aim to reduce the inhibitory activity on the cytotoxic cells and induce cancer cell apoptosis.

However, one side effect of immunomodulation is the risk of *de novo* autoimmune diseases and the creation of autoantibodies, which have been demonstrated in several organ systems including the GI, CNS, and respiratory and cardiac systems [89]. Among the described endocrinopathies are hypophysitis with CTLA4 inhibitors and thyroid dysfunction with PD-(L)1-blockers, [90, 91], type 1 diabetes mellitus (Robert 2016), and primary adrenal insufficiency [92].

The effects of these immune-modulators on fertility may be due to the creation of antiovarian antibodies (AOA). Moreover, the data regarding the effects of immunotherapeutic agents on the human reproductive system is extremely limited and scarce. The official recommendation regarding PD-1 inhibitors is to use contraception during immunomodulation for at least an additional 4–5 months after therapy [92].

Nonetheless, PARP inhibitors such as pembrolizumab (Keytruda) were found to deplete the ovarian reserve in mice by reducing PMF numbers but not effecting AMH levels [93] and also reducing the number of retrieved oocytes at IVF cycles [94]. However, IVF results recovered 3 weeks post exposure [94].

Mechanisms of Chemotherapy-Induced Follicle Loss

Alkylating agents and platins are considered the most highly ovotoxic of the chemotherapy agents, and have therefore been the focus of most of the research attempting to clarify the mechanisms that underlie the loss of ovarian reserve that ultimately results in POI and reduced fertility in many cancer survivors. Studies, primarily using animal models, have proposed several mechanisms, including direct apoptotic death, indirect induction of follicle activation followed by follicle loss ("burn-out" mechanism), and indirect loss as a result of damage to surrounding stroma and/or disruption of blood supply (Fig. 3) [12].

The apoptotic pathway plays a significant role in the therapeutic actions of chemotherapy as well as the ovotoxicity. Chemotherapy-induced apoptosis in nondormant follicles is well established, with numerous studies showing significant apoptotic cell death in granulosa cells of growing follicles [49, 51–53, 68, 95–97]. The death of growing follicles results in a temporary loss of menorrhea experienced by patients immediately following and during chemotherapy treatment [2]. However, it is unclear whether apoptosis plays a direct role in the loss of the dormant PMFs that comprise the ovarian reserve. Cyclophosphamide was shown to induce DNA double-strand breaks, activate the DNA damage response, and induce apoptosis in human PMF oocytes in a xenograft model [98, 99]. Many studies have utilized a mouse model of *in vivo* cyclophosphamide treatment, with some reporting apoptosis [34, 35, 52, 100, 101], while others did not demonstrate apoptosis in the PMF population [36, 40, 102–109]. The role of apoptosis in PMF loss is supported by studies that demon-

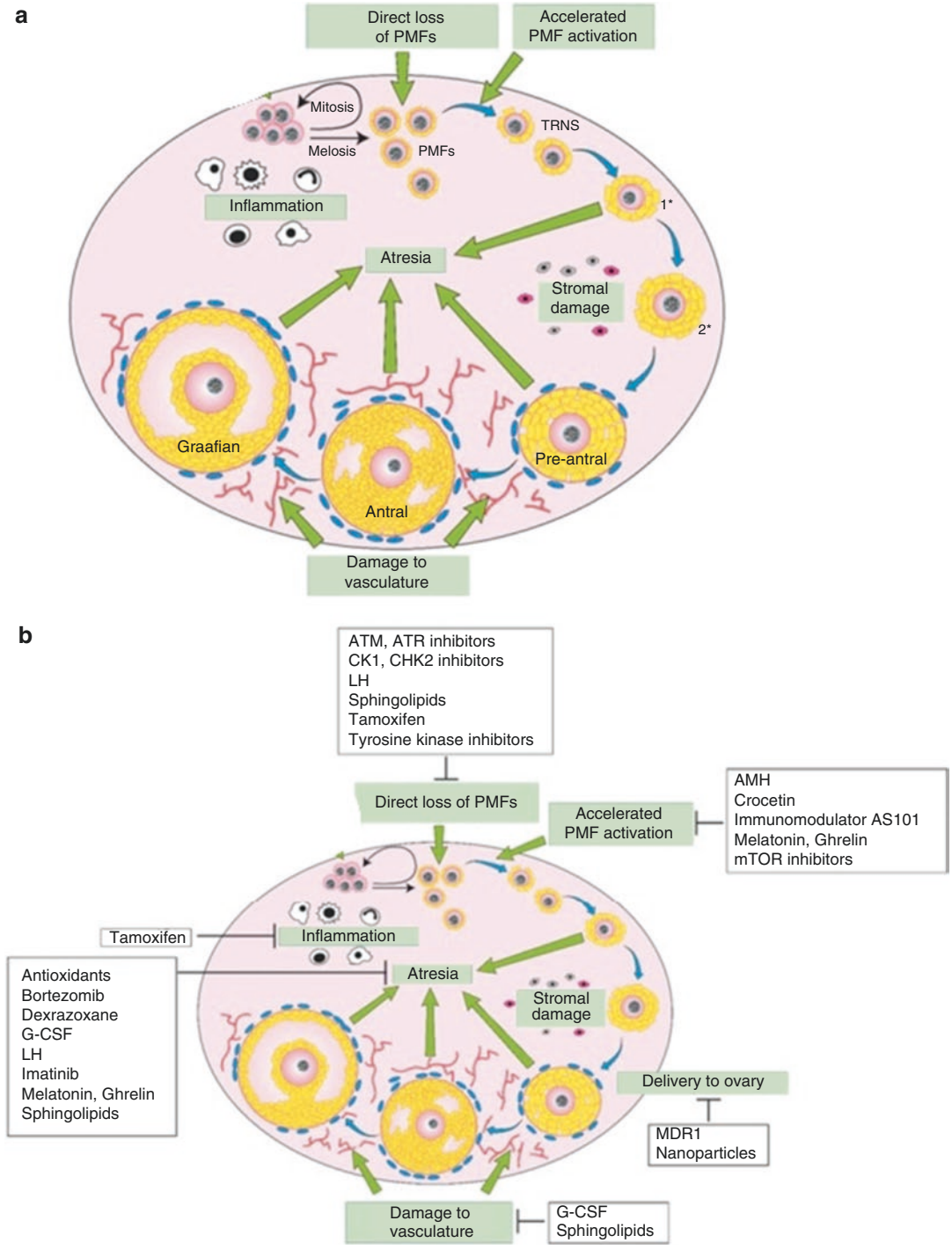

Fig. 3 Summary of proposed mechanisms behind chemotherapy-induced follicle loss. Adapted from Spears et al. Hum Rep Update, 2019 [12]. (**a**) Proposed mechanisms of chemotherapy-induced follicle loss include direct apoptotic loss of primordial follicles, accelerated activation of primordial follicles, follicular atresia, and damage to stromal tissue, vasculature, or inflammation. (**b**) Protectants examined that have been shown to protect against chemotherapy-induced follicle loss via specific ovarian damage pathways or to interfere with drug delivery to the ovary

Table 1 Studies of protective agents (by mechanism of action)

Mechanism of action	Protective agent	References
Prevention of follicle activation	AS101	[37]
	AMH	[107, 110, 112]
	Melatonin	[10]
	mTOR inhibitors	[38, 108]
Anti-apoptosis	Tyrosine kinase Inhibitors	[11, 36, 53–55]
	S1P (sphingosine-1-phosphate) C1P (ceramide 1 phosphate)	[31, 41, 76, 108]
Suppression of pituitary–gonadal axis:	GnRH analogues	[119, 165, 166, 168]
Neovascularization agents	Granulocyte colony-stimulating factor (G-CSF)	[169, 170]
Transport block	Bortezomib	[68]
Upregulation of MDR1	MDR1	[171]

mTOR: mammalian target of rapamycin; GnRH: gonadotropin release hormone; MDR1: Multi-Drug Resistance Mutation-1

strate that agents that block the apoptotic pathway can attenuate the loss of PMF reserve during chemotherapy treatment (Fig. 3, Table 1) [52, 56, 75, 110].

Another proposed mechanism of follicle loss suggests that ovotoxic chemotherapy agents trigger activation and growth of the dormant PMFs, resulting in "burn-out" of the PMF reserve. In *in vivo* animal models, ovotoxic chemotherapy agents were shown to increase recruitment of PMFs into the pool of actively growing follicles that subsequently undergo atresia or apoptosis [66, 102, 108, 111]. This was demonstrated to be mediated by an upregulation in the PI3K signaling pathway, inducing increased phosphorylation of key proteins Akt, mTOR, and FOXO3A [36, 37, 102]. PMF activation and loss were also seen in human ovarian tissue exposed *in vitro* to PM [112]. In contrast, one study observed no evidence of follicle activation using a similar mouse model of chemotherapy treatment [100]. The role of follicle activation in chemotherapy-induced follicle loss is supported by animal studies in which treatment with PI3K pathway suppressors [36, 37, 109] or follicle suppressant anti-Mullerian hormone (AMH) [108, 111, 113] was shown to attenuate follicle activation and loss caused by chemotherapy and preserve fertility (Fig. 3, Table 1).

Stromal damage and focal infarcts resulting from loss of blood supply have been observed in human ovarian tissue retrieved after chemotherapy exposure, and these may indirectly lead to loss of PMFs and increased follicular atresia [114, 115]. However, most of this data was collected from patients exposed to chemotherapy months or years prior to ovarian cortex removal and evaluation, reflecting only the endpoint damage and not the acute mechanisms. There is no evidence to date that prevention of stromal damage protects the ovary from follicle loss.

In addition to antiapoptotic and antiactivation agents, a number of other agents that target mechanisms of chemotherapy-induced ovarian damage have been tested for protection of the ovarian reserve. These are summarized in Table 1 and presented schematically in Fig. 4. Most of these agents were only tested in single studies on animal models, and as such are highly preliminary.

Rather than pointing conclusively to a single mechanism behind PMF loss, the accumulation of data from existing studies leaves unclear to what degree the destruction of the PMF population can be attributed to direct apoptotic damage or the result of indirect loss due to acceleration activation or stromal damage [12]. It is further unclear to what degree the results of animal studies can be extrapolated to reflect on the human ovary since almost all the existing data is derived from animal studies and very few studies have been able to directly examine the impact of chemotherapy on the human ovary *in vivo*. Additional investigation into the mechanisms of follicle loss in human ovaries is a necessary foundation for the development of new methods for fertility preservation in cancer patients.

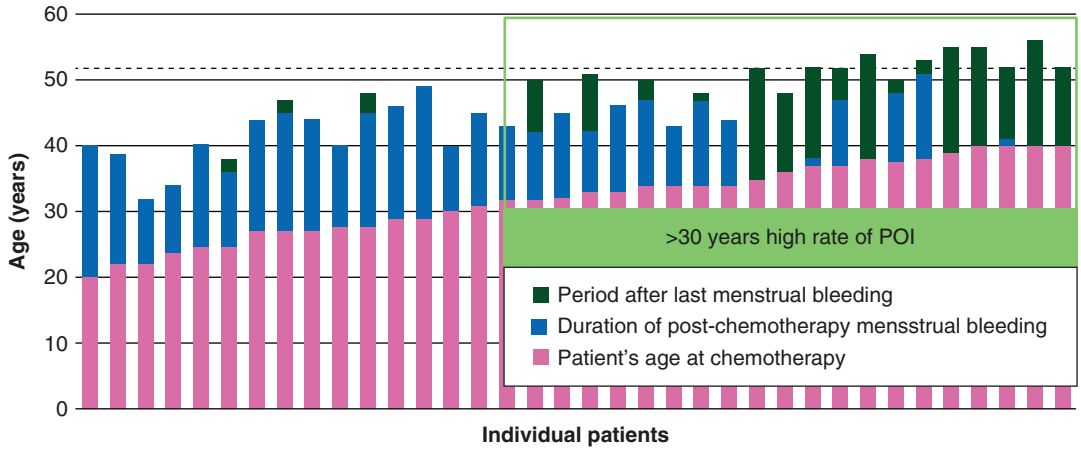

Fig. 4 Long-term ovarian function in women treated with CHOP or CHOP + etoposide for aggressive lymphoma, modified from Meissner 2015 [19], demonstrating significantly higher rates of POI among patients older than 30 years at the diagnosis of the disease

Impact of Diagnosis and Treatment Protocol on Fertility

Breast Cancer

In breast cancer patients, the different treatment protocols have different effects on ovarian function.

BRCA1 (but not BRCA2) carriers have a lower baseline level of AMH. BRCA-1 and 2 genes are responsible for encoding of proteins involved in the DNA damage repair pathway, which has an essential role in the oocyte [116]. Nonetheless, recent studies have demonstrated that BRCA carriers have similar reproductive potential as noncarriers and a normal response in IVF cycles [117, 118].

Cy-containing protocols such as CAF (cyclophosphamide, Adriamycin/doxorubicin, and fluorouracil) were shown to induce high rates of POI (52% amenorrhea 1 month post treatment) [22]. However, the alternative treatment protocol of AC (Adriamycin/doxorubicin and cyclophosphamide) induced significantly less damage to ovarian function [20]. Approximately 50% of patients who were exposed to AC with or without paclitaxel (AC-T protocol) had initial hypomenorrhea, followed by a slow recovery, and eventual restoration of regular menstrual bleeding [22]. However, although menstruation was restored, the postchemotherapy ovarian reserve was still low and the rate of chemotherapy-induced POI ranges from 22 to 38% [117–119].

In contrast to standard chemotherapy, the addition of anti-HER2 blockers (Herceptin – Trastuzumab) and Lapatinib (dual tyrosine kinase inhibitor) for HER-2 positive breast cancer patients does not seem to increase the incidence of POI, and therefore these agents are cautiously regarded as non-gonadotoxic [120].

A relatively new addition to the chemotherapeutic arsenal against breast cancer is carboplatin. The addition of carboplatin to the neoadjuvant protocol for triple-negative BRCA carriers has demonstrated a significant improvement in disease-free survival and overall survival [121]. However, there are as yet no studies examining the gonadotoxic effect of carboplatin.

Hodgkin's Lymphoma

The ovarian damage inflicted by chemotherapy for Hodgkin's lymphoma (HL) varies greatly according to the protocol. Past protocols caused relatively high rates of POI of up to 57%.

However, the ABVD protocol (doxorubicin, bleomycin, vinblastine, and dacarbazine) was found to be significantly less harmful to ovarian function [20, 122] and does not seem to reduce pregnancy rates [123]. The early stages of HL are usually treated with the ABVD protocol, and in some countries the intermediate stages of HL are treated by the ABVD protocol. In other countries, the intermediate stages are treated with 2 × BEACOPP. For advanced stages, 2 × ABVD and 4–6 × BEACOPP escalated [bleomycin, etoposide, doxorubicin (Adriamycin), cyclophosphamide, vincristine (Oncovin), Procarbazine, Prednisolone].

This latter protocol is associated with significantly higher gonadotoxicity than ABVD [124] with a prominent age-dependent effect: amenorrhea rate of 51.4% in women <30 years and 95.0% ≥ 30 years after eight cycles of BEACOPP escalated (higher levels of Cy, etoposide, and Dox) [125]. The gonadotoxic effect was also evident in the AMH levels, as expected: 2.2 vs. 0.1 μg/l at age 18–29 years and 0.7 vs. 0.0 μg/l at age 30–45 years ABVD vs. BEACOPP escalated, respectively [17].

Predictably, 90% of patients with early stages of HL that were exposed to ABVD and/or 2 × BEACOPP regained regular menstruation within 12 months after chemotherapy. This number dropped to 50–75% after 6–8 × BEACOPP escalated, depending on the patient's age [17].

Another concern regarding the treatment protocol for pediatric patients is supplementary radiation.

Current protocols have markedly reduced radiation in first-line treatment approximately 50% of patients received radiation in the EuroNet-PHL-C1 trial and only 25% in the current EuroNet-PHL-C2 trial. Moreover, some patients only received radiotherapy if there was residual tumor as shown by a PET-CT scan [126], and radiation was mainly directed to the patient's chest and upper abdomen, precluding a harmful effect upon fertility. Additionally, radiation has often been replaced by chemotherapy due to less unwanted side effects. These chemotherapeutic regimens include Cy and dacabazine, both alkylating agents. As these irradiation-replacing protocols are relatively new, there is still a paucity of studies regarding their effect on fertility.

The most toxic agent used in the treatment of lymphoma is bone marrow transplantation (BMT). The published rates of POI after BMT can reach 100% [42, 127], although most studies reported an average of 80% [128, 129] and not less than 70% [130]. Consequently, treatment with BMT warrants a preliminary fertility preservation plan for fertility age patients.

Non-Hodgkin's Lymphoma (NHL)

The reported POI rates in NHL patients treated with a variety of drug regimens are relatively high, ranging from between 44% [20] and 60% in women aged >35 years for CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone ± etoposide) [131–133]. However, the toxic effect of the intensified CHOP regimen (Mega-CHOP) protocol found a POI rate of only 8% [134]. The low POI rate may be explained by the use of a different time schedule and dose intensity.

A more recent study has demonstrated a significantly earlier age of menopause, higher rate of moderate or severe menopausal symptoms, and a decreased level of AMH among NHL patients treated with CHOP-like protocols when compared to similar age groups in the general population [135] (Fig. 4).

Leukemia

The two main types of leukemia are acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL).

The common and standard protocol during recent decades is the "7 + 3" induction regimen (a 7-day continuous intravenous cytarabine infusion and three daily doses of daunorubicin). The "7 + 3" is protocol is not gonadotoxic, therefore fertility preservation is not required [136]. After AML treated with chemotherapy, there is normal pubertal development and fertility. However, in 13% of postpubertal patients, there was a decrease in serum AMH [19] and 13–20% of AML patients

need a BMT [137]. BMT may act as an autologous or allogenic stem cell transplantation, causing amenorrhea [138, 139]. Hence, fertility preservation should be individualized according to the severity of the disease.

Pediatric ALL patients receive a remission induction treatment composed of prednisone, vincristine, daunorubicin, asparaginase, teniposide, and cytarabine, followed by consolidation therapy with two weekly doses of high-dose methotrexate at 2 g/m^2. The protocol is a highly gonadotoxic and long-term treatment of 120 weeks of antimetabolite-based therapy with four pairs of drugs (etoposide and cyclophosphamide, mercaptopurine and methotrexate, teniposide and cytarabine, prednisone and vincristine). As expected, the risk for POI depends on the total dosage of alkylating agents in the protocol regimen, specifically Cy, the reported POI rate ranges between 13 and 28%, depending on the Cy dose. Moreover, the commonly used St. Jude protocol [140] for ALL includes BMT, which is known to cause POI [141]. Thus, fertility preservation is a recommended option for patients receiving a high dose of alkylating agents and/or bone marrow transplantation [27]. The need for BMT is approximately 5–10% during primary treatment for ALL and reaches 50% for relapse treatment [142].

Gastrointestinal Cancers

GI cancers, although relatively rare in women of reproductive age, years, have presented in increasing numbers in recent years. Unfortunately, there is no consensus regarding fertility preservation for GI cancers. The current chemotherapy regimen is taxane-based or platinum derivatives-based [143]. As both regimens are considered to be mildly harmful for fertility, we advise pretreatment fertility preservation counseling. Moreover, in most cases of colorectal cancers, the anticancer treatment includes high-intensity pelvic irradiation, which also mandates consideration of fertility preservation, and possible oophoropexy.

Another commonly used chemotherapeutic regimen for GI cancers and metastatic breast cancer is capecitabine (Xeloda), which is an antimetabolite. However, there is a lack of data regarding the possible effect on fertility among women, and, in general, this drug family is not considered gonadotoxic.

Brain Tumors

The commonly used chemotherapeutic agents for brain tumors are based on temozolomide (Temodal), an alkylating agent, usually as a single agent. Other protocols include the use of Cy. Balachandar [144] published a study evaluating POI in children with medulloblastoma, treated by cranial–spinal irradiation and standard/high-dose chemotherapy that included alkylating agents. The POI rates were 60% in patients receiving high-dose chemotherapy compared to 22% in the standard dose and 6% in the irradiation-only group [144]. Moreover, any patient who is exposed to a total dose of irradiation of 20 Gy or more to the hypothalamic–pituitary axis (HPA) is at risk for subsequent hypopituitarism [145]. Multiple hormone deficiencies are common after treatments with 60 Gy [146]. The reported incidence of oligomenorrhea and low estradiol levels after cranial irradiation is 50–70% [147].

Sarcoma

The majority of Ewing sarcoma patients suffer from POI after multimodal treatment protocols. The chemotherapy protocols were VIDE: vincristine, ifosfamide (an alkylating agent), doxorubicin, and etoposide, followed by risk- and response-adapted adjuvant chemotherapy with VAI/VAC protocol: vincristine, actinomycin D, and cyclophosphamide/ifosfamide. Cumulative doses of drugs were VIDE and VAI ifosfamide 102 g/m^2, doxorubicin 360 mg/m^2, and etoposide 2700 mg/m^2. VIDE and VAC cumulative doses were ifosfamide 60 g/m^2, doxorubicin 360 mg/m^2, etoposide 2700 mg/m^2, and cyclophosphamide

10.5 g/m^2. In total, the patients are exposed to high cumulative doses of alkylating agents. Exposure to the above protocol caused a 25% POI rate among girls of a mean age of 12 years. However, this rate rose to 87.5% when an autologous hematopoietic stem cell transplant was performed, and any combination of regimens involving pelvic radiotherapy resulted in 100% POI rate [148].

Despite the paucity of studies examining the risk for POI in osteosarcoma patients, the reported rate of POI these patients is 6.6% with a high-dose protocol of ifosfamide, methotrexate, Adriamycin, and cisplatin [149].

Gynecological Cancers

The common chemotherapeutic regiments for gynecological cancers are the combination of carboplatin and taxane. These two agents are known to have a negative impact on fertility potential.

In cervical cancer, there are two treatment modalities: surgical and nonsurgical that includes irradiation (external beam ± brachytherapy). Multimodality treatment should be avoided due to the increased morbidity [150, 151]. In the nonsurgical approach, cisplatin is usually used instead of carboplatin as an irradiation-sensitizing agent [152]. In cases of pelvic radiation, oophoropexy is indicated if the ovaries are not involved. The damage to the ovaries varies according to the radiation source: 90% in patients undergoing vaginal local brachytherapy had preserved ovarian function compared to 60% in patients undergoing external-beam pelvic radiation therapy [153]. However, fixation of the ovaries out of the radiation field does not prevent the gonadotoxic damage due to chemotherapy, and oophoropexy itself might have a negative impact on the ovarian reserve as ovarian blood supply may be impaired. The incidence of POI has been reported to be up to 40% after ovarian transposition [154]. It should be borne in mind that ovarian transposition is contraindicated if the ovaries have been contaminated by cancer cells. The threshold radiation dose for substantial uterine damage is unknown, but it has been postulated that a dose of more than >45 Gy to the whole pelvis is detrimental to the uterus [155].

Endometrial Cancer

In the early stages of endometrial cancers, progestins are recommended. Progestins are known to be safe and non-gonadotoxic. However, pregnancy is not desirable during treatment and in most cases nonachievable as high levels of progesterone prevent ovulation. Moreover, in many cases, progestins may not suffice and hysterectomy may be required.

Melanoma

Melanoma disproportionately affects young patients and is the most commonly diagnosed cancer in patients aged 25–29 [156]. Approximately one-third of patients with an initial diagnosis of melanoma are of childbearing age, and melanoma is one of the most common malignant tumors diagnosed in pregnant women [157].

New cancer treatments improve survival compared to the more traditional methods of treatment [158]. Although new treatment protocols for melanoma are mainly based on biological agents and immunomodulators, there is morbidity. Walter [159] reported from a literature review that 58% of the systemic regimens for melanoma treatment presented a fertility risk in animal and human studies (categories C and D). Among the first-line therapies, dabrafenib (BRAF inhibitor) has been shown to reduce corpora lutea numbers in rat models. Both MEK inhibitors, cobimetinib and trametinib, have been shown to be associated with fertility toxicity in animal studies, while immunotherapy with ipilimumab and the PD-1 inhibitors has an unclear impact on fertility [160, 161], as discussed above in the "Immunotherapy" section of this review. However, to date there are no human studies in this field.

Rheumatic and Autoimmune Disorders

The current treatment for rheumatic and autoimmune diseases involves immunemodulators and chemotherapeutic agents. As stated above, the most gonadotoxic agent is cyclophosphamide. Studies evaluating the effect on fertility in systemic lupus erythematosus (SLE) showed a substantial rate of POI in young patients of reproductive age. The use of Cy is relatively common in SLE with nephropathy. One study showed that 31% of patients had amenorrhea after Cy treatment of more than 12 months' duration [162]. Predictably, the patient's age was the strongest factor affecting POI. For women older than 32 years, the cumulative dose resulting in sustained amenorrhea in 50% and 90% of the treated women was 8 g/m^2 and 12 g/m^2, respectively. However, only 12% of women aged younger than 31 years developed sustained amenorrhea as the major risk factors for POI were the disease's duration and the presence of anti-U1RNP and anti-Ro antibodies [162, 163].

However, ovarian function and reserve might be influenced by the autoimmune disease itself rather than the therapy [164, 165].

Summary

Chemotherapy imparts an insult to the ovarian reserve of various degree, depending on factors such as regimen protocol, patient's age, and type of cancer. The main damage-inducing mechanisms of chemotherapeutic agents are apoptosis of nondormant follicles, activation and growth of dormant PMFs ("burn-out"), stromal damage, and focal infarcts. Some therapeutic regimens and cancer types pose greater threat to the ovarian reserve than others such as BMT for leukemia and pelvic irradiation. Therefore, it is necessary to identify patients at high risk for postchemotherapy POI in order to implement suitable measures either for protection of the ovaries and/or for fertility preservation.

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Ovarian Function and Fertility Preservation for Young People Treated for Cancer

S. Caprioli, T. W. Kelsey, and W. H. B. Wallace

Introduction

Further advances in the treatment of young people with cancer have led to improved survival, with 85.6% 5-year relative survival for ages 0–14 in the USA for the years 2010–2016 [17]. However, successful cancer treatment during childhood can cause infertility and premature ovarian insufficiency (POI) in some patients [19, 33]. The risk of developing POI is dependent on a number of factors, which include the nature of the underlying disease and the planned therapy. Both chemotherapy and radiotherapy have been shown to affect ovarian function either directly by depleting the primordial follicle pool or indirectly via effects on hormonal regulation of ovarian function.

The aim of this chapter is to review our knowledge of normal ovarian function, discuss and review the assessment of risk of developing POI after modern treatment regimens, and describe both experimental and established technologies currently available for fertility preservation. This chapter is important for the audience, regardless of medical background, to raise awareness on the gonadotoxicity of cancer treatment and the approaches that have been developed to preserve a patient's fecundity. In a clinical environment, this chapter emphasises to doctors the value of communicating with the patient and their family regarding the assessed gonadotoxic risk of the proposed cancer treatment and explains in some detail the different fertility preservation techniques that are currently in clinical practice.

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Ovarian Function

The ovary has two important functions; to produce mature oocytes capable of being fertilised and to secrete the key hormones oestrogen and progesterone. These two functions go hand-in-hand as steroidogenesis regulates the oocyte release.

Formation and Loss of Oocytes

One of the most important functions of the ovary is to form mature oocytes available for fertilisation. Females are born with their full complement of immature oocytes known as primordial follicles (PF), they decline in an exponential fashion, and the menopause occurs when there is insufficient population to sustain recruitment towards maturation, at approximately 750 remaining

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PF. Primordial germ cells – known as oogonia – migrate to the gonadal ridge where the ovaries are formed. During migration, the oogonia undergo continuous proliferation, and upon reaching the ovaries, they stop dividing and form nests of germline cysts. These nests break down, and the individual germ cells form associations with the somatic cells that are present in the area. This individual unit, the PF, comprises a primary oocyte surrounded by a single layer of granulosa cells. The peak PF population occurs at 20–22 weeks' gestation, with about 300,000 PF in the average human foetal ovary [34]. There is a wide range in normal peak PF population size (95% will be between 35,000 and 2,500,000), with this wide range is believed to be responsible for the wide range in ages at menopause (42–58 years) in the healthy population.

In postnatal to pre-pubertal years the PF population declines due to follicle death by apoptosis, with about half the initial peak population lost before puberty is initiated. During and after puberty, PFs are recruited in large numbers towards maturation. A small number of dominant follicles are selected, with some becoming mature eggs. The rest are lost by atresia until fewer than about 750 PF are present in the ovary. This population is insufficient to sustain recruitment to mature eggs, and menopause occurs.

In a clinical environment, premature ovarian insufficiency (POI) occurs when there is an early depletion of the ovarian reserve when compared to the general population. Treatment for cancer can cause this early depletion and predispose to POI and infertility. The proportion of the PF pool that is lost at the time of an insult such as cancer treatment determines the remaining window of opportunity for fertility: the insult effectively increases reproductive age whilst leaving chronological age unchanged. Therefore, minimising the extent of the population loss is crucial for long-term fertility.

Follicle-stimulating hormone (FSH) and anti-Müllerian hormone (AMH) are established biomarkers for the ovarian reserve. FSH is of limited use when assessing POI at pre-pubertal ages since FSH levels can be within the normal age-related range even when there is a total lack of ovarian function. In particular, for pre-pubertal girls, FSH levels do not significantly change throughout cancer treatment and after completion [6]. After puberty early follicular phase FSH is a robust indicator of normal ovarian function, although FSH has low positive predictive value when predicting future menses or POI. AMH, secreted by the granulosa cells of the growing follicles, inhibits primordial follicular activation and has proved to be an accurate indirect representation of the remaining PF pool in children treated for cancer [6]. During infancy, AMH levels are high, plateauing during early adulthood and eventually is inversely correlated with increasing age [20]. Therefore, women with depletion of ovarian reserve due to cancer treatment would have low age-related serum measurements of AMH, and these have robust positive predictive value for future menses or POI [16]. However, it is uncertain if AMH is a robust indicator of ovarian reserve in pre-pubertal children as levels of AMH fluctuate at pubertal ages [20].

Hormonal Secretion

Endocrine control of the ovary is by FSH and luteinising hormone (LH) secreted from the anterior pituitary under control from the hypothalamus. Under stimulation from FSH/LH, the ovary secretes sex steroids which act locally on endometrial tissue in preparation for pregnancy as well as impacting systemically on both the hypothalamus and pituitary gland. The reproductive axis remains relatively quiescent until puberty and after it is active throughout reproductive age. Hormonal secretion of the ovary is governed by the hypothalamic pituitary gonadal (HPG) axis. This tightly regulated cycle is important for the selection of a dominant follicle for ovulation and preparing the endometrium for possible implantation. The hypothalamus releases gonadotrophin-releasing hormone (GnRH) which acts on the anterior pituitary triggering the secretion of LH and FSH. The ovary communicates to the pituitary and the hypothalamus via negative and positive feedback loops. Both oestrogen and inhibin

B suppress the release of hormones from the pituitary and hypothalamus, thus controlling the reproductive axis. However, above a certain threshold, oestrogen can also hyper-activate the HPG axis, stimulating the release of more GnRH and LH. This unique mechanism only applies to females and occurs at a specific point during the menstrual cycle. Consequently, there is a co-dependency of FSH and LH, unlike in males, to produce oestrogen. This female sex steroid is fundamental for ovulation and endometrium proliferation.

Mini Puberty and Puberty

Mini Puberty

When the girl is 3–6 months old, the HPG axis becomes activated, an event known as the "mini puberty" [23]. The role of the mini puberty is less well understood in females than males; nevertheless, it is speculated that this activation stimulates the development of breast tissue [23]. During this event, FSH levels remain high, presumably to stimulate folliculogenesis whilst LH gradually decreases [23]. Although the mechanism is unknown, the HPG axis will subsequently switch off and remain quiescent until puberty.

Puberty

The onset of puberty in girls is largely controlled by leptin, a hormone secreted by adipose fat cells [29]. During puberty, the HPG axis is activated and matures overnight releasing GnRH in a pulsatile manner. In turn FSH/LH are released from the anterior pituitary acting on the ovary to produce sex steroids which trigger menarche, with oestrogen responsible for the development of secondary sex characteristics.

Adulthood

After puberty, the menstrual cycle is continuous throughout adulthood until menopause. This complex cycle shifts from preparing the endometrium for possible implantation each month to shedding and menses if pregnancy does not occur. The hormones FSH and LH influence folliculogenesis, oocyte development, and stimulate ovulation.

Menopause

With each menstrual cycle, the pool of primordial follicles is reduced, and as the female ages she becomes less fertile. Following depletion of the primordial follicle pool, the woman enters menopause. The average age of menopause in the USA and Europe is 50–51 years. Menopause is defined as amenorrhoea for more than 12 months. The absence of menses is due to depletion of the PF pool with insufficient oocytes to continue the normal ovarian cycle. Given that no follicles are growing, oestrogen synthesis cannot occur. The menopause and POI are characterised by oestrogen deficiency which has long-term effects on health, in general, and bone health, in particular, predisposing to osteoporosis.

How Cancer Treatment Affects the Female Reproductive System

There is a large body of evidence that cancer treatment, both chemotherapy and radiation, can be gonadotoxic and cause POI in some patients [24, 33, 35].

Cancer Therapy Affecting the HPG

Ovarian function is dependent on the HPG axis. The HPG axis is the main trigger for puberty; hence, irradiation to any part of the axis can delay the onset of puberty and cause hypogonadotrophic hypogonadism by disrupting release of FSH and LH. [28, 31]. Cranial irradiation may be required for the treatment of a wide range of brain tumours in young people. Other pituitary hormones including growth hormone, ACTH, and TSH may be affected leading ultimately to panhypopituitarism [25] which may develop many years after the initial treatment.

Cancer Therapy Affecting the Ovary

Chemotherapy can also directly affect the ovary. Both ovarian architecture and function can be negatively impacted, resulting in early depletion of ovarian reserve and a reduced fertility window. Although not all chemotherapeutic drugs are gonadotoxic, alkylating agents reduce the pool of primordial follicles. For example, cyclophosphamide, an alkylating agent, disrupts ovarian function by directly accelerating follicular growth [32]. In addition, this drug also induces greater oocyte apoptosis as it rapidly stimulates DNA breaks in the ovary [32]. Alongside ovarian function, cyclophosphamide can also affect the architecture, notably vascular damage which, in turn, will negatively impact the health and function of the follicles [32]. Consequently, chemotherapy can be extremely gonadotoxic having both direct and indirect impacts on the ovarian reserve by accelerating folliculogenesis or increasing apoptosis.

Similar to chemotherapy, radiation directly to the ovaries induces premature reproductive ageing and POI. Radiotherapy induces DNA breaks in the oocyte which, as a result, they either undergo DNA repair mechanisms or apoptosis [3]. The LD_{50} of the human oocyte is <2 Gy [35], making the ovary exquisitely radiosensitive. Radiation to the ovary may also affect the somatic cells, particularly when the granulosa cells mature and develop during folliculogenesis.

Consequently, cancer treatments can directly affect primordial follicles, leading to reduced ovarian reserve. Therapy can indirectly damage follicles that are growing which causes increased recruitment of primordial follicles to replace the damaged dominant follicle (the so-called burnout hypothesis, [27]). However, cancer therapy can also affect different cell types notably the oocyte itself by inducing DNA breaks and the surrounding somatic cells, both of which ultimately lead to cell death.

Different Types of Cancer Treatments Causing Gonadotoxicity

Many cancer treatment regimens have negative effects on ovarian function. However, there are many uncertainties and there is a lack of high-quality evidence to guide the assessment of risk of POI for most of the current chemotherapy regimens in common usage [19]. The majority of the gonadotoxic drugs utilised will have similar risk assessments in both males and females. In this context, risk for females is defined as the chances of developing premature ovarian insufficiency (POI), thus, a reduced fertility window and early menopause. Table 1 highlights the estimated chance of developing POI after treatment exposure. Table 2 is a summary of the estimated gonadotoxicity of regimens currently in use for the most common childhood cancers. The assessment is based on expert opinion (due to a lack of published evidence) from the Childhood Cancer and Leukaemia Group (CCLG) in the UK and remains under regular review.

Therefore, from Table 2, there is a consensus from experts that many cancer treatment regimens can predispose to POI and a reduced window of opportunity for fertility.

As shown in Table 2, most chemotherapeutic drugs present show a dose-dependent gonadotoxicity risk in females. In a recent population-based study from Scotland [2], we have shown in females diagnosed with cancer <39 years of age between 1981 and 2012, cancer survivors

Table 1 The classifications in percentage of the risk assessment

Risk	Percentage
Low	<10
Medium	10–60
High	60–80
Very high	>80

Adapted from the CCLG Oncofertility Consensus Document [7]

achieved fewer pregnancies than the underlying population: standardised incidence ratio (SIR) 0.62 (95% CI: 0.60, 0.63). Reduced SIR was observed for all cancer types. The chance of achieving a first pregnancy was also lower, adjusted hazard ratio = 0.57 (95% CI: 0.53, 0.61) for women >5 years after diagnosis, with marked reductions in women with breast, cervical, brain/CNS tumours, and leukaemia. The deleterious effect of chemotherapy on the ovaries has been confirmed by Chow et al. [8], who compared live birth rates of childhood cancer survivors and their siblings who were used as a control. The rates of live births for the survivors are lower but follow a similar pattern as the control group.

Radiation therapy to a field that includes the pelvis may have adverse effects on the uterus. The uteri of patients who have undergone radiation pre-pubertally will be smaller with an absent endometrium and poor blood flow, all of which affects patients' fertility [5]. On the contrary, patients treated with chemotherapy show that the younger the patient is, the higher the chance they have at recovering full ovarian function and maintain a normal fertility window [24]. This was further shown in Chow et al.'s [8] comparison since the fertility pattern was similar to the

Table 2 A summary of the estimated gonadotoxicity of regimens currently in use for the most common childhood cancers

	Cancer subtype	Drugs that are gonadotoxic	Estimated gonadotoxicity risk in female
Leukaemia	Acute lymphoblastic leukaemia (first line)	Cyclophosphamide or ifosfamide	Low – Medium
	Acute lymphoblastic leukaemia (relapse)	Cyclophosphamide Total body irradiation	Medium – High Very high
	Acute myeloid leukaemia	Nil	Low
	Acute myeloid leukaemia (relapse)	Nil	Low
Lymphoma	Non-Hodgkin's lymphoma (low risk)	Cyclophosphamide	Low
	Non-Hodgkin's lymphoma (standard risk)	Cyclophosphamide	High
	Non-Hodgkin's lymphoma (high risk)	Cyclophosphamide	High
	T-cell non-Hodgkin's lymphoma	Cyclophosphamide	Low – Medium
	B-cell non-Hodgkin's lymphoma	Cyclophosphamide	Medium – High
	High-risk B cell	Cyclophosphamide	Medium – Very high
	Hodgkin's lymphoma	Cyclophosphamide ± dacarbazine	Low – Medium
		Cyclophosphamide + dacarbazine + Procarbazine	Very high
Brain tumours	Ependymoma	Cyclophosphamide + Cisplatin	High – Very high
	Medulloblastoma	Cyclophosphamide + Cisplatin ± Lomustime	Very high
	Pineoblastoma	Cyclophosphamide	Medium
	Atypical teratoid/rhabdoid tumour	Cyclophosphamide + Ifosfamide	Medium
		Cyclophosphamide + Ifosfamide + Thiotepa (no radiotherapy)	Very high
	High-grade glioma	Temozolomide	High
	Intracranial germ cell tumour	Ifosfamide	Low
		Ifosfamide Cisplatin	High – Very high

(continued)

Table 2 (continued)

	Cancer subtype	Drugs that are gonadotoxic	Estimated gonadotoxicity risk in female
Bone and soft tissue	Ewing's sarcoma	Ifosfamide ± Cyclophosphamide Busulphan Melphalan	Very high
	Osteogenic sarcoma	Cisplatin ± Ifosfamide	High – Very high
	Soft tissue sarcoma (low risk)	Nil	Low
	Soft tissue sarcoma (standard risk)	Ifosfamide	High – Very high
	Soft tissue sarcoma (high risk)	Ifosfamide ± Cyclophosphamide	Very high
	MMT	Ifosfamide ± Cyclophosphamide	Very high
	Synovial sarcoma	Ifosfamide	High – Very high
	"Adult-type" soft tissue sarcoma	Ifosfamide	Very high
	Neuroblastoma (low risk)	Cyclophosphamide ± Cisplatin	Medium
	Neuroblastoma (intermediate risk)	Cyclophosphamide ± Cisplatin	High
	Neuroblastoma (high risk)	Cyclophosphamide Cisplatin Busulphan Melphalan	Very high
Wilms tumour	Wilms tumour (low risk)	Nil	Low
	Wilms tumour (high risk/ metastatic)	Cyclophosphamide + pelvic radiotherapy	High – Very high
	Wilms tumour (relapse)	Cyclophosphamide ± Melphalan	High – Very high
Others	Hepatoblastoma	Nil	Low
		Cisplatin ± Carboplatin	High – Very high
	Retinoblastoma	Nil	Low
	Langerhans cell histiocytosis	Nil	Low
		Fludarabine + Melphalan	High
	Extracranial germ cell tumour	Nil	Low
		Cisplatin ± Ifosfamide Vinblastine	High – Very high
BMT	Allogenic bone marrow transplant	Cyclophosphamide busulphan/ Melphalan/Treosulphan	Medium – High
	Allogenic bone marrow transplant	Total body irradiation	Very high
	Allogenic bone marrow transplant	Fludarabine	Low

Adapted from CCLG Oncofertility Consensus Document [7]

control, suggesting that there is an age-specific correlation between the chemotherapeutic insult and the number of primordial follicles that survived.

Conclusion

Consequently, current cancer treatment for some childhood cancers may be gonadotoxic and predispose to POI. Counselling is paramount as undergoing cancer therapy is in general emotionally traumatic. Doctors need to support their patients, but most importantly, before starting treatment they need to discuss the age-specific impacts that the planned treatment is likely to have on the ovaries and HPG axis, and, if indicated, the potential available options for fertility preservation.

Fertility Preservation

Fertility preservation is an emerging field of research into options for maximising the reproductive potential for survivors of cancer. Although most techniques are aimed at post-pubertal women, more recent methods may give pre-pubertal girls the opportunity to preserve their fertility. See figure one for a summary of the currently available established and experimental fertility options for girls and young women.

Ovarian Shielding and Transposition

Non-pharmacologic approaches to protect ovarian function have been used in the clinical environment for many decades. However, these techniques require specific criteria as it can only be utilised for females undergoing pelvic radiation. Nevertheless, if the approaches are executed correctly, they can successfully protect the ovary. One example of this approach is ovarian shielding which involves shielding the ovary during radiotherapy. However, the ability to use this technique depends on the location of the tumour. If the tumour resides in approximation to the ovary, then shielding will not be possible. Improving radiotherapy delivery and techniques which include the development of proton therapy replacing photon radiation may decrease radiation exposure to organs at risk including the ovary [3].

Another potential technique to decrease radiation exposure of the ovaries is ovarian transposition out with the planned radiotherapy field. This laparoscopic surgery is done before cancer treatment and is particularly applicable to the management of cervical cancer if radiation treatment is planned.

GnRH Analogues

A more pharmaceutical approach of fertility preservation is to manipulate th0e HPG axis, suppressing ovarian function with GnRH analogues. The philosophy behind this is to inhibit GnRH release and create a pre-pubertal hormonal environment. GnRH analogues have been proven effective in women undergoing chemotherapy for breast cancer [21]. Only 8% of the women with the analogue had ovarian failure compared to 22% in the chemotherapy-only group [21]. The differences highlight the potential effectiveness of GnRH analogues as a way of protecting ovarian function during chemotherapy treatment for breast cancer. Concerning successful pregnancy outcome, more offspring were born to women in the GnRH analogue compared to the chemotherapy-alone group (21% versus 11%), proving that ovarian function is protected and fecundity level remains similar to pre-treatment [21]. In a recent systematic review and meta-analysis, Lambertini et al. [22] provided evidence for the efficacy and safety of temporary ovarian suppression with GnRHa during chemotherapy as an available option to reduce the likelihood of chemotherapy-induced POI and potentially improve future fertility in premenopausal patients with early breast cancer [22].

A key advantage of this technique is that it is accessible and cost-effective. However, GnRH

analogues do cause menopausal-like symptoms, which can cause great discomfort.

Cryopreservation

Cryopreservation is a process that freezes organelles using vitrification as the main procedure because it is fast and inexpensive compared to the former procedure, slow freezing [18]. Cryopreservation can lead to long-term preservation of cells notably oocytes and embryos. However, these latter approaches are only eligible for post-pubertal women; therefore, an emerging technology is ovarian tissue cryopreservation in order to preserve fertility in prepubertal girls [4].

Embryo Freezing

The most established technique of cryopreservation for fertility preservation is embryo freezing. Ovaries are hyper-stimulated in order to increase the number of ovulatory mature eggs. These are collected and fertilised with partner or donor sperm to produce an embryo. The embryo is then vitrified for future use after the cancer treatment. However, ethical, religious, and social issues are associated with embryo freezing. Additionally, given that a sperm is required to freeze an embryo, there is shared ownership and both biological parents need to consent in order to use this embryo [3]. Therefore, given the criteria, this technique may be useful for couples that are in a relationship or single women who are willing to use sperm donor.

Egg Freezing

Given the limitations and disadvantages of embryo freezing, egg or oocyte freezing was developed which offered women the opportunity to preserve both their fertility and reproductive autonomy as they would have sole responsibility for their egg freezing. Given the requirement for hormonal stimulation and oocyte collection, only post-pubertal women are eligible to cryopreserve their oocytes.

Oocyte cryopreservation comprises daily gonadotrophin injections to stimulate multiple follicles to grow. The oocytes are retrieved and frozen prior to cancer treatment. However, this methodology has a few limitations. Firstly, vitrification negatively affects the oocyte physiology as it may induce osmotic stress [18]. Secondly, hyper-activation of the HPG axis may risk the patient developing ovarian hyper-stimulation syndrome. This complication is due to the build-up of human chorionic gonadotropin-causing ovarian inflammation.

After treatment, artificial reproductive technologies must be used for pregnancy with cryopreserved eggs which may not appeal to all women as these procedures may be expensive and are both emotionally and physically difficult. Data on cancer patients undergoing oocyte cryopreservation remains limited. However, Druckenmiller et al. [14] vitrified oocytes from women with malignant cancers and they had similar fertility rates as non-cancer patients, concluding that long-term freezing or cancer does not impair oocyte quality or function. The main determinant for successful fertilisation is oocyte quality; therefore, Cobo and Diaz [9] compared the quality of fresh egg and thawed/ vitrified egg. There was no difference in fertilisation, embryo quality, and implantation between the two groups, proving to be an effective way of preserving fertility and potentially allowing survivors to have a family after treatment [9].

Tissue Freezing

Embryo and oocyte cryopreservation are only applicable to post-pubertal women, for prepubertal girls ovarian tissue cryopreservation (OTC) remains experimental [4, 36]. OTC involves laparoscopically either an ovariectomy or obtaining 3–5 ovarian cortical strips, removing approximately 70% of the ovarian cortex, cut into fragments and cryopreserved for future use [4]. Cancer treatment can start soon after this process with minimum delay.

OTC for restoration of fertility remains widely regarded as an experimental procedure [26, 30]. In our view, patients having OTC should be counselled and their data should be collected in a research context to improve our understanding of the success rate and complications of this novel

approach. This is particularly important for children and younger adolescents where proxy consent from parents and carers is the usual practice. It remains important to avoid unnecessary procedures of uncertain benefit in unwell children at a time of extreme stress to the patient and their parents and carers. To provide a structure for discussions surrounding whether or not to proceed with ovarian tissue cryopreservation, we have in Edinburgh developed a risk assessment tool, dividing the key issues into those intrinsic to the patient and those extrinsic [36].

Transplantation of frozen/thawed ovarian tissue will lead to normal restoration of hormonal secretion and the menstrual cycle within 20 weeks in the vast majority of patients [1]. An excellent recent review of ovarian tissue cryopreservation from Donnez and Dolmans in the NEJM [11] reports in excess of 130 live births following of frozen thawed ovarian tissue in an orthotopic site. While the denominator of the number of unsuccessful re-implantations remains unknown, there is good evidence that OTC and re-implantation at a later date is effective in adults.

Demeestere et al. [10] reported the first live birth from a woman who underwent ovarian tissue cryopreservation at the age of 13 due to sickle cell anaemia. A few months after transplantation, ovarian activity was present with a normal menstrual cycle and a spontaneous pregnancy occurred 4 years following treatment [10]. Although her diagnosis was not cancer, it still provides the possibility of using pre-pubertal cryopreservation as a fertility preservation technique. Additionally, it highlights that this method is successful in achieving a pregnancy and transplantation restores ovarian function. Whilst pregnancy would suggest normal ovarian function, the ovary is an endocrine organ as well; therefore, induction of puberty would also indicate full restoration of the ovary. A 9-year-old girl diagnosed with Ewing's sarcoma underwent ovarian tissue cryopreservation prior to treatment [15]. This tissue was transplanted back into the patient, and after 4 months, FSH levels were low and oestradiol increased [15]. The following year after transplantation, the patient had menarche and a regular menstrual cycle, indicating that the thawed tissue had restored normal ovarian function [15]. Consequently, this demonstrates that cryopreservation of ovarian tissue can restore both ovarian functions.

Although this approach may be promising for fertility preservation in pre-pubertal girls, there is, however, the risk that the ovarian tissue may contain malignant cells; hence, there is the possibility of transplanting the tumour back into the patient after treatment [12]. This is of particular risk in haematological and disseminated cancers notably leukaemia [13].

In summary ovarian tissue cryopreservation is increasingly available for fertility preservation in children at high risk of developing POI. However, more research is required into optimal patient selection, minimising the risk of contamination by malignant cells and in vitro maturation protocols; nevertheless, there are emerging data as to its efficacy.

Conclusion

Preservation of gonadal function is an important priority for the long-term health of cancer survivors of both sexes and all ages at treatment. Loss of opportunity for fertility is a prime concern for female cancer survivors. Some fertility preservation techniques, such as embryo cryopreservation, are established and successful in adults, and development of oocyte vitrification has greatly improved the potential to cryopreserve unfertilised oocytes. All approaches to fertility preservation have specific challenges in children and teenagers, including ethical, practical, and scientific issues. For young women, cryopreservation of ovarian cortical tissue with later replacement has resulted in at least 130 live births [11] but is still regarded as experimental in most countries. For females, these approaches involve an invasive procedure and have an uncertain risk of tissue contamination in haematological and other malignancies. Decision making for all these approaches needs assessment of the individual's risk of fertility loss and is made at a time of emotional distress. Development of this specialty needs better provision of information for patients

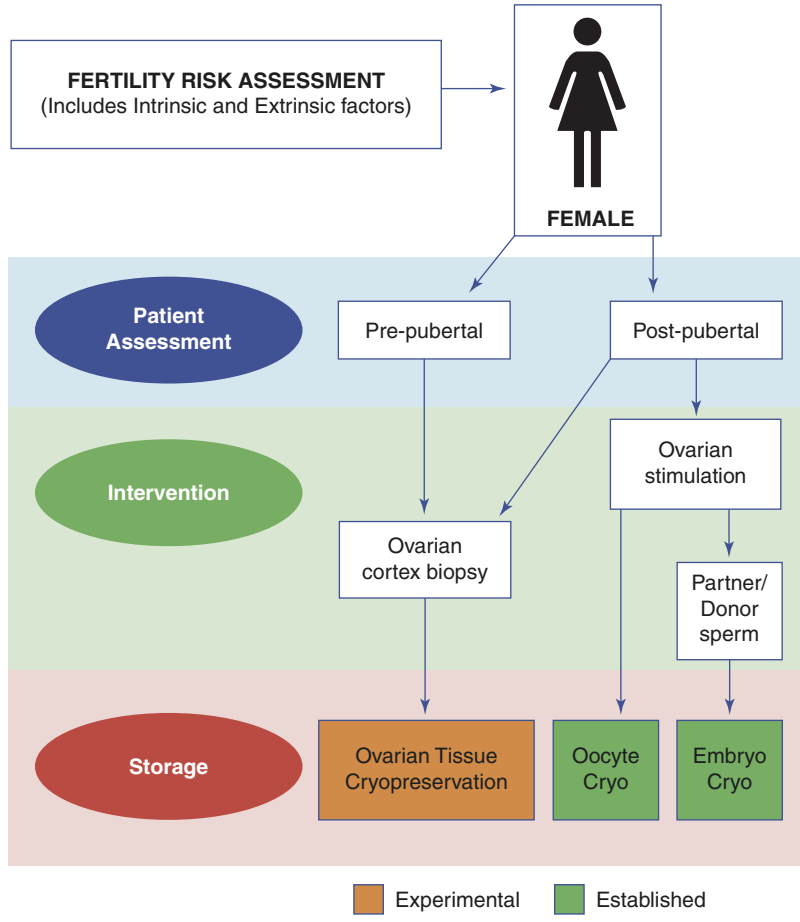

Fig. 1 Ovarian tissue cryopreservation within the wider context of fertility preservation for girls and women. Cryo: cryopreservation. Adapted from Anderson et al. [3, 4]

and their medical teams, and improvements in service provision, to match technical and scientific advances (Fig. 1).

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ART in Cancer Survivors

Elizabeth Ginsburg and Maren Shapiro

Introduction

Fortunately, over the past three decades, advances in cancer treatment have led to significant improvements in survival rates. Unfortunately, many cancer treatments can have permanent effects on reproductive function. With increased survival, there has rightfully been increased attention on survivorship and quality of life, including fertility preservation options [1, 2]. Studies have consistently shown that fertility is important to cancer survivors. In an online survey of young adult cancer patients, 65% had used or would like to have been given information on infertility risks and future reproduction options prior to undergoing treatment [3]. Likewise, in a survey of cancer survivors 3–7 years after treatment, most who had a pretreatment desire for children still wanted children after treatment, and inability to become pregnant was associated with worse mental health and distress [4]. This chapter will explore the relationship between cancer and its treatment on fertility, as well as the use of assisted reproductive technologies (ARTs) to treat the unique population of cancer survivors.

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Cancer and Fertility

Normal Reproductive Function

In order to understand the causes and treatments of infertility, it is important to review the basics of normal reproductive function. In men, normal reproductive function is regulated by the hypothalamic–pituitary–gonadal (HPG) axis. Pulsatile gonadotropin-releasing hormone (GnRH) is released from the hypothalamus in the brain, acting on the anterior pituitary, which subsequently releases follicle-stimulating hormone (FSH) into the bloodstream. FSH travels to the testes and binds to specialized receptors on the Sertoli cells, signaling the release of androgen-binding protein, which initiates spermatogenesis.

In spermatogenesis, specific germ cells in the walls of the seminiferous tubules, called spermatagonia, divide by mitosis to regenerate themselves. A certain portion of them undergo meiosis as well, developing into haploid spermatids, which in turn transform into spermatozoa, which are released into the seminiferous tubules and eventually are transported in semen. Spermatogenesis begins at puberty, and once established, continues to produce spermatozoa throughout a man's adult life, tightly regulated by the feedback cycles of the HPG axis.

FSH also stimulates the Sertoli cells to produce and release inhibin B into the bloodstream, which acts as a negative feedback mechanism to

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inhibit GnRH secretion and thus FSH production. Simultaneously, pulsatile GnRH acts on the anterior pituitary, stimulating the release of luteinizing hormone (LH) into the blood stream. LH in turn acts on the testicular Leydig cells to produce testosterone and on the Sertoli cells to further support spermatogenesis. Millions of sperm are generated daily in the mature male testes, and unlike oocytes, sperm are a renewable resource. Any disturbances in the HPG axis or damage to the spermatogonial germ cells can lead to a decrease in sperm quantity and quality and impairments in fertility.

In women, reproductive function is slightly more complicated and involves the HPG axis, the ovaries, ovarian follicles, and the uterus. Similar to men, the HPG axis regulates reproductive function and the process of oogenesis. The hypothalamus produces GnRH, which signals the release of FSH and LH from the pituitary. These act on the ovaries—specifically on the granulosa and theca cells—to stimulate follicles to grow (in the case of FSH) and to release estradiol (in the case of LH). With a similar negative feedback loop, estrogen then signals to the hypothalamus to reduce the release of GnRH.

In the ovaries, oogenesis begins during fetal development, starting with ovarian stem cells, or oogonia, which divide by mitosis during fetal development, but eventually arrest in meiosis I as primary oocytes prior to birth. In this way, women are born with a non-renewable store of follicles in their ovary, which continuously decline throughout life, starting at about two million in infancy, decreasing to approximately 400,000 by puberty and ultimately reaching roughly 1000 at the time of menopause. When girls reach puberty, the activation of the HPG axis leads to a triggering of ovulation each month. A surge in LH from the anterior pituitary triggers a resumption of meiosis in a primary oocyte, which forms a secondary oocyte that arrests again at metaphase II. That oocyte is released from the ovary during ovulation into the fallopian tube, and ultimately completes the final stage of meiosis immediately after fertilization by a sperm, where the newly formed zygote travels to the uterus and implants.

The term ovarian follicle refers to the developing oocytes and their supporting cells, which support oocytes through the process of oogenesis. Primary oocytes are maintained in small primordial follicles, which are made up of a small layer of granulosa cells. After puberty, every so often a few primordial follicles are recruited to develop into primary follicles. As the granulosa cells surrounding the primary follicles proliferate and divide, they turn into secondary follicles and gain a new outter layer of theca cells, which work along with the granulosa cells to produce estrogens. These follicles continue to grow and develop until they become tertiary follicles, or antral follicles, which ultimately house and expel secondary oocytes during ovulation. While a number of tertiary follicles may develop, the majority of them will undergo atresia and typically only one will develop to the point of ovulation.

In both men and women, there are a number of ways in which cancer and cancer treatment can disrupt normal reproductive function and have an effect on fertility. Cranial treatments can lead to HPG-axis disruption centrally and impaired hormonal signaling, while pelvic and gonadal treatments can lead to decreased or damaged gamete supply, and damage to the uterus can lead to impaired implantation and pregnancy complications.

The Impact of Cancer on Fertility

Cancer itself can have a negative effect on reproductive function and fertility. For example, in men with Hodgkin's lymphoma, semen parameters tend to be poor even prior to treatment, suggesting an intrinsic effect of the systemic illness on fertility [5–8]. Potential explanations for this decline include elevated stress hormones, impact on the HPG axis, and even immune-mediated imbalance [9].

In women, the direct impact of cancer on fertility is less clear. Studies examining how cancer patients respond to IVF stimulation protocols prior to cancer treatments have shown mixed results, with some early studies demonstrating

worse ovarian response [10, 11], and other more recent studies reporting no difference compared with healthy women [12–14]. The most recent study, a 2012 meta-analysis of seven retrospective studies, found that the number of oocytes retrieved after ovarian stimulation was significantly lower in women with cancer compared with age-matched, otherwise healthy women undergoing IVF [15]. When it comes to ovarian reserve parameters, women with cancer prior to gonadotoxic therapy have been found to have lower AMH and AFC values compared to healthy controls [16, 17]. More research is needed to clarify these disparate findings.

Women who carry BRCA mutations represent a unique population in whom fertility concerns should be examined. BRCA 1 and 2 are genes involved in double-stranded DNA repair, and women who carry mutations in these genes have long been known to be at significantly increased risk for breast and ovarian cancer [18]. Newer research suggests that these same mutations may make oocytes more prone to DNA damage, leaving BRCA 1/2 carriers with diminished ovarian reserve, earlier menopause [19–21], and accelerated primordial follicle loss [22]. Not all studies fully support these findings [23], however, and more research is still needed to clarify the role of BRCA in fertility.

The Impact of Cancer Treatments on Fertility

It is very difficult to predict how treatments will ultimately affect an individual patient's fertility—elements such as age at time of treatment, genetic background, type of cancer, medical history, and baseline fertility all factor in.

Radiation

Radiation is a targeted cancer therapy that uses gamma rays, x-rays, and other sources of radiation to destroy cancer cells by breaking up molecules and causing DNA damage. Radiation has known effects on spermatogenesis, the HPG axis, the ovaries, and the uterus. Generally, radiation targets actively dividing cells, but the exact effect depends on dose, site, duration of exposure, frequency or treatments, and age/reproductive function at time of treatment [24].

Radiation is known to have profound effects on uterine function and can compromise the ability of the uterus to support future pregnancies. Notably, radiation can reduce uterine volume and elasticity, damage musculature and endometrium, and decrease vasculature. In girls treated for childhood cancer, whole-body radiation has been shown to impair uterine blood flow and growth, with reduction in uterine size of roughly 60% of that of an adult woman. Ultimate uterine volumes appear to correlate with the age at which radiation was received, and damage may be partially ameliorated by estrogen replacement [25].

Not only can radiation negatively impact embryo implantation, but if a pregnancy successfully takes hold, a history of radiation therapy has been associated with a host of adverse pregnancy outcomes, including abnormal placentation (accreta-spectrum disorders), preterm birth, and intrauterine growth restriction. There has even been a case report of spontaneous uterine rupture at 17 weeks in a patient who received whole-body radiation as a child [26]. Finally, pelvic radiation can cause vaginal fibrosis and stenosis, which can lead to dyspareunia and resultant sexual dysfunction [27].

Cranial radiation for brain tumors has been shown to damage the hypothalamic–pituitary axis and cause endocrine dysfunction regardless of whether treatment occurs in childhood or adulthood [28]. In one series, 61% of patients who received brain radiation for brain tumors showed evidence of hypogonadism, while 70% of premenopausal women became oligomenorrheic [29].

Because oocytes are arrested in prophase of meiosis I, they are more resistant to radiation than actively dividing cells, and the impact of radiation on the ovary depends on both age and dose of radiation. The dose of radiation required to kill 50% of immature human oocytes (also known as the LD50) has been calculated at less than 2Gy [30], but the effective sterilizing dose (ESD) of radiation appears to vary with age, with a decreasing ESD with increasing age at

Table 1 Predicted age at ovarian failure based on age at treatment and dose of radiation

	Dose of radiation (Gy) Mean age of predicted ovarian failure (95% CI)			
Age at treatment (years)	3 Gy	6 Gy	9 Gy	12 Gy
1	35.2 (31.3–39.1)	22.9 (19.0–26.8)	14.3 (10.4–18.2)	8.7 (4.8–12.6)
5	35.8 (31.9–39.7)	24.4 (20.5–28.3)	16.6 (12.7–20.5)	11.6 (7.7–15.5)
10	36.7 (32.8–40.6)	26.5 (22.6–30.4)	19.7 (15.8–23.6)	15.3 (11.4–19.2)
15	37.8 (33.9–41.7)	28.8 (24.9–32.7)	23.0 (19.1–26.9)	19.0 (15.1–22.9)
20	39.0 (35.1–42.9)	31.4 (27.5–35.3)	26.4 (22.5–30.3)	22.8 (20.0–26.7)
25	40.6 (36.7–44.5)	34.2 (30.2–38.1)	29.8 (25.9–33.7)	26.5 (25.0–30.4)
30	42.2 (38.3–46.1)	37.0 (33.1–40.9)	33.2 (30.0–37.1)	30.1 (30.0–34.0)

Adapted from Wallace et al. 2005, Table 1

treatment. This is likely because prepubertal and adolescent girls have a greater number of primordial follicles and thus more robust ovarian reserve [27]. In a 2005 paper, Wallace et al. reported a model that predicts the age of ovarian failure after treatment with varying doses of radiation, which is often used by reproductive endocrinologists to counsel their patients on post-treatment reproductive potential [31]. Based on this model, the ESD at birth is 20.3 Gy; at 10 years 18.4 Gy; at 20 years 16.5 Gy; and at 30 years 14.3 Gy (Table 1).

In males, radiation to the pelvis can cause damage to both the Leydig cells and the germ cell spermatagonia, leading to hormonal disruption and impaired spermatogenesis respectively, although it appears that Leydig cells are more resistant [27]. Germ cell damage occurs in a dose-dependent fashion with radiation, with doses of 1–3 Gy producing reversible azoospermia and doses >3 Gy producing permanent azospermia [32]. Interestingly, unlike female gonads, male gonads appear to be more sensitive to radiation damage in the prepubertal stage. In prepubertal boys treated with <12 Gy of radiation, testosterone production will likely be normal, but elevated levels of LH suggest subclinical damage. In sexually mature males, on the other hand, Leydig cell function is typically preserved up to cumulative radiation doses of 30 Gy [33]. Pelvic radiation not only leads to germ cell and Leydig cell dysfunction, but it can also cause nerve damage, which can lead to anejaculation and erectile dysfunction [27].

Chemotherapy

Unlike radiation and surgical resection which are targeted, chemotherapy is a systemic treatment that impacts most parts of the body. It can be used alone or in conjunction with radiation and surgery depending on the type of cancer and degree of spread. Chemotherapeutic agents are typically classified into a few classes based on mode of action: alkylating agents, antimetabolites, plant alkaloids, and antitumor antibiotics [34]. Frequently, these agents are used in conjunction to take advantage of their various anticancer effects.

Chemotherapy alone does not appear to impact uterine function, but primarily effects the gonads, with some impact on central hormone regulation from the hypothalamus and pituitary [35]. Factors influencing the degree of damage include cumulative dose, the specific agent used, the length of treatment, and the age at treatment [27].

Alkylating agents seem to have the highest risk for infertility, with a well-established dose-related risk of gonadotoxicity. Studies that have tried to assess outcomes typically use the alkylating agent dose (AAD), which is based on a dose score that calculates a patient's cumulative dose per square meter for each alkylating agent used, dividing exposure into tertiles of the study population [27]. Because of this, the ADD is unique to the distribution of drug doses within a specific study population, so cannot be used for comparisons across populations. Green et al. have proposed a new system, called the cyclophosphamide equivalent dose (CED), and validated this tool in

using the Childhood Cancer Survivor Study (CCSS) cohort, which may be an easier tool for patient counseling as it can be directly compared between different cohorts [36]. When using literature that uses the AAD to counsel patients on their individual risk, it is important to correlate their cumulative dose exposure with the tertiles used in a specific study [27, 37–39].

The ovary is highly chemosensitive, with certain types of chemotherapies causing more damage than others. The ovary can be damaged both at the level of the primordial follicles, which is indicative of a patient's ovarian reserve, and at the level of estrogen production [40, 41]. Alkylating agents, such as cyclophosphamide and busulfan, tend to be more toxic than platinum-based chemotherapy, plant alkaloids, and antimetabolites, because they cause DNA damage regardless of cell-cycle stage and thus are more likely to target primordial follicles and reduce ovarian reserve, although both alkylating and nonalkylating agents can harm the ovaries ability to produce estrogen [40].

Like with radiation, age at treatment is an important predictor of the retention of ovarian function, with younger girls more likely to retain ovarian function [27]. Unfortunately, studies show that even if young girls treated with chemotherapy who initially retain ovarian function, which is quite common, they are still at risk for acute ovarian failure (defined as loss of ovarian function within 5 years of diagnosis) and premature menopause (defined as cessation of menses before 40 years old). Long-term follow-up of the multicenter Childhood Cancer Survivor Study (CCSS) cohort found that 6.3% of all participants developed acute ovarian failure, with risk factors being treatment with procarbazine, cyclophosphamide, or high-dose radiation (especially over 10 Gy) [42]. The risk for premature menopause in female survivors was 8% overall compared to only 0.8% in sibling controls—increasing alkylating agent score, increasing radiation dose, and diagnosis of Hodgkin lymphoma were all risk factors [43].

In males, the testes are particularly sensitive to the damaging effects of chemotherapy because of the rapidly dividing germ cell population [44]. Like in women, the most damaging regimens are alkylating agents, which can lead to permanent oligospermia or azoospermia within 90–120 days of treatment. Platinum-based treatments, on the other hand, typically impact spermatagonia and spermatocytes rather than germ cells, so while most men will see a transient decline in semen parameters, the majority will have a return of function within 5 years [44]. Similarly, antimetabolites—like 5-fluorouracil, methotrexate, gemcitabine, and 6-mercaptopurine—also tend to have only a transient effect on spermatogenesis. Chemotherapy can also cause damage to Leydig cell function [27], resulting in low testosterone. Similar to radiation, prepubertal boys appear to be more sensitive to permanent damage [45].

Although traditionally thought to have little impact on the central HPG axis when used without radiation, studies now suggest that chemotherapy alone may in fact have a negative effect on central hormonal regulation. In a review of 31 patients with childhood cancer who received chemotherapy alone without radiation, 81% had some sort of hypothalamic–pituitary dysfunction, ranging from growth hormone deficiency, central hypothyroidism, precocious puberty, and/or gonadotropin deficiency, all of which can lead to fertility issues later in life [46].

Patients undergoing hematopoietic stem cell transplantation for leukemias represent a unique population with a significantly increased risk for gonadal dysfunction. Typically these patients require pretreatment with total body irradiation and/or high-dose alkylating agent chemotherapy, which as discussed previously, can be highly damaging to both germ cells and gonadal stromal tissue. Moreover, typically stem cell transplant is reserved as a second-line treatment for relapsed or refractory cancers, so most patients have been previously treated with chemotherapeutic agents or radiation, which increases their cumulative dose and risk for permanent damage [27].

Surgical Treatment and Fertility-Sparing Surgical Options

For many cancers, surgical excision is a central part of treatment. When these malignancies occur in the reproductive tract—such as is the case for

testicular, ovarian, endometrial, and cervical cancer—the surgeries themselves can be sterilizing. In patients for whom future fertility is important, fertility-sparing surgeries may be an option.

Almost half of all cervical cancer patients are of reproductive age, many of whom have not yet finished childbearing [47]. For women with precancerous lesions [cervical intraepithelial neoplasia (CIN)], the standard of care is a loop electrosurgical excision procedure (LEEP), where a portion of the cervix and transformation zone is removed. Studies have shown that at baseline women with cervical dysplasia appear to be at slightly higher risk for preterm birth than those without disease [48], but when looking at women with a history of cervical dysplasia, one LEEP procedure does not appear to impair fertility [49] or put women at higher risk for preterm birth [50, 51]. The standard treatment for early-stage cervical cancer is a radical hysterectomy, meaning removal of the uterus, cervix, fallopian tubes, and surrounding tissues, which can often be curative without the need for adjuvant chemotherapy or radiation. For certain women with low-risk histology, very early-stage disease less than 2 cm in size (stage IB1 or less), fertility preservation surgery with either a cervical conization, simple trachelectomy (removal of just the cervix), or a radical trachelectomy (removal of the cervix with surrounding parametrial tissue) depending on the extent of disease may be reasonable options [52, 53].

Cervical conization procedures are associated with significantly increased risk for preterm delivery, preterm premature rupture of membranes (PPROM), and possible high risk for cesarean section, low birth weight, and cervical injury at time of delivery [48, 54]. Trachelectomy is a much more invasive procedure than cervical conization and involves removal of a significantly larger portion of the cervix along with surrounding tissue in the case of a radical trachelectomy. Because of the more invasive nature of the surgery, pregnancy complications are more common as well. One of the most common complications from radical trachelectomy is cervical stenosis, with one small retrospective study reporting rates as high as 33%, which can lead to difficulty conceiving and require ART [53, 55]. That same study reported that roughly half of women who tried to conceive after radical trachelectomy required fertility treatment, and 30% of conceived pregnancies resulted in miscarriages before 24 weeks. In larger meta-analyses, live birth rates have been reported as 64–70% and rates of cancer recurrence appear low, at less than 5% [52, 56]. Given the potential fertility and pregnancy complications associated with fertility-sparing surgery for cervical cancer, all women—especially those undergoing radical trachelectomy—should consider a preop consultation with an infertility or maternal–fetal medicine specialist and most experts recommend waiting 6–12 months after a procedure before trying to conceive [57].

Endometrial cancer is the most common gynecologic malignancy in the United States, with over 40,000 cases diagnoses each year, and while most of these cases occur in postmenopausal women, 14% occur in premenopausal women who may want to maintain their fertility [58]. With rising rates of obesity, these numbers are only increasing [59]. The standard treatment for endometrial cancer is total hysterectomy with bilateral salpingo-oophorectomy, but for women with grade I stage I disease confined to the endometrium, treatment with progestins may be an option, albeit certainly not standard of care [58]. Patients should be counseled extensively on the risk for undiagnosed advanced spread, failure of hormone only treatment, and the need for close surveillance. While studies of fertility rates in this population are limited due to the very small sample size, systematic reviews have estimated pregnancy rates of 35% and live birth rates of 28–40% [60, 61]. Aside from their cancer, many women in this population will face difficulty conceiving due to associated comorbidities, such as obesity, polycystic ovarian syndrome, and chronic anovulation, and a majority will require some sort of ART to conceive [58].

There are a wide spectrum of malignancies that affect the ovary, with varying prognoses depending on type. Fortunately, borderline tumors, early-stage tumors, and germ cell tumors are more common in women of reproductive age

and have higher 5-year survival rates than cancers typically diagnosed in older women (78.8% for those <30 years old at time of diagnosis vs. 35.5% for those >60 years old) [62]. The typical management for the majority of ovarian cancers is removal of the uterus, cervix, fallopian tubes, and both ovaries, but according to the National Comprehensive Cancer Network (NCCN) guidelines, fertility-sparing surgery with a unilateral salpingo-oophorectomy is a reasonable alternative for women who want to preserve fertility and have early-stage disease and/or a low-risk tumor (early-stage invasive epithelial tumors, borderline tumors that lack stromal invasion, malignant germ cell tumors, mucinous tumors, or malignant sex cord–stromal tumors) [63].

In the case of borderline tumors, if there is bilateral ovarian involvement but none elsewhere in the abdomen after complete surgical staging, ovarian cystectomy may be acceptable management, but patients should be counseled that recurrence rates are estimated at 30% for unilateral tumors managed with cystectomy and 70% for bilateral tumors managed with cystectomies [64, 65]. In terms of future fertility, in a study of 535 women with borderline tumors managed with fertility-sparing surgery (either unilateral salpingo-oophorectomy or cystectomy), the 15-year overall cumulative incidence of first pregnancy was 84.6% and did not differ based on type of surgery (cystectomy vs. oophorectomy) or surgical approach, although they did find that each additional surgery after the first, either for recurrence or surveillance, reduced the likelihood of pregnancy by roughly 40% [65]. In a meta-analysis of 10 studies involving 626 women with borderline tumors, 58% of those who achieved a pregnancy had a live birth, and while recurrence rates were relatively high at 18%, the overall survival rate was 99.8%, suggesting that conservative management poses minimal mortality risk [56].

Ovarian germ cell tumors are a unique subset of ovarian malignancies because the median age of diagnosis is 19, the majority of patients have stage I disease, and the standard of care is actually fertility-sparing surgery with removal of the affected ovary followed by chemotherapy (typically with vincristine, doxorubicin, and cyclophosphamide) with cure rates of 90–95% [56]. In a multicenter study of 105 patients with ovarian germ cell tumors managed with fertility-sparing surgery, 42 of 45 patients who desired child birth achieved pregnancy with 40 of them ultimately having a live birth [66]. In a meta-analysis of fertility outcomes of 515 patients from seven studies, pooled live birth rates were 80% with recurrence rates of 10% and death rates of 4% [56].

In the case of testicular cancer, treatment typically involves orchiectomy (removal of the affected testes) along with radiation and/or chemotherapy. Removal of one testes may initially impact spermatogenesis, but sperm counts typically recover within a year [67]. Outside of sperm count, orchiectomy does appear to impact fertility, but only in a minimal way. In a study of long-term survivors of testicular cancer, 41% of men who had an orchiectomy and no other treatment had an elevated FSH and 11% had low testosterone. Despite this, fertility rates were still high—of the 39% who tried to conceive, 85% were successful without the use of infertility treatments [68].

Unfortunately, especially in the pediatric population, in order to avoid more radical surgery, many organ-sparing surgical procedures are combined with systemic chemotherapy, which can have its own independent impact on fertility [68]. Surgery can also impact fertility in a more indirect way by causing autonomic nerve damage or vascular injury, especially after pelvic or spinal surgery, leading to sexual dysfunction, which is only exacerbated by androgen or estrogen insufficiency [27].

Psychosocial Impact

Not only can cancer directly impact fertility, it can also have psychological ramifications that can indirectly have an effect. Women who have premature ovarian failure may develop menopausal symptoms—like vaginal dryness and decreased libido—that can make intercourse painful and lead to sexual dysfunction. Moreover, physical body changes after surgeries like mastectomies and orchiectomies or hair loss after chemotherapy can lead to a loss of self-perceived attractiveness and even identity [68–70]. Studies have shown that compared with infertile patients

without a cancer history, cancer survivors with infertility demonstrate significantly greater sexual dysfunction and depression and lower physical quality of life scores, with unmet informational needs about reproductive options exacerbating this distress [71].

Fertility in Cancer Survivors

In recent years, more research focus has been placed on survivorship and quality of life of pediatric cancer survivors, including longer term health effects, like fertility and early menopause. A key factor allowing these studies to be done was the establishment of the Childhood Cancer Survivor Study (CCSS) database, a cohort that includes more than 35,000 five-year cancer survivors from 26 Canadian and US institutions who were younger than 21 at the time of diagnosis (between Jan 1, 1970, and Dec 31, 1986) as well as more than 5000 sibling controls [72]. A number of recent studies have used this cohort to examine pregnancy and infertility rates in cancer survivors.

One analysis found that men were less likely to father a pregnancy than their siblings (HR: 0.56, 95% CI -0.49–0.63), with poor prognostic factors including testicular radiation doses >7.5 Gy, higher cumulative alkylating agent dose score, and treatment with cyclophosphamide or procarbazine [39].

In another analysis, Green et al. found that the relative risk of pregnancy among female survivors was lower than their female siblings (RR 0.81, 95% CI 0.73–0.90)—cranial radiation doses of >22–30 Gy, pelvic radiation doses of >5 Gy, a summed alkylating agent dose score of 3–4, and treatment with lomustine or cyclophosphamide were all associated with decreased likelihood of pregnancy [38]. In a separate analysis, Levine et al. found that female cancer survivors had a more than ten-fold increased risk for non-surgical premature menopause [73]. Treatment combination appears to have an additive effect—in that same cohort, treatment with both alkylating agents and abdominal/pelvic radiation dramatically increased risk for premature menopause to close to 30% [43].

Barton et al. examined 3531 survivors of pediatric cancers and 1366 female sibling controls and found that compared with their siblings, survivors were at an increased risk (RR 1.48, $p < 0.0001$) of self-reported clinical infertility (>1 year of failed conception), which interestingly was more pronounced in early reproductive ages (RR 2.02 for those <24 years, 1.61 for those 25–29 years, and 1.37 for those 30–40 years), suggesting a temporal treatment-related impact on fertility. Factors associated with infertility included uterine radiation and exposure to alkylating chemotherapy. Roughly 70% of survivors saw a doctor for infertility, but only 41% of those with an identifiable cause of infertility were given medication to help them get pregnant, compared to nearly 75% of controls (RR 0.57, 95% CI 0.46–0.70), which raises concern for a provider bias against treating cancer survivors. Reassuringly, although survivors had a greater time to pregnancy than their healthy siblings, more than 60% of those with infertility ultimately achieved a successful pregnancy [74]. As can be seen by Barton's study, while cancer treatments certainly increase a woman's risk for infertility, they by no means guarantee infertility.

Pregnancy Outcomes in Patients with a History of Cancer

Pre-Pregnancy Screening

All women attempting to conceive after a diagnosis of cancer should have a maternal–fetal medicine consultation prior to conception and most often will need to be followed by a maternal–fetal medicine physician during their pregnancy due to the high risk nature of their pregnancies. A maternal–fetal medicine specialist can also determine how extra-reproductive effects of chemotherapy may affect pregnancy risk. For example, some chemotherapy agents—such as doxorubicin, trastuzumab (Herceptin), or bevacizumab (Avastin)—can have cardiotoxic effects, so it may be necessary for women to receive a cardiac evaluation with an echocardiogram and EKG prior to pregnancy. Likewise, radiation therapy

can cause damage to blood vessels, leading to cardiovascular complications, so women treated with chest wall radiation for breast cancer may need pre-pregnancy cardiac screening. Women with cervical cancer who had conization or trachelectomy are at higher risk for preterm delivery due to cervical insufficiency [75] and should have cervical lengths checked early in pregnancy and may even need a cerclage. Finally, women with pelvic or abdominal radiation are at risk for a number of pregnancy complications, including abnormal placentation, preterm labor, fetal growth restriction, and even gestational diabetes, so may need more frequent monitoring during pregnancy [76, 77].

Timing of Pregnancy

The timing of pregnancy after cancer treatment has not clearly been established and depends on many patient specific factors, including the cancer type, treatment type, and prognosis. Most experts generally recommend waiting to conceive until at least 2 years after remission is confirmed as the risk for recurrence is highest in the first two years after diagnosis. One large retrospective study found that women who conceived less than 1 year after starting chemotherapy for any cancer had higher risks for preterm delivery than matched women without cancer. Interestingly, that risk was entirely ameliorated for women who waited at least a year after chemotherapy alone or 2 years after chemotherapy and radiation. When looking at cervical cancer survivors specifically, the risk for preterm delivery largely persisted, likely a side effect of surgeries like LEEPs and cone biopsies used to treat the disease, but did appear to be lower for those who waited more than a year after diagnosis to conceive [78].

Tamoxifen and Timing

In premenopausal women with a history of early breast cancer, adjuvant treatment with daily tamoxifen has been shown to significantly improve survival by about one third. Currently, the best evidence available suggests that women should stay on tamoxifen for 5–10 years [79, 80]. In a small series of women who became pregnant while taking tamoxifen, relatively high rates of congenital anomalies were reported, including ambiguous genitalia, Goldenhar's syndrome, and cleft palate. Because of these potential teratogenic effects, it is generally recommended that women wait 3 months after stopping tamoxifen before trying to conceive [81]. Waiting until the completion of adjuvant endocrine therapy prior to conceiving would leave many women out of their reproductive window. Therefore, after careful discussions with their oncologists, some women will opt to temporarily stop tamoxifen, or other endocrine therapies, in order to conceive. Between the 3-month washout period, the time it takes to conceive, pregnancy itself, and breastfeeding, the time off of treatment is roughly 2 years per child. In order to decrease the time to pregnancy, it may be reasonable to consider infertility treatments, like IVF, in women coming off tamoxifen sooner than you otherwise might. Currently, there is limited data on just how much, if at all, this break in tamoxifen impacts long-term recurrence risk. The Pregnancy Outcome and Safety of Interrupting Therapy for Women with Endocrine Responsive Breast Cancer (POSITIVE) trial, a clinical trial aimed at answering this question, is currently underway and actively recruiting at the time of publication of this chapter (NCT02308085). It is looking at breast cancer-free intervals in women who stop their endocrine therapy for up to 2 years to allow for pregnancy, delivery, breastfeeding, or failure to conceive.

Infertility Evaluation

It is important that female cancer survivors who have undergone chemotherapy undergo appropriate cardiac and pulmonary testing, depending on chemotherapy that was used, prior to conception attempts. We suggest a pre-conception consultation with a maternal–fetal medicine specialist to discuss potential pregnancy risks specifically related to a patient's history. Patients who are carriers of cancer-causing genes should be offered

IVF with preimplantation genetic testing if they wish to avoid having a child who carries their mutation. We suggest infertility evaluation for couples who are unable to conceive within 6 months after cancer treatment. While the return of menstruation is a promising sign of a functioning HPA axis, it does not guarantee that pregnancy will occur due to impacts on other aspects of the reproductive tract discussed previously (i.e., uterus, thyroid, egg quality and age, etc.). The evaluation itself, however, is not markedly different from evaluations in the general population.

History and Physical

Any infertility workup should start with a thorough history, including prior obstetric history, menstrual history (both before and after cancer treatment), family history (including those with known infertility, early menopause, birth defects, developmental delay, genetic mutations, or stillbirth), social history, and thorough medical and surgical history. In women who were premenopausal prior to cancer treatment, complete amenorrhea, irregular menstrual cycles, hot flashes, vaginal dryness, and other symptoms typical to menopause can be signs of ovarian failure, but are not definitive.

Ovarian Reserve Testing

The cornerstone of the female infertility workup, especially in those with prior cancer treatment, is an assessment of ovarian reserve, which reflects oocyte, quantity, and in the setting of ART, response to ovulation induction medications and therefore reproductive potential [82]. There are a number of tests for assessing ovarian reserve available, but none are perfect, so often a combination of tests is used. Anti-Mullerian hormone (AMH) is a hormone produced by the granulosa cells of growing ovarian follicles and can help indicate the size of the pool of growing follicles in the ovary, with lower levels correlating to fewer follicles. It is tested using a blood test that does not vary significantly with the day of the menstrual cycle on which it is drawn [83]. In women with infertility, low AMH levels (less than 1.0 ng/mL) are a useful marker for predicting poor or no response to ovarian stimulation, but is not, however, a sensitive or specific predictor of future pregnancy [82].

AMH is the most studied marker in women who have prior cancer treatments, and a number of studies have shown that AMH is probably the best predictor of diminished ovarian reserve in cancer survivors [84–87], especially in conjunction with antral follicle count (AFC) [35, 88]. AFC is the number of 2–10-mm-diameter follicles measured using transvaginal ultrasound. It is a surrogate measure of the number of dormant primordial follicles in the ovary and has a strong correlation with AMH levels [89, 90]. Importantly, AFC and AMH correlate with the ovarian primordial follicle number independent of chronological age, suggesting that they portray a more specific assessment of ovarian age [91].

While AFC and AMH are probably the best markers of ovarian reserve, other tests are often used in infertility evaluations and can contribute differing information. Follicle-stimulating hormone (FSH) is often used as a marker of menopausal status, with levels in the menopausal range (as defined by the laboratory) in combination with irregular menses prior to age 40, signifying primary ovarian insufficiency (POI) [92]. Borderline FSH levels, typically defined as 10–20 IU/L, can also be used as markers of ovarian reserve and have been shown to have a high specificity for predicting who will respond poorly to ovarian stimulation, but sensitivity is very low and reliability is limited due to high inter- and intra-cycle variability [93]. Cycle day 3 estradiol in and of itself is a poor marker for ovarian reserve, but in conjunction with day 3 FSH can be used to correctly interpret FSH values. As discussed earlier, elevated estradiol levels suppress FSH production. As ovaries age, serum estradiol levels rise in the early follicular phase, which leads to a suppression of otherwise elevated FSH levels, leading to a misinterpretation of the test as "normal." Therefore, normal FSH values in the setting an elevated day 3 estradiol (>60–80 pg/mL, depending on the assay used) is suggestive of diminished ovarian reserve, even in women with normal menstrual function [82].

Other Diagnostic Assessments

Although diminished ovarian reserve is a common finding in women previously treated for cancer, a complete fertility evaluation includes an assessment of tubal patency and the uterine cavity, ovulatory function, and the male partner.

Fallopian tube patency can be assessed in a number of ways. The first is a hysterosalpingogram (HSG), which involves injecting contrast dye through the cervix and using X-ray to assess for flow of dye through the bilateral fallopian tubes. Similarly, hysterosalpingo contrast sonography (HyCoSy) involves distending the uterine cavity with shaken saline and using transvaginal ultrasound to assess the cavity integrity and tubal patency by looking for bubbles flowing through the tubes. If a laparoscopy is being performed for any reason, chromopertubation looks for tubal patency by injecting dye (typically methylene blue) through the cervix and looking for flow through the tubes on laparoscopy. The benefits of an HSG and HyCoSy are that they allow for both assessment of tubal patency and evaluation of the uterine cavity for structural abnormalities (such as fibroids, polyps, or adhesions), whereas chromopertubation requires the addition of hysteroscopy to assess cavity integrity.

Assessing ovulatory function involves determining whether a woman is ovulating. Regular menstrual periods are a good sign of regular ovulatory function; however, ovulation can be confirmed with diagnostic tests, especially in those with irregular cycles. A mid-luteal phase serum progesterone level > 3 ng/mL (obtained roughly 1 week prior to expected menses) is considered evidence of recent ovulation [94]. If progesterone is not appropriately elevated, then a workup for anovulation should be obtained, which includes assessment of serum prolactin, thyroid-stimulating hormone (TSH), FSH, and androgens to assess for polycystic ovarian syndrome (PCOS).

Endometrial Evaluation

In women who have premature ovarian failure after cancer treatment who have been exposed to abdominopelvic or total body irradiation, it is critical to ensure that the endometrium is capable of responding to estrogen priming. Some women and couples are potentially interested in donor egg, but are not interested in or financially capable of having another woman carry a pregnancy for them; it is therefore our practice to perform a "prep cycle." This entails placing a woman on physiologic doses of estradiol, which can be administered orally, vaginally, or transdermally. Ideally the endometrial stripe will thicken to 7 mm or greater. At this time, progesterone is begun and we perform an endometrial biopsy to ensure secretory transformation. In some patients, the endometrial stripe does not reach 7 mm even with different estrogen preparations, but secretory transformation appears adequate and "in phase" based on Noyes criteria [95]. More data are needed to determine if pregnancy and live birth rates are notably reduced in those patients who decide to pursue treatment with donor eggs.

Male Fertility Evaluation

In men, fertility evaluation is much more straightforward and is centered around semen analysis. Semen analysis is performed on an ejaculated semen sample and is composed of ejaculate volume and pH, sperm concentration, count, motility, and morphology. If abnormal, a repeat semen analysis should be repeated at least 1 week later, due to normal variation in sperm concentration and semen characteristics.

Genetic Evaluation

Generally the risk of cancer in offspring of cancer survivors is not increased [96, 97] unless the cancer is a part of an inherited cancer syndrome, such as Li-Fraumeni, BRCA 1 or 2, or Lynch syndrome. In such cases, depending on the inheritance pattern, there can be as high as a 50% chance of passing the syndrome on to offspring, and patients should be counseled on this risk prior to conceiving. Preimplantation genetic testing (PGT) can be used to test embryos for the specific familial mutation in question and only implant those that are not affected, thereby preventing passage of the mutation onto the next generation. PGT is not without its own set of risks, however, and developing the specific probe can take months and additional cost to patients.

Additionally, while PGT is generally accepted as a preventative measure to reduce the risk of passing on monogenic diseases that present early in life, such as cystic fibrosis or Tay–Sachs disease, there has been debate as to whether the technology is appropriate to use in the case of adult-onset disease genes, like the inherited cancer syndromes. In 2013, ASRM released a committee opinion that the use of PGT in such a case should be ethically allowable, but the decision is certainly an individual one [98]. All patients who are known carriers of a heritable cancer syndrome should be offered a consultation with a genetic counselor to discuss options and determine whether preimplantation genetic testing is something they want to pursue.

Infertility Treatment

In Vitro Fertilization

For certain patients with a history of cancer and infertility, trying less invasive methods of ART prior to resorting to in vitro fertilization (IVF) may be appropriate, such as ovulation induction with clomid, letrozole or gonadotropins, or intrauterine insemination (IUI). However, for most, moving directly to IVF is more efficient and is associated with reduced time to conception [99]. IVF can be used to treat a number of causes of infertility, including tubal factor, severe male factor, diminished ovarian reserve, ovulatory dysfunction, and unexplained infertility. Ovarian failure and uterine factor may also be treated with ART, although may require the use of donor oocytes and a gestational carrier, respectively.

Controlled ovarian hyperstimulation using exogenous FSH allows for multiple follicles to develop at once. When these follicles are felt to be mature, based on ultrasound and hormone monitoring, a trigger injection of hCG or GnRH-agonist is used to initiate ovulation and hopefully the production of multiple mature oocytes. The process of stimulation takes roughly 2 weeks. Stimulation protocols are typically determined by the policy and preferences at any given infertility center, and most often are based on ovarian reserve markers, cause of infertility, and other health complications that might necessitate specific timing.

Next, 34–36 h after trigger administration, oocyte retrieval takes place, which is typically performed vaginally under transvaginal ultrasound guidance using intravenous general anesthesia. In patients with complex anatomy, such as those who have had ovarian transposition prior cancer treatment, transabdominal or even laparoscopic approaches may be required.

Oocytes are then combined with sperm in a small volume of culture media and incubated to achieve fertilization in vitro. For patients with severe male factor infertility or in women with low egg numbers, direct injection of the sperm into the oocyte may be performed in a process called intracytoplasmic sperm injection (ICSI), which is discussed later in this chapter. After fertilization, embryos are incubated until either the cleavage stage (roughly day 3) or the blastocyst stage (day 5), at which point they are either transferred back into the woman's uterus, transferred into the uterus of a gestational carrier, biopsied and tested for genetic disease, or frozen for use at a later time.

Ovulation Induction in Cancer Survivors

After chemotherapy, female cancer survivors often have decreased ovarian reserve and even without exposure to chemotherapy, women with malignant disease may have a lower oocyte yield after stimulation [15]. For these reasons, "poor responder" protocols are often chosen. "Poor responder" is a term used to describe women who despite a large dose of medication, mount low estradiol levels, and produce few oocytes, although the specific criteria used are often varied [100]. There are a number of strategies used in this population to increase response to stimulation, including altering pituitary downregulation with varying doses/timing of GnRH agonists or antagonists, modifying ovarian stimulation by varying dose/timing of gonadotropins, or adding adjuvant treatments such as oral contraceptive

pills or steroids. A Cochrane review from 2010 found insufficient evidence to support the routine use of any particular intervention for the management of poor responders [101], so protocol choice is typically left up to the preference of the provider.

The older standard ovarian stimulation protocol, often referred to as the "GnRHa long protocol," uses GnRHa started in the mid-luteal phase or early follicular phase of the cycle before to suppress pituitary function, allowing exogenous gonadotropins to be administered to stimulate follicular growth in a more controlled manner. There is a fear by some, however, that GnRH agonists may be harmful to poor responders by binding to GnRH receptors on the ovary [102]. GnRH also has been shown to decrease ovarian blood flow, which may impact the distribution of gonadotropins to the ovaries [103] and may result in lower responses than alternative protocols. Various protocol alterations have been proposed to minimize or even eliminate GnRHa use.

In a "stop protocol," GnRHa is stopped in the early follicular phase after adequate pituitary downregulation has been achieved, thus decreasing the total dose of GnRHa used [104, 105]. In a GnRH antagonist protocol, a GnRH antagonist is used for pituitary suppression instead of a GnRH agonist. The benefit of a GnRH antagonist is that it abruptly inhibits pituitary function, so only a few days of treatment are required to suppress the endogenous LH surge, rather than multiple weeks. Typically in such protocols, a GnRH antagonist (i.e., Ganirelix, Cetrotide, Cetrorelix, Orgalutron) is added when follicles have reached roughly 12–14 mm in size and is continued until the trigger injection is administered [106].

In a GnRHa flare protocol, a GnRH agonist (i.e., Lupron, Buserelin, Nafarelin, etc.) is administered at the same time as ovarian stimulation with gonadotropins. This protocol takes advantage of the initial agonistic response of GnRH on the pituitary to provide additional stimulation to the growing follicles [107]. A "microdose" protocol has also been proposed, where lower doses of GnRHa are used, typically 40 mcg leuprolide acetate twice daily, to minimize exposure to GnRHa as described above [108, 109].

Besides altering the type of pituitary suppression, protocols that alter the amount of stimulation have been proposed as being beneficial for poor responders. Although somewhat counterintuitive, studies have found that in women with low ovarian reserve higher doses of fertility drugs do not actually produce more oocytes. "Mini stimulation" or "mild stimulation" protocols use either weaker medications (such as clomiphene citrate or letrozole) or lower doses of gonadotropins in the stimulation phase with the goal of producing only a few eggs, but saving cost and medication exposure [110].

Luteal estrogen priming has also been proposed as a way to overcome the natural phenomenon of follicular heterogeneity, to allow for more synchronized follicular growth and a greater mature oocyte yield at the time of trigger administration. Follicular heterogeneity is thought to be due to varying sensitivity of follicles to FSH—some follicles are more sensitive to FSH stimulation and thus are able to start their growth during the late luteal phase when only low levels of FSH are present, whereas the less sensitive follicles do not start growing until the start of the next cycle [111]. Estrogen administered in the luteal phase of the preceding cycle inhibits FSH release, resulting in a more homogenous follicle pool at the start of the next cycle [112].

Ovarian Stimulation in Women with Estrogen-Sensitive Cancers

Controlled ovarian stimulation with gonadotropins typically results in supraphysiological levels of estradiol, which can be of theoretical concern in women with a history of estrogen-sensitive cancers, such as endometrial cancer or estrogen receptor (ER-)positive breast cancers. A number of potential solutions have been proposed to avoid this problem in patients undergoing fertility preservation prior to cancer treatment, including natural-cycle IVF, stimulation with tamoxifen, and stimulation with the addition of aromatase inhibitors [113]. However, studies of pregnancy in cancer survivors do not show that pregnancy, despite very high estradiol and progesterone levels, is associated with a higher risk of recurrence or second cancers. As noted previously, it is

important to ensure that the patient has been approved by her oncologist for pregnancy prior to starting evaluation and treatment of infertility. We do not treat cancer survivors who plan to carry a pregnancy any differently during their IVF treatment.

Outcomes of IVF Treatment in Cancer Survivors

Despite a widely reported increased risk for infertility in cancer survivors, there are very few studies looking at IVF outcomes in this population. Of those that have been conducted, most suggest an impaired response to stimulation and lower pregnancy rates *(see table below for details)* [14, 114–119].

In 2001, Ginsburg et al. found that women undergoing IVF after systemic chemotherapy had worse response to gonadotropins and lower delivery rates than women with locally treated cancers, despite being younger [117]. Similarly, in 2012, Barton et al. found that despite being prescribed more aggressive stimulation protocols, women undergoing IVF after receiving systemic chemo or radiation therapy had fewer oocytes retrieved and a more than three times lower chance of pregnancy and live birth compared to other infertility patients [118]. Moreover, they were five times more likely to have a cycle cancelled because of poor ovarian response. Interestingly, despite having poor response to stimulation, cancer survivors had comparable day 3 FSH levels to the infertility control cohort, suggesting that FSH does not accurately capture the ovarian damage from treatment and that even those with normal FSH should be considered potentially poor responders. AMH was not routinely used for ovarian reserve testing at that time, but as it has been shown to be the best predictor of response to gonadotropins, presumably would have been lower in survivors than in controls.

In 2016, Luke et al. conducted the largest study to date, a population-based analysis linking state cancer registries in Illinois, Texas, and New York to the US national ART database (SART) [114]. They looked at women with a prior diagnosis of cancer undergoing ART treatment and found that live birth rates were significantly lower for women with cancer using autologous oocytes compared to women without cancer (47.7% vs. 24.7%, $p < 0.0001$). Live birth rates did not significantly vary when donor oocytes were used (roughly 60% for both groups). They did not, however, include information on the type of cancer treatments women had and how that impacted their ART outcomes.

The biggest limitation of the data available is the heterogeneity of the studies performed. Most studies combine patients with different types of cancers, varying amounts and types of treatment, and variable time to treatment, making it hard to generalize the results to any one type of cancer. More quality data is needed to adequately counsel patients. Overall, however, it seems that the data that does exist points toward lower pregnancy and live birth rates in cancer survivors undergoing infertility treatments. Given these poor outcomes after cancer treatment, women should be offered fertility preservation prior to treatment whenever possible to increase their chances of a successful pregnancy (Table 2).

Male Factor Infertility

Men with severe oligospermia may require intracytoplasmic sperm injection (ICSI), in which a spermatozoon is injected directly into an oocyte, allowing for fertilization in a more efficient way. A retrospective study of pregnancy outcomes following ICSI with cryopreserved sperm of both cancer survivors and non-cancer patients found live birth rates in the survivor cohort to be at 62.1%, which was at least comparable, if not better, than the non-cancer population [100].

If men have azoospermia and did not bank sperm prior to treatment, they may be a candidate for testicular sperm extraction (TESE)—a surgical procedure in which sperm are extracted from testicular biopsies. Two small studies of male cancer survivors with azoospermia found that sperm retrieval by TESE was successful in about 39–43% of patients, and rates of success did not

Table 2 Studies looking at in vitro fertilization in cancer survivors

Study	Population	Number of patients/cycles	Number of oocytes/embryos retrieved (avg., min–max)	Pregnancy/live birth rates
Ginsburg et al. [117]	Women undergoing IVF/GIFT after systemic and local cancer treatment	71 fresh cycles (56 local therapy, 15 systemic therapy)	14.5 oocytes, 7.5 embryos (local therapy) 10.3 oocytes, 7.0 embryos (systemic therapy)	14 live births (25% live birth rate) (local therapy) 2 live births (13% live birth rate) (systemic therapy)
Barton et al. [118]	Female cancer survivors after chemotherapy/ radiation	53 women (39 attempted fresh cycles)	8 oocytes (0–36) 4 embryos (0–18)	5 live births/ 39 attempted fresh cycles (12.8% live birth rate)
Das et al. [14]	Women who had undergone chemotherapy prior to IVF treatment	14	4.5 oocytes (2–7) 71.4% fertilization rate	Not reported
Chan et al. [115]	Women with a history of chemotherapy for cancer or autoimmune disease	35 women (35 fresh cycles)	10 oocytes (2–27) 58.6% fertilization rate (0.5–100%)	Not reported
Luke et al. [114]	Women with cancer history undergoing ART (SART database)	441 women (393 using autologous oocytes)	Not reported	28.8% pregnancy rate; 24.7% live birth rate (85.8% live birth per conception)
Su et al. [116]	Female lung cancer survivors after targeted therapies: Crizotinib (anaplastic lymphoma kinase inhibitor) or denosumab (RANKL antibody)	2 women (3 cycles)	10.6 oocytes (7–17) 2 embryos (0–4)	1 live birth of twins (100% live birth rate per embryo transferred)

differ significantly by age, serum FSH, or time from chemotherapy. Moreover, of those who were able to retrieve sperm, pregnancy and live birth rates with IVF/ICSI were high (50–64% and 42–59%, respectively) [120, 121].

Donor Egg

For patients who were unable to preserve gametes prior to gonadotoxic treatment and are left with complete ovarian insufficiency or severe azoospermia, donor gametes—either oocyte or sperm—can be used. Occasionally, even women with diminished ovarian reserve who carry a dominant hereditary cancer syndrome, such as a BRCA mutation, may opt for donor egg IVF rather than going through preimplantation genetic testing, knowing that roughly 50% of the oocytes they retrieve will be affected.

Typically, the process begins with identifying a donor, either someone known to the couple trying to conceive or an anonymous donor. In the United States, donors are often compensated anywhere from $2500 to $10,000. Younger donors are preferred (<34 years old) for higher success rates and screened for physical and mental health and risk for infectious diseases. The donor undergoes ovarian stimulation (as described above), typically using a stimulation protocol that minimizes the risk for OHSS. When the oocyte is recovered, it is fertilized with sperm provided. To be able to implant the fresh embryo into the recipient, her menstrual cycle is synchronized with the donor's stimulated cycle using a combination of estrogen and progesterone. With improvements in cryopreservation technologies in recent years, egg banks are also an option and can simplify the process by obviating the need for cycle synchronization.

The benefits are that donor gametes can allow for a child to be at least partially biologically related to the couple, and as long as there is no uterine compromise, can give female cancer survivors with ovarian compromise the ability to carry

the pregnancy. The downsides are that they do not allow for complete biological parenthood and donor oocytes can still be quite costly for patients. In some states, however, a large proportion of the cost of donor egg is covered for female cancer survivors who have significantly decreased ovarian reserve and who are less than 40 years old [122]. These insurance companies cover the cost of the donor's ovarian monitoring and egg retrieval, and the uterine preparation and embryo transfer for the recipient. Screening the donor and compensating the donor are not covered. For women purchasing previously banked donor eggs, which require ICSI, the cost of ICSI and uterine preparation are covered, but not the cost of the eggs.

Even in women who do not appear to have diminished ovarian reserve, there may be a benefit to using donor oocytes. A large population-based cohort study of women with a history of cancer treated with ART in three US states found that live birth rates for women undergoing IVF with autologous oocytes within 5 years of a cancer diagnosis had a significantly lower chance of live birth than women undergoing IVF without a history of cancer (47.7% vs. 24.7%, $p < 0.0001$). The live birth rates with autologous oocytes also differed significantly by cancer diagnosis, with women with a history of melanoma having the highest live birth rates at 53.5% and those with breast cancer having the lowest live birth rates at 14.3%. Interestingly, when donor oocytes were used, live birth rates were comparable, regardless of cancer diagnosis or type [114].

Gestational Carriers

For women with uterine factor infertility, such as those who underwent hysterectomy for endometrial or cervical cancer or those who underwent pelvic radiation, the use of a gestational carrier may be the only option for biological parenthood. Gestational carriers can even be an option for women with no uterine compromise if there are concerns about the stress of pregnancy on a woman's body, such as in those who have severe cardiac disease from chemotherapy, or for those who need to continue long-term treatments, such as breast cancer survivors on tamoxifen. The biggest limitation of surrogacy is the high patient cost, which is unlikely to be covered by insurance, as well as potential ethical concerns surrounding coercion and exploitation of potential carriers. Laws surrounding gestational carriers vary by state and typically require close assistance from a reproductive law specialist.

Finally, traditional adoption of nonbiological children is always an option that can be considered and has the benefit of not relying on a patient's natural fertility. Unfortunately, adoption is not a guarantee—it remains expensive and competitive in the United States and adoption agencies are not always welcoming to cancer survivors. Although there are theoretical legal protections preventing the discrimination against cancer survivors, qualitative studies in the United States have shown reluctance among adoption agencies to consider cancer survivors as potential adoptive parents and both formal and informal discrimination throughout the process [123, 124].

Conclusions

Infertility is one of the most common and distressing long-term complications of cancer treatment [27]. Fortunately, advancements in assisted reproductive technologies, such as fertility preservation, and our understanding of how cancer treatments impact normal reproductive function have led to an era where future fertility is a possibility for many survivors. Treating infertility in cancer survivors is complex and certainly requires an interdisciplinary approach, but with close collaboration by oncologists, surgeons, maternal–fetal medicine physicians, and reproductive endocrinologists can be safe and effective.

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Fertility-Sparing Strategy in Ovarian Tumors

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Introduction

Conservative and functional surgery is increasingly used in surgical oncology. Its aim is to preserve organs' functionality and reduce radical resection. Development of new surgical procedures in oncologic gynecologic surgery is a perfect example of this evolution. Although radical surgery remains the gold standard in the treatment of ovarian cancer, a conservative approach can be considered in patients with early stages disease in order to preserve their fertility function. These procedures were proposed to selected patients, depending on histologic subtypes and prognostic factors. Ovarian cancers are classified into epithelial (including borderline and malignant tumors) and non-epithelial cancer.

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Borderline Ovarian Tumors (BOT)

Taylor described low malignant potential ovarian tumors for the first time in 1929. This ovarian disease is defined as lacking overt stromal invasion at histological examination; it is characterized by a less aggressive behavior than invasive epithelial ovarian tumors [1]. The two main histological groups are mucinous and serous borderline tumors, the latter being the most frequent in Europe and North America [2]. Unlike mucinous diseases, serous borderline tumors are bilateral in 15–25% and associated to extraovarian diseases in 25–35% in the form of "implants" [2, 3]. During three decades, peritoneal implants were divided into noninvasive (exhibiting no evidence of "destructive stromal" invasion) and invasive (showing invasion of the underlying tissue or solid nests), with the morphological aspects being very close to those observed in patients with peritoneal carcinomatosis from low-grade serous carcinoma (LGSC). In 2014, the classification of gynecological tumors has been revised, calling the "previous" serous "borderline tumors" (or "low malignant potential") "atypical proliferative serous tumors" (APST) [4]. Noninvasive implants are so called because "implants" and invasive implants are now considered as extraovarian LGSC [4].

The previous characterization between both implants had been considered as strategically and therapeutically the most important issue in stage

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II or III APSTs. Anyhow, the crucial therapeutic step is the removal of all peritoneal implants, with surgery being then the exclusive treatment in patients with noninvasive implants (implants) [2, 5]. During the last three decades, adjuvant treatment was considered in patients with invasive implants [2]. Nevertheless, as we had no level A evidence of the efficacy of chemotherapy in this context, surgical resection (without adjuvant treatment) is now considered to be the standard management in patients with invasive implants (extraovarian LGSC) [6]. But the prognosis and outcome of patients with peritoneal spread continue to fuel debate, particularly in respect of both subgroups of peritoneal disease [5, 7–14].

Modalities of Conservative Surgery and Clinical Outcomes

The standard treatment of BOT consisted of a total abdominal hysterectomy and bilateral salpingo-oophorectomy, peritoneal cytology, omentectomy, and multiple peritoneal biopsies. These procedures allowed to perform an adequate staging and eventually propose an adjuvant therapy only in patients with invasive peritoneal implants. The prognosis of BOT is excellent. But late recurrences (after 5 or 10 years) could be observed [2, 15]. Conservative surgery is defined as preservation of the uterus and at least part of one ovary in order to preserve fertility. BOT arise in a young population, where fertility is a major issue. So, the analysis of conservative management data is crucial in such patients. The risk of relapse is increased after this type of surgery. The global recurrence risk is estimated at 13% (10–16% IC 95%) [15]. The rate of recurrence is correlated with the type of conservative treatment used (salpingo-oophorectomy or cystectomy) with a higher rate of between 10 and 42% in patients undergoing cystectomy [15]. Nevertheless, the real impact of performing a cystectomy only (as opposed to a salpingo-oophorectomy) on recurrence rates is a subject of debate, and some authors report similar recurrence rates for both treatments. In the only randomized trial regarding BOT, Palomba et al. reported 32 patients with bilateral BOT were randomized between bilateral cystectomies versus unilateral salpingo-oophorectomy on the largest lesion and contralateral cystectomy [16, 17]. After a follow-up period of 81 months, there was no difference between these procedures in terms of the cumulative recurrence rates. On one hand, the cumulative pregnancy rate and cumulative probability of a first pregnancy were higher in patients treated with bilateral cystectomy compared with unilateral salpingo-oophorectomy and contralateral cystectomy [16, 17]. On the other hand, patients undergoing bilateral cystectomy had a shorter time to first recurrence and a higher rate of radical treatment of the recurrence [16, 17]. This study implies that in the case of bilateral serous BOTs, if technically feasible, bilateral cystectomies should be performed to improve pregnancy rates.

The high rate of relapse implies that the optimal treatment in patients with intraoperative diagnosis of BOT is unilateral adnexectomy, which reduces the risk of relapse. Cystectomy should be performed only in cases of bilateral tumors and/or in patients with only one ovary (previous history of adnexectomy). In case of relapse on the remaining ovary under borderline form, another conservative management (cystectomy) may be proposed to these patients in order to preserve fertility. In that case, complete evaluation must be performed preoperatively including MRI with assessment of possible safe functional ovarian tissue and oncofertility evaluation and consultation to discuss if fertility preservation technique before surgery.

Survival of Patients after Conservative Surgery

Fertility-sparing surgery is associated with a higher recurrence rate compared to radical treatment (bilateral salpingo-oophorectomy), although it does not affect survival rates, because most of these recurrences are of borderline nature and easily cured by a second surgery (possibly conservative). Nevertheless, the outcomes of

Table 1 Literature review of series reporting >50 stage II or III serous borderline ovarian tumors

	N patients	N (%) noninvasive implants	N (%) invasive implants	Median Follow-up (months)	N recurrences		N Deaths	
					Non-invasive implants	Invasive implants	Non-invasive implants	Invasive implants
Bell et al. [3]	56	50 (89)	6 (11)	72	NR		3	5
Seidman et al. [9]	65	52 (80)	13 (20)	99	2	7	1	6
Gerhenson et al. [7, 8]	112	73 (65)	39 (35)	139	14	9	6	6
Zanetta et al. [13]*	53	28 (52)	16 (30)	70	3		0	
Longacre et al. [10]**	113	85 (75)	14 (12)	60	3	4	2	5
Du Bois et al. [14]	155	132 (85)	23 (15)	41***	NR		NR	
Shih et al. [11]*	85	NR	36	44	14		7	
Vang et al. [12]	133	114 (86)	19 (14)	180	18	5	NR	
Maria et al. [5]	212	170 (80)	33 (20)	115	20	8	10	4
Total	**829**	**572**	**176**	**97**	**57**	**33**	**22**	**26**

*All subtypes mixed; ** type of implants in three patients who died uncertain; ***: median Follow-up for the entire series; *NR* not reported

patients treated conservatively for stage II and III disease remain discussed [3, 7–14] (Table 1). A nomogram has been proposed to predict recurrence in patients with early- and advanced-stage mucinous and serous BOT. Surgical procedure (radical vs. fertility sparing) was associated with an increased risk of recurrence, as were International Federation of Gynecology and Obstetrics stage, age at diagnosis, histologic subtype, and completeness of surgery [18]. Nevertheless, the main issue is to evaluate the risk of invasive recurrence, which is at lethal risk.

The risk of progression to invasive carcinoma could be estimated to be 2–3%, mainly in mucinous tumors treated by cystectomy [2]. Due to the higher risk of lethal recurrence with mucinous BOT, it seems logical for mucinous BOT to recommend initial unilateral salpingo-oophorectomy rather than cystectomy, while for serous BOT cystectomy is an acceptable option in the absence of high-risk factor [19].

In the German large series including 950 patients, two-thirds with serous BOT and 30.5% with mucinous BOT, among the 74 patients with relapse, 30% had malignant transformation to invasive ovarian cancer. Five-year progression-free survival and overall survival were 12% and 50%, respectively. Overall, invasive cancer following initial BOT was diagnosed in 2.3% of all 950 patients with confirmed diagnosis [14].

Fertility Results after Conservative Surgery

Pooled estimate for pregnancy rate is after conservative treatment of early-stage BOT 54% [15]. Different factors could have influenced fertility rates. The first one is the type of conservative treatment. As previously mentioned, Palomba's trial demonstrated that the use of cystectomy improves fertility results [16, 17]. This treatment should thus be the preferred option particularly in patients at high risk of bilateral tumors (serous BOT).

The second factor is the age of the patient. Fauvet et al.'s [20] study clearly demonstrated that spontaneous fertility results were worse in patients over 40 years. Trillsch et al. reported in

the large German series that despite favorable survival, young patients of childbearing age with BOT relapsed were at higher risk for disease recurrence [21].

Two other factors that could have a potential impact on fertility rates are the use of a laparoscopic approach and the use of a two- or three-step surgery (initial, restaging, iterative surgeries) [22]. Further studies analyzing these potential factors would be interesting. A model has been proposed to predict live birth rate after fertility-sparing surgery for patients with BOT, including FIGO stage, age at diagnosis, histological subtype, and surgery type [23].

ART is an option for woman with BOT-associated infertility. However, only a few series have reported their experiences [15, 24]. IVF was more often used than simple ovarian stimulation. But we have very few data to confirm the safety of such management in patients with implants at initial management. There is a real need for fertility preservation referral centers associating oncologists and fertility experts who can evaluate conservative management of BOT along with alternative therapeutic options to preserve fertility as well as ART.

Epithelial Ovarian Cancer (EOC)

Indications of Conservative Surgery

Standard surgical procedure in EOC is radical (hysterectomy with bilateral salpingo-oophorectomy).

Di Saia first proposed conservative treatment for EOC in highly selected cases, that is, in patients with fertility desire, willing to undergo close gynecologic follow-up and stage IA, well-encapsulated ovarian cancer without peritumoral adhesions, ovarian capsule lymphatic channels and/or mesovarium invasion, and negative peritoneal washings [25].

In 1994, Colombo et al. and in 1997 Zanetta et al. published the first series specifically dedicated to EOC [26, 27]. Their series comprised 56 patients, and the authors performed conservative treatment in selected cases – stage IA–IC disease (any grade) – with excellent survival (96% 5-year survival rate in Colombo et al.).

An American multicenter study comprising 52 patients was reported in 2002 [28]. In that study, the estimated overall survival of patients with early-stage EOC who underwent conservative treatment was 98% at 5 years and 93% at 10 years. The authors proposed that conservative treatment be performed in stage I EOC of any grade.

In 2005, a French multicenter study reported a series of 34 patients with strict inclusion criteria (systematic review of slides, complete staging surgery, and chemotherapy for patients with stage ≥IC) [29]. The results reported were 1 recurrence out of 13 patients in case of stage I grade 1, and 8 out of 20 patients in case of grade 2 and 3 stage IA to IC.

The study by Park et al. in 2008 comprised 62 patients with EOC, 59 of whom had early-stage disease [30]. Patients with stage IC or grade 3 tumors had significantly poorer survival (5-year survival 88%). The authors reported that FSS could be considered in young patients with stages IA–C, grades 1–2.

A Japanese multicenter study included a total of 211 patients from 30 institutions who underwent conservative treatment for EOC [31]. Five-year recurrence-free survival rates was 97.8% for stage IA with favorable histology (grade 1, grade 2, not clear cell), 100% for stage IA clear cell, 33.3% for stage IA grade 3, 92.1% for stage IC with favorable histology, 66% for stage IC clear cell, and 66.7% for stage IC grade 3. The authors recommended conservative treatment in stage IA disease either with a favorable histological subtype or clear-cell histology, in stage IC only with a favorable histology, but specified that conservative treatment should be avoided in the case of grade 3 tumors.

A large analysis in the Surveillance, Epidemiology and End Results (SEER) database of FSS with preservation of the ovary in stage IA or IC disease seems to confirm the absence of impact on survival rates [32]. Nevertheless, as stated by the authors, “To detect a 20% difference in survival for patients with stage IC disease, a cohort of 1282 pts with 52 deaths is required.”

Therefore, since none of the published series involved such large numbers of patients, it is not possible to conclude definitively about the safety of conservative management in this situation.

The largest series was published by Fruscio et al. in 2013 with an update in 2016 [33, 34]. This Italian retrospective study evaluated 240 patients treated with conservative treatment. Oncological prognosis was the same for patients with radical or conservative treatment. For grade 3, prognosis was worse but did not seem to depend on the type of surgery. The authors concluded that conservative treatment can be proposed to all young patients when the tumor is limited to the ovaries. Distant recurrences were considered more frequent in the case of grade 3 tumors, and the patients should be closely monitored.

The results reported in those studies suggested that such conservative surgery could be safely performed in patients with stage IA, IC grade 1, and probably grade 2 diseases. In patients with stage IA, IC, and grade 3 diseases, the rate of recurrence is increased, but it is impossible to precise if this increased rate is related to the preservation of the uterus itself or the natural history of the high-grade lesion. According to these uncertainties, many teams consider that the use of conservative surgery is contraindicated in stage IA IC grade 3 tumors [35].

Fertility Results

Fertility rates after such management are between 60 and 70%. In case of persistent infertility, ovarian stimulation or IVF remains contraindicated.

Follow-up of patients is based on clinical examination, blood markers, and the use of a systematic imaging (abdomino-pelvic ultrasonography).

Completion of surgery after childbearing (or after 40 in patients who have not been pregnant) remains discussed. Nevertheless, a case of relapsing EOC 10 years after conservative treatment could suggest discussing the removal of the remaining ovary in order to reduce the risk of recurrence on the spared ovary.

Non-Epithelial Ovarian Cancer

Non-epithelial malignant tumors are characterized (compared to epithelial cancers) by [1] the occurrence of disease in younger patients and [2] the (overall) good prognosis of this tumor (even in case of extra-ovarian disease) due, in most of the cases, to an excellent chemocurability of these tumors. They could be classified mainly into two different groups: germ cells tumors (MGCT) and sex cord stromal tumors (SCST).

MGCT

Most papers concerning the results of conservative surgery in non-epithelial cancers concerned this group of tumors [36–38]. Most frequent tumors in this group are dysgerminomas, endodermal sinus tumor (EST), malignant teratoma, and mixed subtypes. Chemotherapy used in such tumors is the "BEP" regimen (bleomycin, etopioside, and cisplatin). Such conservative management of a part of one ovary could be discussed in patients with bilateral involvement of both ovaries (case of teratomas) or in patients with peritoneal disease treated using adjuvant chemotherapy (particularly in dysgerminomas or malignant teratoma) [36, 38]. Risks of bilateral involvement of both ovaries are observed mainly in dysgerminoma and less frequently in teratoma. Biopsies of the contralateral ovary if macroscopically normal are not recommended.

Fertility results are well evaluated and are high, very close to that observed in patients without history of malignancy. Menstruations and endocrine ovarian functions are maintained in a very large majority of these young patients treated using the BEP regimen. In the largest and more recent series from Satoh et al. [39], all patients who underwent fertility-sparing surgery recovered their menstrual cycles. Also, 16 of 23 patients receiving BEP (70.0%) and 13 of 17 patients receiving non-BEP (76.5%) who were nulliparous at fertility-sparing surgery and married at the time of investigation (although these criteria can be criticized) gave birth to 21 and 19 healthy children, respectively.

SCST

Most frequent subtypes of the tumors were granulosa cell, Sertoli–Leydig, and thecal cell tumors. Very few papers are devoted to the conservative management in such tumors (most of them were case report or short series). In the series of Zhang et al. involving the analysis of the SEER database, 376 women treated for SCST, 71 young patients were treated using uterine preservation for stage I disease (75). The survival of patients treated conservatively and radical was similar [40].

Two important characteristics are observed in granulosa tumors and impact the conservative surgery: bilaterality is unusual (between 2% and 8% of bilaterality – 41); such tumors are frequently associated to endometrial disorders (hyperplasia or cancers). So random biopsies of contralateral ovary (if macroscopically normal) are not needed, but uterine curettage should be systematically performed. The overall prognosis of granulosa cell tumor is good in early-stage disease (stage IA) and conservative management could be discussed in young patients with similar stage. But conservative management should not be proposed in higher stage (or if ovarian rupture during the initial surgery) because the prognosis is more reserved.

Gouy et al. recently published a series of 23 patients with Sertoli–Leydig cell tumors (SLCT) and with centralized pathological review of these tumors by two expert pathologists [41]. They also reviewed the literature. The results suggested that, for stage Ia disease, conservative surgery must be proposed in children and in women of reproductive age. The difficulty in managing stage Ia is determining whether to use an adjuvant treatment. The risk of recurrence for stage Ia was around 7.5% (27/362), but the risk of death in case of recurrence was an important 70% (19/27). The rate of recurrence was similar, regardless of the type of surgery (among all cases reported in the literature: 21/265 – 8% in the conservative surgery group and 6/97–6% in the radical surgery group). The prognosis of SLCT is known to be correlated with the FIGO stage, but prognosis also depends on tumor differentiation, the presence of heterologous elements, and the presence of a retiform pattern 1. ESMO guidelines identified poor differentiation and the presence of heterologous elements as indicators of a poor prognosis [42]. Schneider et al. showed that, in addition to those two prognostic factors, the presence of a retiform pattern was a third indicator of a poor prognosis [43].

The prognosis of advanced stage disease (stages II and more severe) is poor; advanced stages are associated with a high rate of death. In the review of the literature by Gouy et al. [41], 19 patients with advanced stage disease were reported. Of these, 14 experienced a recurrence and 11 died. The use of completion surgery after childbearing remains debated in SCST [41].

Conclusions

Conservative treatment gives good results on fertility and does not affect the survival of patients with borderline ovarian tumor. It must be considered for young women willing to conceive even if peritoneal implants are discovered at the time of initial surgery. In case of infertility, medically assisted procreation techniques may be proposed to patients with stage I BOT with a limited number of stimulation cycles.

In patients with epithelial ovarian cancer, conservative surgery of an ovary and the uterus can only be considered in adequately stratified patients, with serous, mucinous, or endometrioid tumor and a careful follow-up and with a stage IA, IC, grade 1 (and probably 2) disease.

In patients with non-epithelial ovarian cancer, conservative surgery has a large place, particularly in patients with malignant germ cell tumors.

Practical Clinical Tips

- Pathologic review of the tumor by an expert pathologist.
- Patient in agreement with a careful follow-up.
- Fully staged patients particularly in epithelial cancer.
- Removal of the retained ovary should be considered only in epithelial cancer.

Take Home Messages

- Conservative treatment gives good results on fertility and does not affect the survival of patients with borderline ovarian tumor.
- In case of infertility, medically assisted procreation techniques may be proposed to patients with stage I BOT with a limited number of stimulation cycles.
- In patients with epithelial ovarian cancer, conservative surgery of an ovary and the uterus can only be considered in stage IA, IC, grade 1 (and probably 2) disease.
- In patients with non-epithelial ovarian cancer, conservative surgery has a large place.

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Ovarian Transposition

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Definition

Ovarian transposition corresponds to the surgical displacement of one or both ovaries from their anatomical location to a situation away from the radiation field for women with cancer requiring pelvic radiation therapy and willing to preserve their fertility or their endocrinal function.

Introduction

In the field of preservation of fertility, ovarian transposition (OT) has been advocated as a solution when ovarian function and subsequent fertility may be impaired by pelvic radiation therapy (PRT) [1]. First described by McCall et al., the objective is to protect one or both ovaries by moving them away from radiation field for reproductive but also endocrine function preservation [2]. The American Society of Clinical Oncology (ASCO) and the National Comprehensive Cancer Network (NCCN) recommend offering OT as an option for optimizing fertility preservation in patients with cancer [3, 4].

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OT is proposed in case of PRT or brachytherapy (BT) that will deliver more than 6 Gy to the ovaries. It is currently considered more as a method of preserving endocrine ovarian function. With the development of other techniques for the preservation of female fertility, its indications have become rarer. Transposition is generally done in the flanks in accordance with the positioning of the irradiation beams and/or the chosen irradiation technique in order to reduce the dose received by the ovaries to less than 2 Gy. The protection is only partial; however, the efficacy to preserve endocrine function is well demonstrated and spontaneous pregnancies or after in vitro fertilization (IVF), with or without repositioning, have been described. The goal of this chapter is to present technical aspects and results of OT for ovarian function preservation in patient with cancer.

Indications

Ovarian transposition is indicated for women from 11 to 40 presenting with pathology requiring PRT and willing to preserve their fertility or their endocrinal function. For women older than 40, the higher risk of decreased ovarian function is not in favor with OT [5]. According to Hoekman et al., OT prior to PRT is effective in women until the age of 35 years and needs to be discussed in patients aged 36–40 years [6]. OT should not be performed

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for women with cancer at risk of ovarian metastases or who will receive chemotherapy agents with a high level of gonadotoxicity that would lead to premature ovarian failure. In the systematic review by Hoekman et al., the main indications for OT were cervical cancer (375/765 patients, 49.0%) and lymphoma (233/765 patients, 30.5%).

Usual indications are

- Gynecological cancer, including [5].
  - Uterine cancer.
  - Ovarian dysgerminomas.
  - Vaginal and cervical cancers (if there is a low risk of ovarian metastasis or recurrence), but in standard practice, that kind of procedure is not recommended for advanced stage due to the risk of ovarian recurrence, and, for early stage, there is no indication for PRT.
- Rectal and anal cancers [7].
- Pelvic lymphoma: Hodgkin and non-Hodgkin tumor in the pelvis.
- Ewing sarcoma of pelvis, osteosarcoma, rhabdomyosarcoma, medulloblastoma.

Surgical Technique

Transposition of the ovaries is used to minimize ovarian follicle exposure to radiation (Figs. 1 and 2). The objective of the procedure is to move one or both ovaries away from the radiation field [8]. Laparotomy or laparoscopic approach is possible, but the procedure is nowadays mostly performed by a minimally invasive approach giving the advantage of a prompt healing, if no open surgery is needed for the surgical treatment of primary cancer [9]. This avoids the postponing of radiotherapy [10].

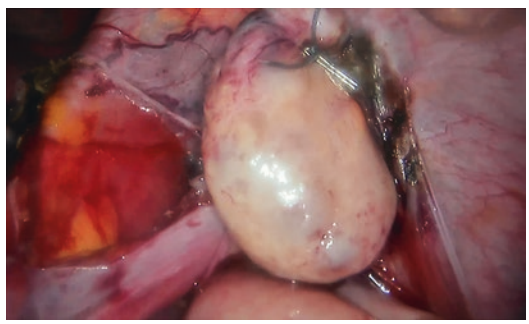

Fig. 1 Laparoscopic view of a right ovary transposed in the right iliac fossa, fixed with a non-absorbable suture and marked with a clip

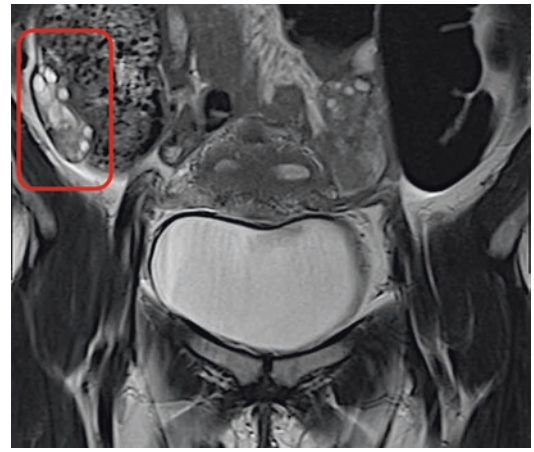

Fig. 2 Magnetic resonance imaging, pelvic coronal view, right ovary after transposition in the right iliac fossa and uterine anterofixation before pelvic radiation therapy for a rectal cancer in a young woman

The transposition could be medial (behind the uterus) or lateral (above the pelvic rim), usually to paracolic gutter [11], depending on the pathology presented by the patient and the planned radiation therapy treatment. Indeed, in case of pelvic cancer, a location above the pelvic brim and as lateral as possible will be adapted, but in case of lymph nodes involvement along the common iliac or para-aortic vessels or in patients with Hodgkin lymphoma, medial transposition should be preferred [12–14].

Several techniques have been proposed. The laparoscopic OT is performed under general anesthesia. The patient is placed in the Trendelenburg position. The trocar placement should consider the need to place ovaries in iliac fossae. Then lateral trocar should be placed in the flanks in an upper place than usual position. The location of the midline incision for the optical trocar varies in the literature, and it could be inserted through the umbilicus or at a more cephalic location.

Laparoscopic OT on the homolateral abdominal side wall, along the homolateral paracolic

gutter, implies the section of the utero-ovarian ligament and to liberation of the ovary from the fimbria. In some situations, the fallopian tubes could be preserved in order to allow spontaneous conception, but better transposition could be achieved when transecting ovaries from mesosalpinx and fallopian tubes. Then, the dissection of ovarian vessels is required to mobilize the ovary. As described by Arian et al., it seems relevant to open parietal peritoneum to create a retroperitoneal tunnel for passing the ovary through in order to prevent the ovarian vessels from taking a sharp turn into the pelvic cavity and thereby preventing alteration of ovarian blood flow. This technique has been reported to have a success rate of 88.6% for preservation of ovarian function. The ovary is fixed with a nonabsorbable suture and marked with a clip that allows its location during radiotherapy, especially given the risk of ovarian migration, the radiation oncologist then needing to adjust the radiation field.

The identification with a clip allows also the correct estimation of the dose received during dosimetry, and ovarian surveillance, required if there is a risk of secondary ovarian localization of the disease. Several video articles that illustrate surgical technique are freely available on the net [15, 16].

Transposition of the ovary into subcutaneous tissue is another option but it is associated with higher risk of cyst formation [17]. Transposed ovaries could by later safely punctured for oocyte retrieval [18]. In certain cases, ovaries could be returned to their original location after irradiation.

Christianson et al. proposed several tips to perform OT. They suggest, for pediatric patients, to associate OT and port placement for chemotherapy under general anesthesia. For older women, if oocyte retrieval has been undergone, to wait 1–2 weeks, as an enlarged stimulated ovary may be difficult to transpose. It seems pertinent to discuss with radiation oncologist before the procedure to determine the best placement according to the planned treatment for the pathology [1].

Gonadal shielding during radiotherapy is a common method when the field of gonads is shielded by lead blocks reducing the expected radiation dose to 4–5 Gy [14]. The minimum free margin is 2 cm in order to reduce the risk of irradiation due to the movement of inner organs.

Unilateral or Bilateral

It is possible to transpose only unilateral ovary, but better results will be achieved with bilateral procedure [19]. In fact, OT has been performed unilaterally in most cases as the transposition of only one ovary was sufficient to insure endocrine function. With the development of cryoconservation, harvesting one ovary may be discussed as the time of contralateral OT in a combined approach. Some teams propose to associate OT and cryopreservation of ovarian cortex that suppose the OT of one ovary and the harvesting of the second one [20]. This strategy should be undertaken with the vision of the expected fertility restoration modalities that should be discussed at the beginning of the management of the disease.

Loss of Function and Radiation Therapy-Related Toxicity

PRT is responsible for a dose- and age-related reduction of the follicular pool.

Iatrogenic premature ovarian failure (POF) is one of severe complications of oncologic treatment, leading to early menopause and infertility. Hormonal depletion and decrease of the number of oocytes are responsible for impairment of fertility potential of the patient. The influence of radiotherapy in case of pregnancy is also pronounced in an elevated risk of abortion and premature delivery and higher risk of intrauterine growth retardation.

POF can be evaluated by several methods: clinical symptoms, measuring FSH (>10 IU/L) and estradiol levels ($\leq$ 80 pg/mL), by calculation of antral follicles at ultrasonographic examination or by evaluation of levels of anti-Müllerian hormone (AMH) [21].

Oocytes are particularly sensitive to radiotherapy [22]. It seems that a dose of 20 Gy

would provoke complete ovarian failure while 2 Gy would be the lethal dose for an oocyte [23]. Ovaries of children are less sensitive to PRT with a dose of 10–20 Gy to observe the loss of at least half of ovarian follicles, whereas this dose would be 4–6 Gy in adults [24–26]. As stated by Hoekman et al., there is a great heterogeneity in radiation techniques (EBRT, brachytherapy or a combination of both techniques, fields arrangement, use of Cobalt or photons, additional chemotherapy, etc.), leading to difficulties to assess ovarian toxicity from a single protocol [13]. Most recent techniques of irradiation, like intensity-modulated radiotherapy (IMRT), may allow to decrease dose applied on transposed ovaries. In their retrospective study on 105 patients with cervical cancer treated by radical hysterectomy and OT before PRT, Yin et al. show that IMRT was likely to make possible normal ovarian function preservation in 39% of cases. In this study, the best threshold for ovarian preservation was less than 9.985 Gy for the maximal dose and less than 5.32 Gy for the mean dose. Best results were obtained for patients younger than 38 years old [27].

Ovaries are sensitive organs to radiotherapy as radiation therapy affects all rapidly dividing cells such as primordial and maturing follicles. Various factors are involved in PRT-related toxicity (volume, total radiation dose, fractionation technique, field arrangement, patient age) [28]. The most important factors are age and dose (Table 1) [29], while there is a clear reduction of necessary sterilizing dose with an increased age [30]. There is also difference based on the fractionation schedule (single dose or repeated). A single dose of radiation is more toxic than multiple fractions of the same total dose [31]. In total body irradiation, fractionated irradiation is less toxic than on single-dose irradiation [31].

Table 1 Sterilizing dose based on the age delivered to the ovaries by radiotherapy (adapted from Lambertini et al. [32])

Age (years)	Dose (Gy)
At birth	20.3
10	18.4
20	16.5
30	14.3
40	6.0

The benefit of OT does not prejudge the effect of radiotherapy on the uterus and its impact on subsequent fertility, with in addition an individual radiosensitivity variation for the same dose. Indeed, outside of endocrine consideration, a fertility-sparing approach implied to preserve both ovarian and uterine function. PRT is associated with reduced uterine volume, impaired uterine distensibility owing to myometrial fibrosis, uterine vascular damage, and endometrial injury [33]. Data to determine a threshold of radiation dose on the uterus that surely compromise successful pregnancy are limited. It seems that the consequence of pelvic radiation therapy starts from 14 Gy with a fibrosis that would be marked above 30 Gy [34]. Data suggest that a dose of 45 Gy would be incompatible with further pregnancy [35]. According to Signorello et al., the risk of preterm birth for children of patients in whom the uterus had been radiated started from 50 cGy and from 250 cGy for low birth weight [36].

In this context, uterine fixation may be discussed in some selected cases (Fig. 3). Indeed, a simple surgical technique may decrease side effects related to radiotherapy in young women wishing sparing their fertility. An anterior uterine fixation can be associated to ovarian transposition and performed by laparoscopy [16]. To decrease the tension between uterus and abdominal anterior wall, a uterine manipulator can be placed inside the uterus to expose the uterus in an anterior position. The assistant operator applies a constant pression on the abdominal wall to avoid any tissue tears during laying sutures. As proposed by Kölher et al., three points of resorbable sutures are sufficient to maintain uterus in contact with the abdominal anterior wall [37]. Uterine fixation could enable to spare uterus from high-dose irradiation to limit it to less than 30 Gy in case of posterior pelvic tumor (e.g., rectal cancer).

With this technique, Köhler et al. published the first case of successful delivery in a 39-year-old patient with anal cancer after fertility-preserving surgery followed by primary

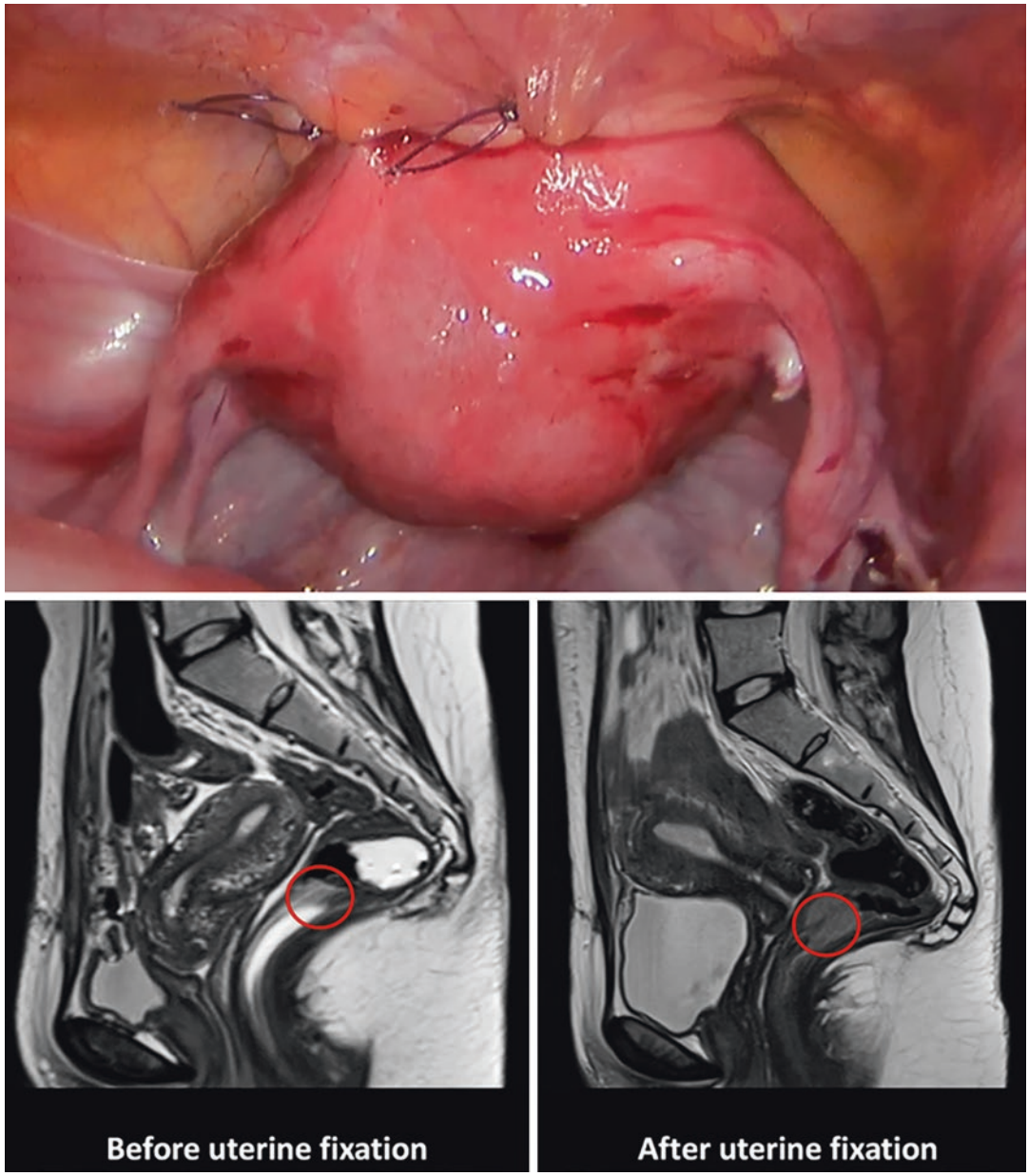

Fig. 3 Laparoscopic uterine anterofixation to move away uterine cavity from radiation field for rectal cancer in a young woman (red circle) [16]

chemoradiation [37]. In 2017, Ribeiro et al. described a surgical technique of laparoscopic uterine transposition before radiotherapy for rectal cancer [38]. Beyond the surgical feat, studies that assess viability, effectiveness, and safety of this technique are needed. Uterine transposition could be part of management of young patients presenting with rectal or anal cancer as it requires uterine pedicles ligation, and its impact on pregnancy needs to be evaluated.

Ovarian Transposition: Clinical Results

The overall success rate of retained ovarian function is around 90% [39]. One must take into account that success rate is influenced by methods of evaluation (presence of menstrual cycle, FSH levels, AMH levels) and the length of follow-up as it decreases throughout the time. Factors that can negatively impact OT success are increased patient age, radiation dose and protocol, ovarian protection during PRT, and concomitant chemotherapy [40]. Pregnancy rates after laparoscopic ovarian transposition were found around 30% [41].

Safety

Radiotherapy-induced cancer in the ovary is one of the concerns that has actually not been confirmed as significant [39]. The risk of surgical ovarian transposition is similar to other gynecological procedures associated with the risk of bowel and vessel injury. The risk of ovarian carcinoma in a transposed ovary is extremely low [39]. This is even more pronounced when fallopian tubes are being resected during the surgical procedure.

Ovarian Function

The first objective of OT is to preserve ovarian endocrine activity to reduce the burden of treatment and subsequent quality of life. To assess ovarian function, authors can observe the presence of climaterial symptoms, the need for add-back hormonal therapies, hormonal measurements, radiological evaluation, or fertility.

Hoekman et al. published in 2019 a systematic review. Thirty-eight studies (765 fertile patients undergoing OT prior to PRT) were selected. No meta-analysis could be performed due to the lack of homogeneity among the studies. With a mean follow-up ranging from 7 to 102 months, authors report a preservation of ovarian function after OT in 15.4 to 100% of patients after any kind of PRT, in 20 to 100% after external beam radiotherapy (EBRT), with or without brachytherapy, and in 63.6 to 100% after brachytherapy only. Ovarian function, in case of addition of chemotherapy with PRT, was preserved in 0 to 69.2%, showing the toxicity of chemotherapy and combination of treatment for ovarian function. In the systematic review and meta-analysis by Gubbala et al. based on 24 reports of 892 women undergoing ovarian transposition, the rate of preserved ovarian function was 94% among women who underwent brachytherapy. After EBRT with or without brachytherapy, the rate of preserved ovarian function was 65% [39]. In a retrospective study comparing patients with cervical cancer before 45 years who underwent OT before PRT versus women treated with hysterectomy or trachelectomy and PRT, the 5-year rate of preserved ovarian function was 60.3% in the transposition group versus 0% in controls ($p < 0.001$) [6].

Fertility

Oocyte retrieval from a transposed ovary is possible transabdominally. Ovary can also be repositioned back into the pelvis in order to attempt spontaneous pregnancy [12]. Nevertheless, few cases of spontaneous pregnancy have been reported after PRT and OT. Most often, those cases concern patients treated for a Hodgkin lymphoma, with an inverted Y irradiation protocol. Ovaries were attached to the uterus and shielded from radiation [13].

In the study of Morice et al., the pregnancy rate after PRT and OT for pelvic cancer was 15% (4/27) for patients treated for a clear cell adenocarcinoma of the vagina and/or the cervix, and 80% (8/10) for those treated for an ovarian pure dysgerminoma or a para-uterine soft tissue sarcoma. Twelve patients were pregnant for a total of 18 pregnancies of which 16 were obtained spontaneously. In 12 cases, pregnancies occur without replacement of ovaries in the pelvis. The conclusion of the authors is that the prognosis for fertility is excellent after OT and PRT in patients with morphologically normal genital tract and that repositioning of the ovary is not essential to achieve pregnancy [41].

Surrogate pregnancy is the only option in some situations and has been reported in case of injured uterus after PRT [13].

Follow-Up and Complications

Failure rate of transposition is about 10–14% [42]. Even with a careful technique, failure in OT may occur due to scattered radiation [43–45], or in case of secondary displacement of the ovaries in the pelvis before cessation of PRT [12, 45].

Complications reported after OT include infarction of the fallopian tubes and chronic pelvic pain [40, 46]. Hoekman et al. reported 12.8% of complication after OT in their systematic review [13]. The risk of metastases within the transposed ovary is rare. Five cases have been reported among 920 patients [13].

There is no expected specific pain in the postoperative period. The risk of damaged vascularization may lead to ischemia and subsequent ovarian reserve impairment. A higher incidence of functional ovarian cyst has also been reported (about 25% of cases) [46, 47].

Clinical Case

A 26-year-old woman without any medical history and who never had been pregnant presented with an adenocarcinoma of the rectum and liver metastases. A neoadjuvant chemotherapy was administered with FOLFOX regimen and then a partial right hepatectomy was performed. Before the surgical excision of rectal cancer, a pelvic concurrent chemoradiation therapy was indicated.

Imaging (Fig. 3) emphasized the fact that tumor was located just behind the uterine corpus and was very close to the ovaries. In this context, PRT was at high risk to definitively compromise patient fertility, by its toxicity on ovarian function on the one hand, and by uterine injuries on the other hand. On preoperative pelvic magnetic resonance imaging (MRI), expected applied radiation isodose was attempted to be high on uterus, in particular in this case of retroverted uterus. Indeed, posterior wall of the uterus was close to the 45 GY isodose, and the entire endometrium, myometrium, and cervix were within the 30 Gy dose volume.

We undertook to reduce PRT consequences by proposing a fertility sparing surgical strategy based on an OT and an uterine fixation according to the technique published by Köhler et al. in 2016 [37]. The procedure was performed by laparoscopy without any perioperative complication.

In comparison with preoperative pelvic MRI, imaging demonstrated that uterine fixation enabled to spare uterus from high-dose irradiation to limit it to less than 30 Gy.

Postoperative pelvic MRI illustrates how this simple procedure concomitant to ovarian transposition allows to bring uterine away from rectal tumor. During the entire PRT period, imaging controls assessed that uterus was correctly fixed.

The patient did not report any specific pain or complication in the postoperative period. Three months after the completion of pelvic concurrent chemoradiation therapy, patient had normal menstrual periods. Hormonal blood test was satisfactory. Pelvic ultrasound showed persistent ovarian function, with the presence of macrofollicles. Endometrial thickness was preserved, and uterine artery Doppler did not present abnormalities.

Conclusion

Despite the efficiency of OT to ensure ovarian protection, this technique seems underused. In the study published by Selter et al., the prevalence of OT was only 6.9% (CI 95% [5.2–8.6]) among 828 patients aged of 35 years or younger who were treated by PRT for cervical, anal, or

uterine cancer [48]. In the study by Phelippeau et al. concerning 553 patients between 15 and 49 years old and treated with a cancer between 2005 and 2014 with a risk of ovarian failure, 62 (11.2%) received PRT only 7 underwent OT [1]. According to Han et al., the reasons for this observation are multifactorial and secondary to the fears of patients and caregivers concerning oncological safety of OT and the lack of availability of surgeons trained in this procedure [49].

An early counseling concerning options to preserve fertility is extremely needed in young patients presenting with pelvic cancers. Oncologist, gynecologist, and specialist in reproductive medicine should collaborate to offer a personal approach adapted to each specific situation, including assisted reproduction techniques or surrogate mothers [50].

A close collaboration is required between surgeons and radiation oncologist to optimize the treatment and to decrease side effects of radiotherapy. Intensity-modulated radiation therapy may contribute to enhance a multimodal fertility-sparing strategy in young patient global management. Indeed, we have to keep in mind that results reported in the literature did not take into account the significant improvement of radiation techniques that may increase performance of fertility-sparing approaches.

Patients' selection according to ovarian preservation objectives, tumor type, treatment regimen, and OT technique remains essential to prevent complications and ensure optimal prevention of ovarian function without compromising cancer management. Long-term follow-up that can evaluate the risks of OT but also fertility rates after completion of PRT and chemotherapy is still needed.

Take Home Messages

- Ovarian transposition indication: women from 11 to 40 with pathology requiring pelvic radiation therapy and willing to preserve their fertility or their endocrinal function.
- Pelvic radiation therapy is responsible for a dose- and age-related reduction of the follicular pool.
- The overall success rate of retained ovarian function is around 90%.
- Pregnancy rates after laparoscopic ovarian transposition were found around 30%.
- Uterine fixation may be associated to ovarian transposition.

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Hormonal Suppression for Ovarian Protection

Eva Blondeaux, Stefano Spinaci, and Matteo Lambertini

Introduction

Survival after cancer diagnosis has significantly improved over the past years thanks to advances in both the use of more effective anticancer treatments and early detection of cancer lesions at preclinical stages [1]. Therefore, survivorship issues are of crucial interest in order to limit the potential serious long-term consequences of anticancer treatments [2]. On this regard, for newly diagnosed premenopausal patients, chemotherapy-induced gonadotoxicity is of particular concern. In fact, this potential adverse event of chemotherapy administration is associated with negative side effects such as menopause-related symptoms and the possible risk of infertility [3]. Thus, all premenopausal women, at the time of diagnosis, should be informed about the potential chemotherapy-induced gonadotoxicity and should receive information on the available strategies to reduce the risk of developing this side effect and its negative consequences [4–8].

The administration of a gonadotropin-releasing hormone agonist (GnRHa) during chemotherapy induces a state of temporary ovarian suppression. This intervention aims at reducing chemotherapy-induced gonadotoxicity. Until now, this is the only medical option available for protecting gonadal function during chemotherapy [9]. Despite many research efforts over the last 30 years both in the preclinical and clinical settings, the use of GnRHa during chemotherapy remains highly debated in the literature [10–13]. Nevertheless, recent larger studies have clarified the role of temporary ovarian suppression with GnRHa during chemotherapy in premenopausal women with cancer. Temporary ovarian suppression with GnRHa should not replace the well-established fertility preservation procedures, but it is recommended as an option to reduce the risk of chemotherapy-induced gonadotoxicity, especially in breast cancer patients [6, 8, 14–16].

This chapter aims to review the biological rationale and the available preclinical and clinical data on the use of temporary ovarian suppression

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with GnRHa during chemotherapy in premenopausal women with cancer. In addition, it describes clinical cases of young premenopausal women facing the need of reducing the long-term consequences of chemotherapy-induced gonadotoxicity. Moreover, the still existing grey zones requiring further research efforts in this field are highlighted.

Mechanism of Action

There are two main hypotheses on the potential mechanism of action for the protective gonadal effect of temporary ovarian suppression with GnRHa during chemotherapy [17, 18]. However, these hypotheses have not been fully validated and its mechanism of action remains to be fully clarified.

The first hypothesis is based on the indirect effect of GnRHa. The induced prepubertal hypogonadotropic state is reached by suppressing the activity of the hypothalamic–pituitary–gonadal axis. It is expected that, in this state, the follicles would be maintained at the quiescent stage, being less vulnerable to chemotherapy-induced gonadotoxicity [19]. The reduction in follicle-stimulating hormone (FSH) levels obtained by GnRHa administration may reduce the proliferation rate of follicular cells in growing follicles with subsequent indirect prevention of an accelerated recruitment of the quiescent follicular pool [20]. However, the relative resistance of prepubertal girls to chemotherapy-induced gonadotoxicity may be also due to their significantly larger pool of primordial follicles and not only to the low gonadotropin level [21]. The anti-Mullerian hormone (AMH) is secreted by the growing follicles, and it can downregulate the recruitment of primordial follicles [22, 23]. Chemotherapy administration damages the growing follicles leading to a rapid reduction in AMH levels. The reduction in AMH levels leads to a recruitment of primordial follicles into the growing pool (i.e., "burnout effect" of chemotherapy) [24]. The administration of GnRHa during chemotherapy may limit the burnout effect by reducing the recruitment of primordial follicles [25]. However, more robust experimental evidence is needed to confirm this hypothesis. Another indirect effect of the GnRHa administration is the decrease in utero-ovarian perfusion with subsequent possible reduced exposure of the follicles to chemotherapy [20]. Experimental models of both rat [26] and human [27, 28] demonstrated that estrogens induce an increase in ovarian perfusion, although some studies did not confirm these results [29, 30]. Notably, reduced ovarian exposure to chemotherapy may increase the risk of persistent ovarian disease or metastasis, but this theory is not supported by clinical evidence [21].

Besides the indirect effect, it has been hypothesized a direct effect of GnRHa in the ovaries. It has been hypothesized that GnRHa could bind to GnRH receptors (GnRHR) that are present in both interstitial cells and granulosa cells of ovarian follicles at different stages [31]. The stimulation of GnRHR, mediated by GnRHa, seems to have an inhibitory action on immature follicles as well as a stimulatory effect on mature follicles [32]. Moreover, GnRHa can upregulate the anti-apoptotic molecules such as sphingosine-1-phosphate (S1P) [33], thus protecting ovaries by decreasing apoptotic events and mitochondrial stress [34, 35]. However, this mechanism is poorly understood. Finally, GnRHa can play a role in protecting ovarian primordial germ cells from gonadotoxic chemotherapy. This can be partially explained by the fact that GnRHR are implicated in pathways of cell growth/survival and primordial follicle activation [36–38]. However, this theory remains to be proven in the ovaries.

Experimental Evidence

Over the past years, many experimental studies on GnRHa administration have been conducted in both female mice [25, 39] and rats [40–43]. However, only three studies have been conducted to evaluate the role of GnRHa use during chemotherapy in female primates or human models [35, 44–46]. Three studies demonstrated a protective effect of GnRHa administration during chemotherapy. The first was conducted in rhesus mon-

keys treated with cyclophosphamide [44], while the second was an in vitro study on human granulosa cells exposed to doxorubicin [45]. Another in vitro experiment evaluated the role of co-administration of GnRHa and cyclophosphamide on human immature cumulus cell-oocyte complexes [35]. Results suggested that the protective effect of GnRHa is probably mediated by an anti-apoptotic effect on cumulus cells rather than a direct effect on oocytes that do not express GnRHR [35].

On the contrary, another in vitro study on human granulosa cells and ovarian tissue fragments exposed to various chemotherapy drugs (cyclophosphamide, paclitaxel, 5-fluorouracil, or docetaxel plus doxorubicin plus cyclophosphamide) failed to demonstrate protection of GnRHa co-administration [46].

Clinical Evidence

Several randomized trials evaluating the efficacy and safety of temporary ovarian suppression with GnRHa during chemotherapy have been conducted among premenopausal women with breast cancer. The aim of these trials was to evaluate the possibility to preserve ovarian function and potential fertility among patients undergoing chemotherapy administration (Table 1). In this setting, a total of 14 different randomized trials were conducted [47–61]. Globally, 10 out of 14 trials demonstrated a protective effect of administration of GnRHa during chemotherapy in reducing the risk of chemotherapy-induced premature ovarian insufficiency (POI). Consistently, similar results have been demonstrated in the three largest trials (POEMS/SWOG S0230 [58, 62], Anglo Celtic Group OPTION [60], PROMISE-GIM6 [52, 59]). Notably, the majority of the trials had a short follow-up and pregnancy rate was a preplanned secondary endpoint only in the POEMS/SWOG S0230 trial [58, 62].

Fewer studies have been conducted among women with tumors other than breast cancer (Table 1). Four trials were conducted among women with hematological malignancies and only one trial was conducted in patients with ovarian cancer [63–68]. No protection from POI with GnRHa was observed among any of the hematological trials. The only trial conducted in 30 women with ovarian cancer demonstrated a reduced risk of developing chemotherapy-induced POI with the concomitant use of GnRHa.

In order to draw more robust conclusions, several meta-analyses have been performed [69–89]. Out of 21 meta-analyses conducted, 19 demonstrated a significantly reduced risk of chemotherapy-induced POI in premenopausal women with cancer treated with concurrent use of GnRHa. A larger benefit was demonstrated in the meta-analyses that included only breast cancer patients.

Taken together the evidence on the protective effect of GnRHa during chemotherapy, some considerations can be made on this option as a strategy to preserve ovarian function and potential fertility in premenopausal patients.

First, among premenopausal women with breast cancer, the majority of the trials demonstrated a reduced risk of chemotherapy-induced POI. However, no benefit was shown for women with hematological malignancies. This could be partially explained by the largest number of breast cancer patients (1647 patients) enrolled in the trials compared to those (154 patients) included in the hematological studies. Moreover, breast cancer patients tend to be older at diagnosis (usually around 40 years) compared to women with hematological cancers that are usually diagnosed before the age of 30 years. Furthermore, premenopausal women with breast cancer are candidates to receive a chemotherapy regimen with an intermediate gonadotoxic risk mainly due to the use of cyclophosphamide [90], whereas patients with hematological malignancies are candidates to receive chemotherapy regimens with low (e.g., ABVD) or high (e.g., conditioning regimens for hematopoietic stem cell transplantation) gonadotoxic risk [3].

Second, different definitions and timepoints of chemotherapy-induced POI were used among the different randomized trials. Although no standard definition of chemotherapy-induced POI is available, according to experts the best definition should take into account both amenorrhea for ≥2 years and a postmenopausal hormonal profile

Table 1 Clinical evidence from the randomized clinical studies evaluating temporary ovarian suppression with gonadotropin-releasing hormone agonists during chemotherapy in premenopausal cancer patients

Authors	Type of disease	Treatment arm	POI definition (timing)	No. patients	Median age (years)	Overall results
Li M et al. [47]	Breast cancer	CT + goserelin vs. CT	Amenorrhea (12 months)	31 vs. 32	40 vs. 39	Protection
Badawy A et al. [48]	Breast cancer	CT + goserelin vs. CT	Amenorrhea and no resumption of ovulation (8 months)	39 vs. 39	30 vs. 29.3	Protection
Sverrisdottir A et al. [49]	Breast cancer	CT + goserelin vs. CT	Amenorrhea (up to 36 months)	51 vs. 43	45 vs. 45	Protection
Gerber B et al. [50]	Breast cancer	CT + goserelin vs. CT	Amenorrhea (6 months)	30 vs. 30	35 vs. 38.5	No protection
Sun JB et al. [51]	Breast cancer	CT + goserelin vs. CT	Amenorrhea (12 months)	11 vs. 10	38 vs. 37	Protection
Del Mastro L et al. and Lambertini M et al. [52, 59]	Breast cancer	CT + triptorelin vs. CT	Amenorrhea and postmenopausal levels of FSH and E2 (12 months)	148 vs. 133	39 vs. 39	Protection
Munster P et al. [53]	Breast cancer	CT + triptorelin vs. CT	Amenorrhea (24 months)	27 vs. 22	39 vs. 38	No protection
Elgindy EA et al. [54]	Breast cancer	CT + triptorelin vs. CT	Amenorrhea (12 months)	50 vs. 50	33 vs. 32	No protection
Song G et al. [55]	Breast cancer	CT + leuprolide acetate vs. CT	Amenorrhea and postmenopausal levels of FSH and E2 (12 months)	89 vs. 94	40 vs. 42	Protection
Jiang FY et al. [56]	Breast cancer	CT + triptorelin vs. CT	Amenorrhea (NR)	10 vs. 11	Not reported	Protection
Karimi-Zarchi M et al. [57]	Breast cancer	CT + triptorelin vs. CT	Amenorrhea (6 months)	21 vs. 21	37 vs. 37	Protection
Moore HCF et al. [58, 62]	Breast cancer	CT + goserelin vs. CT	Amenorrhea and postmenopausal levels of FSH (24 months)	105 vs. 113	38 vs. 39	Protection
Leonard RCF et al. [60]	Breast cancer	CT + goserelin vs. CT	Amenorrhea and postmenopausal levels of FSH (between 12 and 24 months)	103 vs. 118	38 vs. 39	Protection
Zhang Y et al. [61]	Breast cancer	CT + goserelin vs. CT	Amenorrhea and postmenopausal levels of FSH and E2 (36–72 months)	108 vs. 108	37 vs. 39	No protection
Waxaman JH et al. [63]	HL	CT + buserelin vs. CT	Amenorrhea (up to 36 months)	8 vs. 10	28 vs. 26	No protection
Loverro G et al.[a] [64]	HL	CT + triptorelin vs. CT	Amenorrhea (NR)	14 vs. 15	24 vs. 24	No protection
Behringer K et al. [65]	HL	CT + goserelin vs. CT + OC	AMH levels below normal range (12 months)	11 vs. 12	25 vs. 25	No protection
Demeestere I et al. & Demeestere I et al. [66, 67]	HL and NHL	CT + triptorelin + OC vs. CT + OC	Postmenopausal levels of FSH (12 months)	65 vs. 64	26 vs. 27	No protection
Gilani M et al. [68]	Ovarian cancer	CT + triptorelin vs. CT	Amenorrhea and postmenopausal levels of FSH (6 months)	15 vs. 15	21 vs. 22	Protection

aThe inconsistencies in methods and results pose strong doubts about the randomized nature of the study

POI premature ovarian insufficiency, *CT* chemotherapy, *FSH* follicle-stimulating hormone, *E2* estradiol, *HL* Hodgkin lymphoma, *NHL* non-Hodgkin lymphoma, *AMH* anti-Mullerian hormone, *OC* oral contraceptive

[6, 91]. However, the majority of the trials considered only amenorrhea for the definition of chemotherapy-induced POI and only few trials reported results of AMH variation during chemotherapy and follow-up of cancer patients.

Third, paucity of data is available on the potential use of GnRHa during chemotherapy for fertility preservation. This is both due to the fact that desire of a pregnancy was not an inclusion criteria for any of the trials and the studies were not powered to detect differences in post-treatment pregnancies. Moreover, the majority of the trials reported results at a follow-up that is too short to evaluate differences in the potential fertility preservation efficacy of this strategy. Although it is worth noticing that a significantly higher number of post-treatment pregnancies in breast cancer patients treated with GnRHa was observed in the most recent meta-analyses [82, 83, 85, 86, 88], no benefit was observed among women with hematological malignancies [86].

Clinical Cases and Practical Clinical Tips

1. A 32-year-old premenopausal woman was diagnosed with stage II breast cancer. After mastectomy and sentinel lymph node dissection, the pathology reported a diagnosis of ductal carcinoma pT2 (3.5 cm) pN1a (1 lymph node with metastatic carcinoma out of three dissected lymph nodes) grade 3. Additional immunohistochemical analysis showed negative estrogen and progesterone receptor expression, and negative human epidermal growth factor receptor 2 (HER2) overexpression.
   According to current guidelines, adjuvant amthracycline- and taxane-based chemotherapy should be administered. Considering that the patient is 32 years old, her risk of developing POI can be considered low (<20%). However, the patient is childless and strongly desires to have children after anticancer treatments. As a strategy for fertility preservation, the patient underwent oocyte cryopreservation before starting anticancer treatments. In addition, administration of GnRHa during chemotherapy (starting at least 1 week before the first chemotherapy cycle) was proposed in order to reduce the risk of POI. After 8 months from the last chemotherapy and GnRHa administration, the patient experienced menstrual resumption.
2. A 41-year-old premenopausal woman was diagnosed with stage I breast cancer. After lumpectomy and sentinel lymph node dissection, the pathology reported a diagnosis of ductal carcinoma pT1b (0.8 cm) pN0 (no metastatic carcinoma detected in the dissected sentinel lymph node) grade 2. Additional immunohistochemical analysis showed positive estrogen and progesterone receptor expression, and positive HER2 overexpression.
   According to current guidelines, adjuvant chemotherapy with paclitaxel and the anti-HER2 agent trastuzumab should be administered. The patient has already two children and was not interested in fertility-preserving strategies. However, she was concerned about the possibility of developing POI. Although this regimen has a low risk of gonadotoxicity (<20%) [92], GnRHa during chemotherapy was proposed. After chemotherapy, the patient received radiotherapy to the remaining breast tissue and antihormonal therapy by continuing GnRHa and adding exemestane for 5 years. After the completion of endocrine therapy, the patient was 46 years old. At that time GnRHa were stopped. After 1 year from the completion of anticancer treatments, no menstrual resumption was observed although premenopausal levels of follicle-stimulating hormone and beta-estradiol were observed.

3. A 31-year-old premenopausal woman was diagnosed with stage 3 Hodgkin lymphoma. She presented with supra- and sub-diaphragmatic lymphadenopathies. A biopsy revealed classic Hodgkin lymphoma with nodular sclerosis. She received chemotherapy with ABVD for six cycles. She already had two children before diagnosis. Because the risk of POI with ABVD chemotherapy regimen in young (<32 years) patients is very low and considering the controversial data with the use of GnRHa in this setting, this strategy was not proposed. During chemotherapy, the patient experienced amenorrhea but menstrual cycles resumed after 3 months following the end of anticancer treatments.

Conclusions

Recent clinical trials in breast cancer patients have provided evidence favoring the use of GnRHa during chemotherapy as a strategy for reducing the risk of chemotherapy-induced POI. Based on these findings, current guidelines recommend the use of temporary ovarian suppression with GnRHa during chemotherapy for premenopausal breast cancer patients interested in preserving ovarian function irrespectively of their pregnancy desire [6, 8, 14]. Contrariwise, for patients interested in fertility preservation, cryopreservation strategies should be offered as first technique and GnRHa during chemotherapy can be given as adjunctive (and not as alternative) option.

For women with tumors other than breast cancer, there is limited evidence on the protective role of this option. In this setting, the use of temporary ovarian suppression with GnRHa during chemotherapy may be discussed considering its favorable safety profile and its other potential medical benefits (e.g., prevention of menometrorrhagia) [66, 93]; however, the controversial protective effect should be clearly highlighted.

Further research efforts in this field to better elucidate its protective mechanism of action are needed.

Definitions

- GnRHa: gonadotropin-releasing hormone agonist.
- FSH: follicle-stimulating hormone.
- AMH: anti-Mullerian hormone.
- GnRHR: gonadotropin-releasing hormone receptor.
- POI: premature ovarian insufficiency.
- CT: chemotherapy;
- E2: estradiol;
- HL: Hodgkin lymphoma;
- NHL: non-Hodgkin lymphoma;
- OC: oral contraceptive;
- HER2: human epidermal growth factor receptor 2.

Take Home Messages

- GnRHa during chemotherapy can be offered to breast cancer patients interested in preserving ovarian function irrespectively of their pregnancy desire.
- GnRHa during chemotherapy is not an alternative to cryopreservation strategies that remain the first options to be offered to cancer patients interested in fertility preservation.
- GnRHa during chemotherapy may be discussed in patients with tumors other than breast cancer considering its favorable safety profile.

Key Readings

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Oocyte and Embryo Cryopreservation: Methodology and Clinical Results

Ana Cobo, Aila Coello, Megan Hassane, and José Remohí

Introduction

The cryopreservation of biological materials is the process at which they are subjected to cryogenic temperatures below zero enabling a complete stopping of biochemical reactions in order to preserve the viability of the cells. Freezing itself is lethal for living systems. However, knowledge of the mechanisms involved in the freezing process results highly useful to provide the stable conditions necessary to preserve life and prevent possible adverse effects derived from the process. Consequently, different cryopreservation protocols able to preserve life at subzero temperatures have been developed and are applied in different fields, including medicine.

The essential role of cryopreservation in ART has become obvious since the establishment of the infertility treatment, bringing flexibility and efficiency to the practice. Thus, efficient cryopreservation protocols have been developed either spermatozoa, as well as oocytes and embryos at all developmental stages.

The transfer of frozen and thawed embryos has been widely and successfully applied following the first pregnancy achieved with this strategy. Conversely, oocytes cryopreservation has proven much more challenging, traditionally producing results that have not always been reproducible, especially during the 1980s and 1990s. However, during the early 2000s, vitrification brought efficient and reproducible outcomes to clinical practice, which has allowed the establishment of egg-banks [1], are currently benefitting infertile patients as well as women who are seeking for fertility preservation (FP) or oocytes recipients enrolled in egg-donation programs. Beneficiaries of this approach include cancer patients who need an option to preserve fertility before undergoing their oncological treatment [2], or women who wish to delay their motherhood for a variety of reasons [3, 4], government restrictions on IVF [5], ethical reasons against embryo cryopreservation, and practical reasons such as unavailability of the male gamete on collection day [6, 7].

Therefore, the aim of this chapter is to provide an overview of the main cryopreservation strategies and an appraisal of its efficacy when applied for different clinical indications in ART practice.

Cryobiology Background

Cryopreservation protocols are devoted to prevent the cell death caused by the formation intracellular ice crystals. The use of cryoprotectants (CPAs) allows the depressing of the freezing and eutectic points, thereby lowering the probability of ice formation [6]. The mechanisms by which

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intracellular ice formation causes damage obey to the aggregation of small crystals during thawing, caused by their high activation energy, and becoming larger structures. These large crystals cause damage mainly by mechanical effect, destroying cell structures. The deleterious effects of partial crystallization of cells and tissues depend on the cell type and the number of cells. In a tissue, the lysis of several cells is compensated by those that survive, ultimately rendering a functional tissue. That is the case for spermatozoa or ovarian cortex, or even embryos; but not in the case of the oocytes, where it is "all or nothing" because the cell survives or does not survive.

There are two main strategies in cryobiology: slow freezing and vitrification. During the former, the cells are gradually dehydrated in the presence of CPAs, and the temperature is lowered at a very slow cooling rate (−0.3 °C). In this way, the cells are exposed to low temperatures (between +15 °C and − 5 ° C) during an extended period of time, which can lead to what is known as chilling injury [8]. This detrimental event affects mainly the lipids of the cell membrane [9], the microtubules of the meiotic spindle [10], and causes hardening of the zona pellucida [11].

During vitrification, ice formation is circumvented in both the intra- and extracellular spaces due to the direct conversion from liquid to a vitreous solid [12]. The combination of the exposure to very high cryoprotectant concentrations and very high cooling rates, from −15,000 to −30,000 °C per minute, causes significant dehydration of the cell with a partial intracellular diffusion of cryoprotectants [13], thus the equilibrium between the intra- and extracellular compartments is not reached, explaining why this procedure is also known as "non-equilibrium freezing." Therefore, the probability of vitrification will rise by increasing the cooling rate and the viscosity of the solution and by lowering the volume containing the cells [13]. Very high warming rates are also related to success due to the prevention of recrystallization during warming and are considering even more critical than cooling rates [14]. At the end of the vitrification process, the liquid becomes a very viscous solid with vitreous aspect, giving the name to the phenomenon. Vitrification efficiently avoids chilling injury because the passage of the cells by the temperature range in which the injury happens (+15 °C and − 5 °C) is completely circumvented by plunging the cells directly into liquid nitrogen, passing from room temperature straight to −196 °C. In this way, the depolymerization of the meiotic spindle does not befall before or during the vitrification process. Initial studies have attributed a stabilizing effect of CPAs on tubulin fibers that has been confirmed by noninvasive studies. These studies have also shown that depolymerization occurs at warming during the dilution of cryoprotectants at room temperature. During this step, the inner cryoprotectant is replaced by water during rehydration, thus rendering the cytoplasm exposed to room temperature in the absence of CPAs, which explains why depolymerization takes place at this point. It has also been shown that complete spindle restoration happens depending on the incubation time post warming [15–17] and happens faster following vitrification than after slow freezing [17]. These observations have counteracted the initial concerns related to the possible generation of unbalanced gametes after cryopreservation due to the disassembly of the meiotic spindle and the possibility of scatter chromosomes [18]. The restoration of a functional meiotic spindle is supported by the findings showing comparable aneuploidy rates between embryos developed from vitrified and fresh oocytes from infertile patients [19] and by the analysis of the perinatal outcomes of babies born from vitrified oocytes [20].

The main drawback of the vitrification technique is the use of elevated concentration of cryoprotectants, which can damage oocytes and embryos through chemical toxicity and the osmotic stress [21]. Nonetheless, an appropriate and phased composition of CPA could mitigate the toxic and osmotic consequences of extremely concentrated CPAs mixtures [6]. In this way, a combination of two or three of these agents can decrease the individual specific toxicity. The most common mixture used for this purpose consists of ethylene glycol (EG), dimethyl sulfoxide

(DMSO), and sucrose or trehalose [6]. To optimize the results, in addition to an appropriate selection of CPAs, it is also helpful to use these agents at lower concentration as possible, while maintaining the necessary composition able to achieve vitrification. As a result, an extreme cooling rate prevents chilling injury and allows the reduction of the concentration of CPA, thereby preserving the cells at nontoxic concentrations of cryoprotectant.

The "minimum drop vitrification" method proposed by Arav uses a very small volume of vitrification solution by placing the samples on a specific device that must be cooled very quickly [22]. Extremely high cooling rates are achieved when the samples are loaded in a minimum volume are directly immersed in liquid nitrogen (LN). These methods are also known as open systems.

Hermetically closed vials achieve lower cooling rates compared with open devices. Nevertheless, it is worth mentioning that the direct contact of samples with liquid nitrogen has raised some concerns due to the theoretical risk of cross-contamination mainly because the LN itself is considered as a potential source of pathogen agents. A study aimed to assess the presence of viral sequences in different samples including follicular fluid, culture media, and liquid nitrogen used for vitrification and storage in seropositive patients for human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV) undergoing IVF cycles failed to detect the virus in all the samples analyzed [23]. This is extremely interesting evidence for low or absent cross-contamination possibilities when using open vitrification systems even when dealing with samples from seropositive women, who in some cases showed positive blood viral load. However, there are some measures that can be taken to make the tool safer, such as sterilization of liquid nitrogen [23, 24], the storage in vapor-phase systems [25], or store in a closed device even after open vitrification [26]. Needless to say, the use of efficient closed devices avoids any possibility of cross-contamination while providing efficient outcomes either for embryos [27, 28] or for oocytes [29, 30].

Vitrification technology has evolved in last years, providing different devices, protocols, and methodologies. Thus, we can count on open or closed systems, named due to the necessity to get in contact with liquid nitrogen in the formers or not in the latest. Different combinations of cryoprotectants have also been described as well as different osmotic agents and protein substitutes [11].

Cryopreservation Protocols and their Implications in Survival of Oocytes and Embryos

Embryo Cryopreservation

The embryo cryopreservation protocol was standardized 20 years ago and has been widely applied with minimal modifications, providing successful outcomes since its introduction in 1985 [31]. The method of choice has been the slow freezing procedure, in which the embryos are cooled at very slow rates after being treated with cryoprotectants at a relatively low concentration (1.0–1.5 M), thus limiting the toxic and osmotic damage [31]. In this protocol, the dehydration of the cells and the diffusion of cryoprotectant agents into the cells, takes place very slowly during a long period of time. At the end, the procedure allows the balance of extra and intracellular fluids, which explains why this method is also known as "equilibrium freezing." When the process is completed, the extracellular fluid crystallizes, while intracellular ice formation cannot be completely avoided, being one of the greatest shortcomings of this strategy. As a result, the hyperosmolarity produced can cause severe osmotic damage "solution effect" [32].

Intracellular ice formation is one of the causes responsible for the partial blastomeres loss observed in a high proportion of embryos after slow freezing. It is well known that partial lysis in frozen and thawed embryos results in an impaired implantation potential. Conventionally, survival after slow freezing was defined when 50% or more blastomeres survived the process. Due to the association between lower implantation and

partial blastomere loss, the efficacy of cryopreservation programs was more accurately assessed by evaluation of the proportion of fully intact and partially lysed embryos after the freezing/thawing process instead of the evaluation of survival [33].

Prior to vitrification, the freezing at early cleavage stage rather than blastocyst stage was the most common approach [33], probably due to a lack of consistency after slow freezing of D5–6 embryos, combined with lower survival rates. Nevertheless, the absence of crystallization achieved with vitrification leads to an extremely high proportion of fully intact embryos of 95% [34], which may explain the comparable implantation rates observed when results were evaluated between vitrified/warmed embryos versus fresh embryos belonging to the same morphological category [35]. This finding and the increased survival rates achieved for embryos cryopreserved both at the cleavage and at the blastocyst stages represent a clear advantage of vitrification over slow freezing. Besides, a meta-analysis published in 2008 confirmed the efficacy of embryo vitrification over the slow freezing procedure, which explains the shift to vitrification observed in many IVF centers [36].

Table 1 shows results from some publications, showing efficient and comparable results obtained with embryos at different developmental stages while using different vitrification devices. Comparable survival, implantation, and delivery rates were shown in a large series of cryo-transfers of vitrified embryos from cleavage to blastocyst stage [34], as well as similar clinical outcome can be accomplished with both closed and open devices.

Different vitrification approaches have also proven effective when considering biopsied or non-biopsied cleavage stage or blastocyst embryos, thus making vitrification a valuable complementary tool to PGS analysis [37]. In our hands, blastocysts survival after the trophectoderm biopsy is 96.3% and clinical results after the transfer of chromosomally normal embryos indicate that vitrification does not affect its potential for implantation (45.2% of the implantation rate; 62.5% of clinical pregnancy rate; 54.2% of ongoing pregnancy rate), all these outcomes highlight the usefulness of vitrification as adjunct to PGS programs.

Table 1 Survival and clinical outcome of early cleavage and blastocysts stage embryos following vitrification with open and/or closed devices

Author	Developmental stage	Survival rate	Pregnancy rate	Observations
Liu et al. 2013 [38]	Cleavage (day 3)	97.6%	36.3%	
Panagiotidis et al. 2013 [39]	Blastocyst	84.1%	45.9%	Open system
		82.1%	42.4%	Closed system
Chen et al. 2013 [40]	Blastocyst	98%	47.6%	Open system
		95.8%	42.2%	Closed system
Hashimoto et al. 2013 [41]	Blastocyst	96.9%	46.8%	Open system
		97%	45.4%	Closed system
Kang et al. 2013 [42]	Blastocyst	96.6%	41.8%	Single ET
		97.8%	48.1%	Double ET
Van Landuyt et al. 2013 [43]	Cleavage (day 3)	94%	–	20.7% IR
Muthukumar et al. 2013 [44]	Blastocyst	85.5%	52.6%	Day 5
		79.6	32.6%	Day 6
Roy et al. 2014 [45]	Blastocyst	94.4%	58.8%	
Murakami et al. 2014 [46]	Blastocyst	98.7%	51.5%	HSA supplement
		98.9%	56.0%	rHA supplement
Levron et al. 2014 [47]	Cleavage (days 2–3)	81.6%	20.0%	
Reed et al. 2015 [48]	Blastocyst	96.3%	46.2%	Non-biopsied
		97.6%	58.2%	Biopsied
Iwahata et al. 2015 [49]	Blastocyst	96.1%	–	49.4% IR
		96.5%	–	49.7% IR
Total		**96.3%**	**45.9%**	

The availability of vitrification for embryo storage has also made possible the segmentation of IVF leading to the so-called "freeze-all strategy." Controlled ovarian and stimulation results in extremely high levels of estrogen and progesterone that leads to an asynchrony between the embryo developmental stage and the endometrium, situation that ultimately may impair the endometrial receptivity [50, 51]. As a result, delaying the embryo transfer to a different unstimulated cycle, in which the uterus has not been exposed to supra-physiological doses of reproductive hormones, it would appear a reasonable solution. In addition, the rationale of transferring the embryos in a more physiological environment of a "non-stimulated" endometrium is for many the reason why frozen embryo transfers lead to fewer obstetric and perinatal complications as compared to fresh embryos transfers [52]. Moreover, some studies have reported improved IVF results when all embryos are electively frozen for later transfer [50, 53, 54]. However, this evidence should be taken with caution since some of the available studies have been criticized due to the serious flows in their study design. In fact, in our routine practice we have not found evidence of improved IVF outcome after freeze-all strategy in terms of ongoing pregnancy or live birth rates (36.2% vs. 33.8%) in women with a normal response to ovarian stimulation. When adjustments were made for patient and other variables likely to affect results, there was still no evidence of any impact of freezing. Although these findings do not support a change in the practice of IVF moving to a freeze-all strategy, there are some cases such as patients at risk of ovarian hyperstimulation syndrome (OHSS) [51] or in cases with elevated serum progesterone levels [55].

Oocyte Cryopreservation

Table 2 summarizes the survival and clinical outcomes of human oocytes vitrification reports from the first pregnancy achieved in humans [56]. Although it is difficult to draw conclusions with a great variety of protocols, devices, and types of oocytes (patients' own/donated), if we focus on oocyte donors an overall survival rate of ~90% is observed, and curiously the great majority of studies used open devices, except for two studies [29, 30]. Approximately 60% clinical pregnancy rate has been achieved with donated vitrified oocytes in the literature. Overall, the comparison between fresh and vitrified oocytes showed similar results as they were higher with vitrification compared with slow freezing. When focusing on studies that report data on own vitrified oocytes, a mean survival rate of 80% is observed with ~40% clinical pregnancy rate per transfer.

The efficiency of oocyte vitrification was demonstrated in a large clinical trial that showed comparable outcomes in terms of embryo development and clinical results of vitrified versus fresh oocytes [74]. In this large randomized clinical trial, the superiority of fresh donations over cryo-donations was not proven. In contrast, the noninferiority of egg storage strategy was established through vitrification [74].

In the same way, others have reported their experience showing very similar results [69, 85]. These outcomes were consistent with those from a study involving shared vitrified and fresh oocytes, which found similar developmental parameters and clinical results between fresh and cryo-donations [88]. Other authors evaluated the combined oocyte vitrification and embryo transfer strategy in the blastocyst stage of their ovum donation programs [89]. Both blastocyst formation (41.3% vs. 45.3%) and pregnancy rates (61.8% vs. 60%) between vitrified and fresh oocytes were similar. Another prospective observational study reported a 90% survival rate and a 43.5% ongoing pregnancy rate (OPR) by considering "fresh" and cryo-transfers of surplus embryos [30]. Unlike most studies published to date, this work used a closed vitrification system. In line with this, another study has shown comparable results between oocyte donation cycles conducted with vitrified oocytes using open versus closed systems [29]. No differences were observed in terms of implantation (13.8% vs. 10.1%), clinical pregnancy (36.0% vs. 28.0%), ongoing pregnancy (33.3% vs. 24.0%), and live

Table 2 Human oocytes' vitrification, survival, and clinical outcomes of different studies from 1999 to 2015

Author	Study design	Device	Own / donated	No. cycles (no. oocytes)	N° warming cycles (no. oocytes)	Survival rate (%)	Fertilization/ inseminated (%)	IR (%)	CPR/ transfer (%)	OPR/ transfer (%)	LBR (%)
Kuleshova L, 1999 [56]	Case report	OPS	Own-donated	4 (17)	4 (17)	64.7	45.4	33.3	33.3	33.3	1 (5.9)
Yoon T, 2000 [57]	Case report	EM grid	Own	7 (90)	7 (90)	63.3	43.3[a]	9.4	42.9	42.9	
Yoon T, 2003 [58]	Descriptive	EM grid	Own	34 (474)	34 (474)	68.6	71.7	6.4	21.4		7
Katayama KP, 2003 [59]	Case report	Cryotop	Own	6 (46)	6 (46)	94	91			33.3	
Kyono K, 2005 [60]	Case report	Cryotop	Own	1 (5)	1 (5)	100	100	100	100		1
		SF	Own	1 (4)	1 (4)	25	100				
Kuwayama M, 2005 [61]	Cohorts	Cryotop	Own	–64	–64	90.6	89.6		41.4		7 + 3OPR
		SF	Own	–9	–9	22.2	0				
Yoon T, 2007 [62]	Descriptive	EM grid	Own	28 (426)	30 (364)	85.1	77.4	14.2	43.3		5+
											7OPR
Selman H, 2006 [63]	Descriptive	OPS	Own	6 (53)	6 (24)	75	77.7	21.4	33.3		
Antinori M, 2007 [64]	Cohorts	Cryotop	Own	120 (463)	120 (330)	99.4	93	13.2	32.5	23.3	
		Fresh	Own	251 (1755)			96.7	10.3	28.6		
Lucena E, 2006 [65]	Descriptive	Cryotop	Own	40 (370)	4 (28)	92.9	87		100		
		Cryotop	Donated	33 (337)	18 (131)	89.3	87.6		57.1		
Cobo A, 2008 [2]	Cohorts	Cryotop	Donated	30 (231)	30 (231)	96.9	76.3	40.8	65.2	47.8	
		Fresh	Donated	30 (219)			82.2	100	100	100	
Chang C, 2008 [66]	Cohorts: IVO	Cryotop	Donated	10 (240)	18 (137)	85.4	86.3	61.9	83.3	27.8	19 (13.9)
	Cohorts: IVM-MI	Cryotop	Donated			82.3	89.3				
	Cohorts: IVM-VG	Cryotop	Donated			79.3	60.8				
Sher G, 2008 [67]	Descriptive	Cryoloop	Donated	16 (111)	19 (78)	96.1	90.7	61.3	81.2		17 (21.8)

Chian, 2009 [68]	Cohorts: OS	Cryoleaf	Own	38 (463)	38 (463)	81.4	75.6	19.1	50[b]		15 (39.5)[c]
	Cohorts: IVM	Cryoleaf	Own	20 (215)	20 (215)	67.5	64.2	9.6	20[b]		4 (20.0)[c]
Nagy ZP, 2009 [69]	Cohorts	Cryotop	Donated	20 (153)	20 (153)	89	87	55.3	75		26
		Fresh	Donated	9 (182)			75.3	47.4	55.6		
Cao, 2009 [70]	Randomized	Cryoleaf	Own-donated	−292	−292	91.8	67.9				
		SF	Own-donated	−123	−123	61	61.3				
Schoolcraft WB, 2009 [71]	Descriptive	Cryotop	Own	12 (160)	12 (160)	76.9	78.9	51.9	72.7		12 (7.5)
Fadini R, 2009 [72]	Cohorts	Cryoleaf	Own	59 (285)	59 (285)	78.9	72.8	9.3	18.2		
		SF	Own	286 (1348)	286 (1348)	57.9	64.6	4.3	7.6		
Grifo, 2010 [73]	Cohorts	Cryotip	Own-donated	−163	−163	95.1	74.2				
		SF	Own-donated	−159	−159	88	84.3				
Cobo A, 2010 [74]	Randomized	Cryotop	Donated	295 (3286)	295 (3286)	92.5	74.2	39.9	55.4	49.1	
		Fresh	Donated	289 (3185)			73.3	40.9	55.6	48.3	
Rienzi L, 2010 [75]	Randomized sibling-oocytes	Cryotop	Own	40 (124)	40 (124)	96.8	79.2	20.4	38.5	30.8	
		Fresh	Own	40 (120)			83.3		43.2		
Kim TJ, 2010 [76]	Descriptive	EM grid	Own	19 (483)	20 (395)	81	72.3	45.3	80[b]	65	20 (5,1)
Almodin CG, 2010 [77]	Cohorts sibling-oocytes	Vitri-inga	Own	46 (252)	46 (252)	84.9	80.8	14.9	45.6		
		Fresh	Own	79 (413)			81.4	21.3	51.9		
Ubaldi F, 2010 [78]	Cohorts sibling-oocytes	Cryotop	Own	182 (1132)	115 (487)	89.7	85.4	16.1	31.5	25.2[d]	
		Fresh	Own	173 (511)			87.1	23.2	44.8	37.4[d]	

(continued)

Table 2 (continued)

Author	Study design	Device	Own / donated	No. cycles (no. oocytes)	N° warming cycles (no. oocytes)	Survival rate (%)	Fertilization/ inseminated (%)	IR (%)	CPR/ transfer (%)	OPR/ transfer (%)	LBR (%)
Smith GD, 2010 [79]	Randomized	Cryotip	Own	48 (349)	48 (349)	80.5	76.9		37.5[e]		
		SF	Own	30 (238)	30 (238)	66.8	67.1		13.3[e]		
Noyes N, 2010 [18]	Cohorts	Cryotip & Cryolock	Own-donated	−167	88	77.2					
		SF	Own-donated	−148	85	89.7					
Trokoudes KM, 2011 [80]	Cohorts sibling-oocytes	Cryotop	Donated	36 (210)	36 (210)	91.4	84.4	24.7	55.6	47.2	17 (8.1)
		Fresh	Donated	36 (247)	41 (247)		86.6	25.6	48.8	43.9	17 (6.9)
García JI, 2011 [81]	Randomized	Cryolock	Donated	20 (283)	34 (283)	89.4	76.1	43.9	61.8		
		Fresh	Donated	58 (696)	85 (696)		87,5	42.9	60		
Paffoni A, 2011 [82]	Cohorts	Cryotop	Own	53(268)	53(268)	82.8	73.0	13.4	26.4[e]		11 (4.1)
		Cryotip	Own	51(261)	51(261)	57.9	57.6	5.8	7.8[e]		3 (1.1)
Parmegiani L, 2011 [26]	Cohorts sibling-oocytes	Cryotop + hermetical cryostorage	Own	31(168)	31(168)	89.9	84.9	17.1	35.5		7 (4.2)
		Fresh		31			88,3		13,3		
Cobo A, 2012 [34]	Cohorts	Cryotop	Own	384 (1192)	384 (1192)	84.9	66.1	25			73 (6,1)
		Fresh	Own	587 (1170)			64.9	25.6			108 (6)
Stoop D, 2012 [30]	Descriptive	CBS straw	Donated	14 (123)	20 (123)	90.2	77.5	33.3	50	45	
Forman EJ, 2012 [19]	Randomized	Cryotop	Own	44 (294)	44 (294)	81.6	77.9			53,9	
		Fresh	Own	44 (294)			90.5			57.7	
García-Velasco JA, 2013 [83]	Descriptive: Non–oncological	Cryotop	Own	725 (5498)	26 (191)	84.8			42.3[f]	30.7[g]	4 (2.1)
	Oncological	Cryotop	Own	355 (2939)	4				25[f]	25[g]	1
Chang C, 2013 [84]	Randomized sibling-oocytes	Cryotop	Own	22 (186)	22 (186)	79.6	66.6[a]	30.1	45.4		11 (5.9)

		Fresh	Own	22 (204)			75				
Papatheodorou A, 2013 [29]	Randomized sibling-oocytes	Vitrisafe	Donated	75 (608)	75 (608)	90.9	73.4	10.1	28.0[b]	24.0[d]	18 (3)
		Vitrisafe+ HSS	Donated	75 (598)	75 (598)	82.9	82.5	13.8	36.0[b]	33.3[d]	27 (4.5)
Solé M, 2013 [85]	Randomized sibling-oocytes	Cryotop	Donated	99	99	85.6	78.2	34	53.5	44.4	42 (4.2)
		Fresh	Donated	99	99		80.7	33.3	47.5	39.4	38
Siano, 2013 [86]	Cohort sibling-oocytes	Cryotop		14 (83)	14 (83)	72 (86.7)	69.4	7 (25)	53.8		6 (46.1)[h]
		Fresh		14 (81)	14 (81)		78.2				
Martinez, 2014 [87]	Descriptive: Oncological	Cryotop	Own	375	11 (65)	60 (92.3)	46 (76.7)	7 (31.8)	6 (54.5)	4 (36.4)	4 (6.1)[h]
Cobo, 2015 [1]	Retrospective, observational	Cryotop	Donated	3146 (42152)	3610 (42152)	38,087 (90.4)	26,869 (71.2)	2220 (39)	1678(48.4)	1382 (39.9)	1674 (4.0)

IR implantation rate, *CPR* clinical pregnancy rate, *OPR* ongoing pregnancy rate, *LBR* live birth rate, *a2PN*/warmed oocyte, *CPR* cycle started, *cLRB*/cycle started, *dOPR*/cycle, *eCPR*/warming cycle, *fCPR*/patient, *gOPR*/patient; hLBR/

birth rates (36.0% vs. 24.0%) between closed and open groups, although survival rate was statistically lower when the closed system was used (82.9% vs. 91.0%, P < 0.05). Therefore, all these studies definitively confirmed our previous observations about the nonalteration of vitrified oocytes potential to develop into embryos capable of generating competent ongoing pregnancies in a similar proportion to fresh oocytes [74].

Another publication shows our experience after 6 years of systematic donation of vitrified oocytes, confirming the clinical results reported previously, but also offering a complete picture of the scope of the technology [1]. After analyzing more than 40,000 oocytes, we were able to confirm over 90% survival rate. Nevertheless, differential survival rates among donors or among different stimulation cycles from the same donor were also observed. Thereby, a small proportion of cases in which survival was very low (10–50%) or even 0% (in 1.4% of cases) (Table 3). In an attempt to calculate a model to predict survival, it was found that any variable was useful to that purpose. However, the awareness of the fact that in some cases unexplained low outcomes are achieved and the low incidence of these adverse results is of interest to all who apply egg-banking. The evaluation of this large series has confirmed our previous findings related to the clinical outcome (Table 4).

Kaplan–Meier analysis observed in Fig. 1 provides interesting information about the number of vitrified oocytes consumed by a recipient (either in one or more ovum-donation cycles) necessary for a newborn. The analysis showed that the live birth rate increases exponentially, and the patient can achieve a baby with a probability of almost 100% when approximately 3–4 of egg donation cycles (around 35–40 oocytes) are completed. The health of infants and the obstetric evolution of pregnancies conceived with vitrified oocytes are comparable to those observed in our population of children conceived with fresh oocytes, thus endorsing the safety of the technique [20].

The benefits of oocyte vitrification in two infertile populations include having to avoid the risk of hyperstimulation [90] and low responders (LR) [91] among others. A potential alternative to the management of low responder is to create a larger oocyte stock by accumulating vitrified MII oocytes over several stimulation cycles and inseminating them simultaneously. Theoretically, this could help to increase the chances of success by "turning" the poor responders situation into a "normo-responder"status. We have addressed the benefits of oocyte accumulation from different COS cycles in low response patients, and they were shown in terms of higher newborn rates per patient initiated as compared to standard-treated poor responder patients. More than 1000 vitrified oocytes retrieved were accumulated by vitrification, which led to improved cohorts to be inseminated (mean 7.02 MII oocytes) [91].

Advanced maternal age LR patients (>40 years old with ≥6 metaphase II oocytes) undergoing

Table 3 Distribution of survival rate in warming procedures finally not donated due to low survival (74)

	No. warming procedures (%)	No. surviving oocytes / total warmed oocytes	Mean survival rate/ warming procedures %	Mean no. surviving oocytes/ warming procedures
SV 0%	45 (31.5)	0/412	0,0	0,0
SV 10–15%	4 (2.8)	7/59	11.9 (3.6–20.1)	1.8 (1.7–1.9)
SV 16–20%	7 (4.9)	12/65	18.5 (9.0–27.9)	1.9 (1.8–2.0)
SV 21–30%	17 (11.9)	52/208	26.0 (19.1–30.9)	3.2 (3.2–3.3)
SV31–50%	62 (43.4)	254/602	42.2 (38.3–46.1)	4.1 (4.0–4.1)
SV54–67%	8 (5.6)	35/65	53.8 (41.7–66.0)	4.4 (4.3–4.5)
Total	143	362/1411	25.7 (23.4–27.9)	2.5 (2.5–2.5)

Table 4 Clinical outcome in an ovum donation after 6 years of egg-banking practice [1]

		95% CI
No. cycles	3467	
MII oocytes injected/donation cycle (mean)	37,725 (10.9)	10.7–11.1
Fertilization rate	26,869 (71.2)	70.8–71.8
Number of fresh embryo transfers/donation cycle	3050/3467 (87.9)[a]	86.8–89.0
Number of embryos replaced (mean)	5695 (1.9)	1.8–1.9
Implantation rate (no. sacs/no. embryos transferred)	39,0 (2220/5695)	37.8–40.5
Clinical pregnancy rate/transfer	1678/3050 (55.0)	53.2–56.8
Clinical pregnancy rate/cycle	1678/3467 (48.4)	46.7–50.1
Clinical miscarriage	274 (16.3)	14.5–18.1
Ectopic pregnancy	22 (1.3)	0.8–1.9
Ongoing pregnancy / transfer	1382 (45.3)	43.5–47.3
Ongoing pregnancy /cycle	1382 (39.9)	38.3–41.5
Delivery rate /donation cycle	1357/3467 (39.1)	37.5–40.7

Numbers in parentheses are percentages unless otherwise indicated

aIn 89 cases, the fresh embryo transfer was deferred for a future cryotransfer

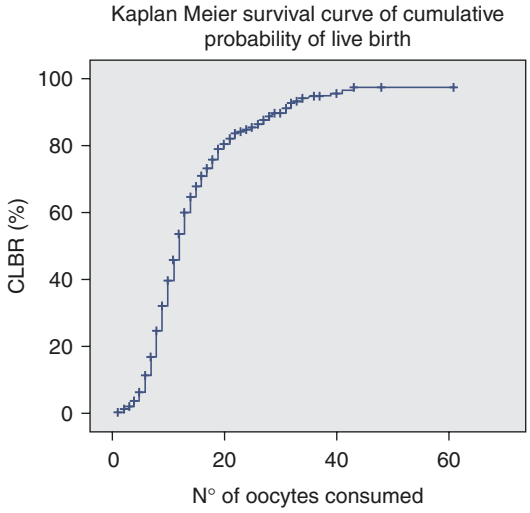


Fig. 1 Kaplan–Meier curve for the cumulative delivery rate of at least one baby, depending on the total number of oocytes consumed [1]

PGS analysis also benefited from this strategy: implantation rate 24.4% versus 19.8% in vitrification versus fresh oocytes [92]. A later report also showed that the accumulation strategy in advanced maternal age patients requiring PGS can be conducted at MII oocytes or early cleavage embryos sowing similar advantages [93].

It is worth mentioning that the true advantage of the "accumulation" strategy relies in cost-effective issues rather than in the clinic benefit itself. From the clinical point of view, it is highly likely that after an undetermined number of consecutive fresh cycles, the cumulative rates achieved would be comparable to those achieved after accumulation. However, the high dropout incidence in poor responder patients plays a key role due to the limited capacity of couples to cope with consecutive failures. Instead, patients quit, seek another clinic, or switch to ovum donation. On the other hand, the accumulation strategy may be of greater advantage from a cost-effective standpoint since special packages considering successive COS and vitrification cycles with just one ICSI procedure could result in a lower economic burden than in the case to assume the cost of consecutive complete IVF cycles [91].

Fertility Preservation

Growing evidence on the efficiency of oocyte vitrification has encouraged professionals to offer this option for women who wish to preserve their gametes to allow them to have the opportunity to conceive in the future and to have their own genetic offspring [4, 94].

Although fertility preservation (FP) initially was intended to oncological patients because of the occurrence of ovarian failure caused by gonadotoxic treatments, many other medical conditions may compromise fertility, such as autoimmune and iatrogenic diseases that also require an intervention to safeguard gametes for their future use [95].

Another population that may benefit from this technique is the group of women that preserves fertility for other motivations known as "social reasons." These women decide to postpone moth-

erhood for different personal reasons. In these cases, the greatest drawback is the patients' age, thus this indication is also known as elective fertility preservation (EFP) due to the age-related fertility decline, as a consequence of the natural process of depletion of the ovarian reserve [96]. EFP for nonmedical conditions is increasingly accepted as an option to postpone motherhood [97]. In turn, EFP can also help women to endure some medical conditions that may reduce their future fertility, such as endometriosis or other conditions that lead to premature menopause. In these cases, the condition itself is not an impediment to become pregnant at the time of diagnosis. However, for several reasons, these women decide to postpone maternity, opting to have their oocytes vitrified for future IVF treatments.

Currently, the suggested options for FP also include embryo and ovarian tissue cryopreservation. Nevertheless, the need for male gamete, as well as different ethical and religious issues, does not make embryo cryopreservation the most preferred option by single women. Ovarian tissue cryopreservation is the technique of choice for prepubertal patients [98] and for women with hormone-dependent diseases [99]. Despite a recent publication suggesting that it is an effective method for preserving fertility [100] and offering real possibilities for future motherhood, it is still considered an experimental technique. Consequently, oocyte vitrification has become the technique of choice for FP on adult patients, being the best option to maintain women reproductive autonomy [100].

Oocytes Vitrification in Oncological Patients

The first case reported in Europe of a pregnancy after FP using oocyte vitrification was from our group in a patient, whose ovarian cortex was cryopreserved [101]. After grafting, four stimulation cycles were performed to accumulate and vitrify mature oocytes, leading to a successful twin pregnancy. Since then, several studies have reported clinical results with cryopreserved oocytes for FP in cancer patients (Table 5).

In 2011, Kim et al. reported the birth of the first baby born after oocyte vitrification in a patient with chronic myeloid leukemia, whose oocytes were vitrified for FP before chemotherapy and radiotherapy, and they were stored for 9 years until the patient returned to seek a pregnancy [102].

García Velasco et al. published in 2013 the first clinical results of the IVI FP program. As for oncological patients, only four returned to use their vitrified oocytes, two of them becoming pregnant. Unfortunately, one suffered a miscarriage in week 6 of gestation. Therefore, only one who was recovered from a non-Hodgkin lymphoma managed to become a mother [103]. A year later, Alvarez et al. reported on the birth of a healthy newborn with devitrified oocytes in a young patient with ovarian carcinoma [104]. The same year da Motta et al. reported a successful delivery of a patient who had overcome breast cancer and whose oocytes had been vitrified prior to treatment and stored for 6 years [105].

In 2014, Martínez et al. published the obstetric results of the first pregnancies achieved after FP due to oncological causes using oocyte vitrification [106]. Of the 493 women who consulted for FP, 357 cryopreserved their oocytes and 11 returned after being cured for IVF treatment. Four pregnancies were achieved with healthy newborns [106]. Two years later, Perrin et al. reported the first live birth in France after an FP in an oncological patient [107]. A very recent study published by our group shows the outcome achieved by 80 patients who returned to use their oocytes vitrified prior to the oncological treatment in the largest series published so far (in all, 1077 women choose oocyte vitrification for FP prior to the oncological treatment) [95]. Overall survival rate was 81.8% and cumulative live birth was 35.2%. Cancer patients showed poorer reproductive outcome when compared to elective freezers [95].

Table 5 Live birth reported after fertility preservation in cancer patients: slow freezing and vitrification of oocytes

	Sánchez Serrano et al. 2009 [101]	Kim et al. 2011 [102]	García-Velasco et al. 2013 [103]	Álvarez et al. 2014 [104]	Da Motta et al. 2014 [105]	Martinez et al. 2014 [106]	Perrin y col. 2016 [107]
Type of malignancy	Breast cancer	Chronic myeloid leukemia	Non-Hodgkin lymphoma	Invasive ovarian carcinoma	Breast cancer	Non-Hodgkin lymphoma, breast cancer	Hodgkin lymphoma
Cryopreservation technique	Combined OTC-SF + OV (Cryotop)	Vitrification (EMG)	Vitrification (Cryotop)	Vitrification (Cryotop)	Vitrification (Cryotip)	Vitrification (Cryotop)	Vitrification
Age at FP	36	22	31	28	36	33/30/33/37	36
No. of cryopreserved oocytes	16	7	4	14	28	4/5/3/8	5
Storage time (years)	2	9	2	1	6	2/3/5/3	2
Pregnancy	Twin	Single	Single	Heterotopic	Triplet	Single (4)	Single
No. of live births	2	1	1	1	1	1 (4)	1
Weeks of gestation	34	35 + 3 days	39	38	–	40/40/40/38	37,5
Weight of babies	1650 and 1830	2410	3440	2650	2970	3440/2850/3220/2950	3180
Sex of baby	Males	Male	Male	Male	–	–/male/female/male	Female

Vitrification of Oocytes in Non-oncological Patients

Oocytes cryopreservation is the most valuable option for women who want to delay motherhood, an option that is becoming increasingly frequent, especially in developed countries and in women at advanced age, reproductively speaking, who want to become pregnant. In fact, the maternal age of the first pregnancy has continuously increased since the 1970s [97]. Different reasons are behind a new lifestyle; the most frequent causes are the difficulty of finding a suitable partner or the inability to find a well-paid job. Without a doubt, the sociocultural environment leads women to seek the personal, professional, and economic stability before embarking on motherhood [3]. Consequently, the possibility of cryopreserving their gametes may provide them with extended period before starting the project of becoming mothers.

In 2016, we published a study describing in detail our experience with oocyte vitrification as an elective FP approach [97]. After analyzing the distribution of cycles in this population, we found that many women who came to cryopreserve oocytes were motivated by age-related issues (94.2%). While 2.1% of them did it for the diagnosis of endometriosis, 1.7% of them due to a decreased ovarian reserve and 1.6% needed ovarian surgery. Other reasons were behind the remaining 0.5%. When we analyzed demographic characteristics of these patients, we found that their profile was quite interesting. Most of the women were single heterosexuals (75.6%) and mostly reported a high-level education.

The data showed us a clear and strong effect of female age on the outcomes as it happens in the fresh cycles. In the younger group, more oocytes were recovered and vitrified, and better survival rates (94.6% vs. 82.4%) and pregnancy (61.5% vs. 31.8%) were obtained compared to women with >35 years. The increase in chromosomal abnormalities with maternal age correlates strongly with the decrease in embryonic viability, and it is considered that almost 80% of the oocytes are aneuploid at the age of 40 years. Unfortunately, many patients came to preserve their gametes from the age of 37, thus exceeding the optimal range to obtain better results after IVF treatment. In fact, it was shown the impact of oocytes number used according to the age of the patient, being necessary a greater number of them to obtain a live newborn in the case of older women (≥36 years). The results led us to suggest that, as a minimum, at least 8–10 mature oocytes should be vitrified to obtain a reasonable success rate, and in older women this individualized treatment should be administered considering the possibility of using a genetic diagnostic treatment (PGT-A).

A recent study published by our group, which summarizes the largest casuistry published to date, confirms these findings providing valuable information on the determinants of reproductive success in FP patients [95]. This study included around 6300 women who performed more than 8000 cycles of FP, of which 700 returned to seek pregnancy, achieving 162 healthy babies in the EFP group and 25 in Onco-FP group. Age was the main motivation in the EFP group. In fact, we have witnessed a dramatic increase in the number of EFP cases over a decade from 2% to 22% of vitrification procedures in our clinics [95]. This growing demand in EFP reflects the increasing evidence for the possibility of achieving pregnancy in this group of patients and is probably due to more women are aware of age-related fertility decline issues. Contrary to what happened in the EFP population, the demand for FP in cancer patients represented only 2% of all oocyte vitrification procedures and was steady over a 10-year period. This could be because of the number of women diagnosed with cancer is being smaller than comprises women who opt for EFP. Additionally, part of the reason could also lie in oncologists' attitudes toward FP, most probably due to misinformation about the approach.

Factors that Affect Clinical Outcomes in Elective and Oncological FP

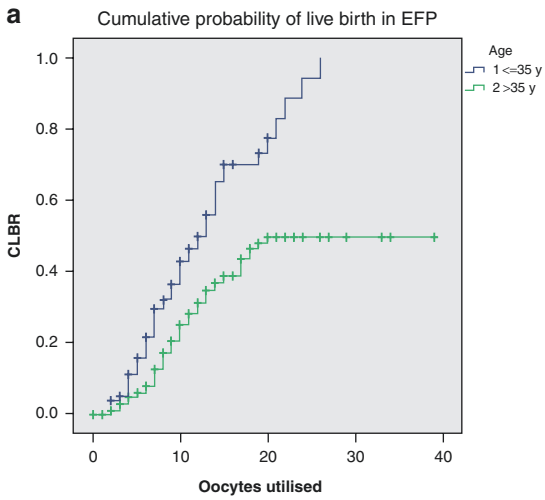
Due to the increasing interest in preserving fertility, it is crucial to be aware of the factors associated with clinical outcomes in order to provide patients with proper counseling. In this line, in our last study, we analyzed the impact of the indication for FP on the results by comparing IVF data, oocyte survival, clinical results, and live birth rates, and in how factors such as the stimulation protocol, number of oocytes available, indication, and the age of the patient impacted the outcome [95].

Special attention was drawn to age as it is recognized as one of the strongest confounders in assisted reproduction. In this study, 70% of EFP women were older than 35 years and 15% were aged ≥40 years by the time of vitrification. The distribution was the opposite in the Onco-FP group, where 70% of the patients were younger than 35 years. This fact confirmed our previous observations, showing the impact of the number of utilized oocytes according to patients' age at vitrification.

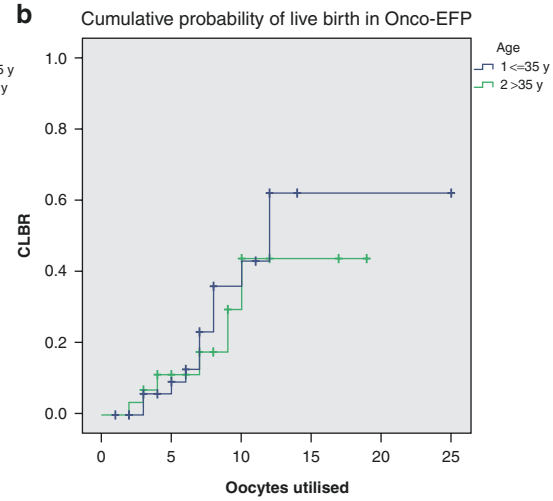
The most widely used protocol for ovarian stimulation in the EFP was the antagonist, while the antagonist and letrozole was used in many cancer patients. The reasons for using different COS protocols obey the presence of two very different populations. Nevertheless, we observed more retrieved and vitrified oocytes when the antagonist protocol was administered to both EFP and Onco-FP patients. The efficiency with letrozole was associated with lower levels of estradiol observed in Onco-FP, which confirms that it may be applied safely [108]. Differences for ovarian response were not observed in the number of retrieved oocytes when the letrozole-based protocol was used in the Onco-FP group. However, fewer MII oocytes were vitrified in the cancer patients stimulated using letrozole versus cancer patients treated with the antagonist protocol, which is consistent with our previous observations [109]. Probably, the lower doses of gonadotropins used in these patients contribute to explain our MII yield, although the possible effect of the ovarian stimulation protocol remains to be ruled out [95].

Regarding the clinical results attained by FP patients when they returned, perhaps our most reliable finding was the poorer outcome achieved by cancer patients compared to who preserved fertility electively, especially when analyzed by age. The ~70% cumulative newborn rate in the EFP group for young patients (≤35 years), compared to ~40% in the age-matching Onco-FP population, was particularly noteworthy as it was the significantly lower oocyte survival in young cancer patients. All this made us speculate that the underlying disease in cancer patients could probably impair reproductive outcome. Although the effect of the sole presence of cancer on survival and the cumulative live birth rate was not statistically confirmed (OR for oocytes survival = 1.484 [95%CI = 0.876–2.252]; P = 0.202 and OR for cumulative live birth rat = 1.275 [95%CI = 0.711–2.284]; P = 0.414). The fewer cancer patients returning to use their oocytes may most probably explain the lack of confirmation of the impact of the disease on reproductive outcomes. Conversely, age strongly affected outcomes (adj.OR for survival = 1.922 [95%CI = 1.274–2.900]; P = 0.025 and adj.OR for CLBR = 3.106 [95%CI = 2.039–4.733]; P < 0.0001). By any means, the clinical results achieved by the Oncological-FP patients herein suggest the possible effect of underlying malignancy [95].

Finally, the impact of the number of utilized oocytes according to patients' age at vitrification was confirmed. According to our findings, young Elective-FP patients (≤35 years) with 8–10 oocytes yield a cumulative probability of having a baby (CLBR) of ~30% and 45%, respectively, which may be considered a reasonable success rate. Furthermore, with ~25 oocytes, the cumulative probability rises to ~95%, which reveals the great impact of increasing the number of oocytes available in



Age ≤35. N = 123		Age >35. N = 518	
N°oocytes	CLBR(95%CI)	N°oocytes	CLBR(95%CI)
5	15.8 (8.4-23.1)	5	5.9 (3.6-8.3)
8	32.0 (22.1-41.9)	8	17.3 (13.3-21.3)
10	42.8 (31.7-53.90)	10	25.2 (20.2-30.1)
15	69.8 (57.4-82.2)	15	38.8 (32.0-45.6)
20	77.6 (64.4-90.9)	20	49.6 (40.7-58.4)
24	94.4 (84.3-100.4)		



Age ≤35. N = 42		Age >35. N = 38	
N°oocytes	CLBR(95%CI)	N°oocytes	CLBR(95%CI)
5	9.1 (-0.7-19)	4	11.1 (-0.8-23.1)
8	35.8 (14.3-57.2)	9	29.3 (3.7-54.8)
10	42.9 (19.7-66.1)	10	43.4 (11.3-75.3)
12	61.9 (35.4-88.5)		

Fig. 2 Kaplan–Meier plotting of cumulative probability of live birth (CLBR) for elective fertility preservation (EFP) (**a**) and Onco-FP (**b**), according to the number of oocytes consumed and patients' age at vitrification (≤35 year, in blue). Overall comparisons (log-rank (Mantel–Cox), Breslow (generalized-Wilcoxon) and Tarone–Ware) for EFP = $p < 0.0001$. Log-rank (Mantel–Cox); P = 0.577; Breslow (generalized-Wilcoxon); P = 0.833; and Tarone–Ware; P = 0.703 for onco-FP group. Tables below the figures slow CLBR and 95%CI according to the number of oocytes consumed in each case [95]

younger patients. Although this is also true for older patients, the impact of adding oocytes is much weaker (Fig. 2) [95].

Definitions

- *Vitrification:* Physical phenomenon by which an aqueous solution solidifies without ice formation turning into a glassy state.
- *Chilling injury:* Irreversible cell damage following exposure to low temperatures, from +15 °C to −5 °C before the nucleation of ice.
- *Cryoprotectant agents:* Substances that prevent cellular damage caused by freezing. Cryoprotectants can permeate into the cell or act as osmotic agents from the extracellular space.
- *Fertility preservation:* Strategy used to safeguard fertility, useful in women whose reproductive capacity is threatened due to a drastic reduction in ovarian reserve, whether due to a medical condition, gonadotoxic treatment, or age.

Practical Clinical Tips

- Increased social awareness about the impact of age on fertility is highly advisable when elective fertility preservation is considered.
- In order to avoid unrealistic expectations, women should be informed about their individual chances of oocyte survival, which depends heavily on their age, and the possibilities of having a live birth according to the number of frozen eggs.

Take Home Messages

- Vitrification brought efficient and reproducible outcomes to the clinical practice, which has allowed the establishment of eggs-banks that are currently benefitting infertile patients and women who are seeking to preserve their fertility or oocyte recipients enrolled in egg-donation programs.
- Egg banking has easier the logistics of donation programs: there is no need for synchronization between the donor and the recipient, elimination of without the waiting lists, and the absence of the quarantine period.
- The availability of an efficient embryo cryopreservation program also allows the practice of single embryo transfer as a strategy to minimize the incidence of multiple pregnancy rates, as well as IVF segmentation by allowing the freeze-all strategy.
- Age is the most powerful factor that affects outcome in FP cycles.
- The number of oocytes used by the patient is closely related to the success of the treatment, with a considerable advantage in the result by adding a few oocytes, especially in young patients with EFP.
- The type of ovarian stimulation has no clear impact on outcomes.
- Cancer patients seem not to "benefit" from young age in terms of reproductive outcome.
- Cancer patients achieve lower outcome, but the effect of the disease needs further clarification.

Clinical Cases

- Case 1

  A 31-year-old woman diagnosed to have staged III breast cancer was referred to our fertility clinic for oocyte cryopreservation prior to starting oncological treatment. Detailed counseling regarding the methodology and experimental nature of the technique was done. After checking the oncologist's report, oocyte vitrification cycle was programed using an antagonist protocol with letrozole. Letrozole (5 mg/day; Femara; Novartis, Switzerland) was initiated on day 2 of the cycle and was maintained until oocyte retrieval. After 2 days of letrozole administration, rFSH was added (150 IU). When the leading follicle reached ≥14 mm, 0.25 mg/day of the GnRH antagonist was added. Final oocyte maturation was triggered with a single bolus of a GnRH when the leading follicle reached >20 mm 1 day later than when letrozole was not used. Oocyte retrieval was scheduled 36 h after triggering and letrozole was reinitiated until menstruation appeared. A total of 11 oocytes were retrieved of which 9 at MII stage. Vitrification was performed following the routine laboratory protocol. Once oocytes were vitrified, they were stored in a vapor liquid nitrogen tank for unlimited time. After 5 years and 3 months, when the disease was overcome, the patient came back to attempt pregnancy. All the vitrified oocytes were warmed up at once, among which seven survived and were microinjected (ICSI) using cryopreserved partners' semen sample. Five

oocytes fertilized. On day 5, two embryos developed to good quality blastocyst stage (class B). One of them was transferred, while the remaining supernumerary embryos were vitrified.

Pregnancy was not achieved and the couple was counseled to proceed with the frozen embryo transfer, which was carried out 3 months later. The endometrium was prepared using estradiol valerate and progesterone. A serum beta hCG performed 13 days later confirmed the pregnancy (320 mUI/ml). A baby boy weighing 3450 g was born in 39th week of gestation.

- Case 2

A 35-year-old single woman came to our center seeking for fertility preservation. Her ovarian reserve was estimated by her AMH level (7.4 pmol/L). The option of oocyte vitrification was proposed, estimating that approximately six MII oocytes would be recovered per stimulation cycle. The woman was informed about her individual probabilities of live birth according to the number of vitrified oocytes: according to our experience, 10–15 oocytes would give a 40–70% chance of having a baby. Therefore, the clinician recommended two stimulation cycles. Ovarian stimulation (OS) was initiated on day 2 of a spontaneous cycle. An initial dose of 225 IU recombinant FSH (rFSH) (Gonal-F, Merck-Serono, Spain; Puregon, MSD, Spain) and 150 IU highly purified hMG (Menopur, Ferring Pharmaceuticals, Spain) were administered until triggering. When a leading follicle reached ≥14 mm, a GnRH antagonist (Cetrotide, MerckSerono; Orgalutran, MSD) was administered at 0.25 mg/day. Final oocyte maturation was triggered with a single dose of a GnRH agonist (0.1 mg of Decapeptyl, Ipsen Pharma) when the mean diameter of two follicles was ≥18 mm. Oocyte retrieval was scheduled 36 h later. A total of 12 oocytes were retrieved (7 oocytes were at MII stage and 5 were GVs). MII oocytes were vitrified and stored. Three months later, the patient underwent another vitrification cycle following the same ovarian stimulation protocol. Nine oocytes were retrieved of which 8 MII were finally vitrified.

Three years later, the patient returned to attempt pregnancy with her vitrified oocytes. Thirteen oocytes survived and were subsequently microinjected using semen bank sample. Ten oocytes were fertilized. On day 5, a good quality blastocyst was transferred and three more were vitrified. Two additional blastocysts were available for vitrification on day 6. Pregnancy was achieved and the ultrasound revealed a singleton pregnancy. A healthy baby boy weighing 3.1 kg was delivered by cesarean section at 39th week of gestation.

Key Readings

– Cobo A, Garcia-Velasco JA, Coello A, Domingo J, Pellicer A, Remohi J. Oocyte vitrification as an efficient option for elective fertility preservation. Fertil Steril, 105:755–64 e8, 2016.
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Ovarian Stimulation for Fertility Preservation (Different Protocols)

Lilli Zimmerman, Stephanie Willson, Robert Setton, and Glenn Schattman

Patients who want to preserve their potential for having genetic children *in the future* represent a unique and increasingly expanding cohort of consultations seen at fertility centers around the world. Regardless of the indication—whether it is a cancer diagnosis requiring urgent life-saving treatments that will damage the ovaries, chronic illness like lupus or sickle cell disease, or planned oocyte cryopreservation to counteract the effects of the natural decline in fertility that occurs due to aging—the approach utilized for ovarian stimulation has unique considerations relative to the approach used when the goal is imminent pregnancy. The most common methods of fertility preservation in postmenarchal females currently include controlled ovarian stimulation (COS) for oocyte or embryo cryopreservation [27]. COS has even been reported to be successful for fertility preservation in a pre−/peri-menarchal teenage cancer patient [24] and may thus be utilized in this pediatric population as well. In some cases, ovarian stimulation must be performed when elevated levels of estradiol commonly seen may be harmful to the individual (e.g., breast cancer or a hypercoagulable state). Ovarian stimulation for fertility preservation may be accomplished utilizing a variety of individualized and specialized protocols depending upon each patient's unique clinical circumstances. In this chapter, we will discuss the many unique and individualized considerations surrounding the selection of ovarian stimulation protocols for patients undergoing fertility preservation for a variety of indications.

The ultimate goal of ovarian stimulation is to optimize mature oocyte yield and quality in a safe manner and, in the case of cancer patients, often on a tight time schedule. As long as the option exists for chemotherapy or radiation to be delayed by approximately two weeks, women desiring fertility preservation may choose to undergo ovarian stimulation for the purpose of cryopreservation of oocytes or embryos. With infertility patients, waiting for the early follicular phase to start ovarian stimulation—in order to synchronize endometrial development with follicular maturation, allowing for fresh embryo transfer—is commonplace. Most reproductive endocrinologists feel quite comfortable proceeding with ovarian stimulation using this approach, which follows a logical pattern consistent with the natural ovarian life cycle. However, cancer patients can get diagnosed or present at any time during their menstrual cycle. That is okay, as we now understand that follicular development does not initiate only at a specific time in a woman's menstrual cycle; rather, it occurs continuously and incessantly from before birth until

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menopause. Given that COS specifically for fertility preservation does not involve an embryo transfer in that same menstrual cycle, endometrial development is irrelevant. From our own work and that of others, "random-start" stimulation cycles (cycles that are initiated at any phase of a woman's cycle, i.e., early or late follicular, peri-ovulatory, or luteal) yield similar outcomes with regard to total as well as mature oocyte yield [4, 20, 21, 23, 32]. This allows for immediate initiation of ovarian stimulation as soon as the patient is seen and cleared by the oncologist, thus minimizing the time from first visit to oocyte retrieval and subsequent medical treatments.

Due to the limited window of time before the initiation of potentially gonadotoxic chemotherapy or radiation treatments, the ability to begin ovarian stimulation at any point in a woman's menstrual cycle is critical to minimizing the delay in life-saving treatments and optimizing outcomes. Since patients are often able to complete just one or occasionally two cycles of stimulation prior to initiating cancer treatment, *"getting it just right"* from the outset is a high priority. Complicating the matter even further is that some patients are diagnosed during pregnancy; this unique situation will also be discussed in this chapter.

Traditional Ovarian Stimulation Protocols

Stimulation protocols utilized in COS for assisted reproductive technology (ART) fall into specific categories (luteal GnRH agonist protocols, flare protocols, antagonist protocols, etc.) and have been extensively described in the literature [8, 25]. The basic principle of COS protocols involves ovarian stimulation with either endogenous or exogenous gonadotropins, along with suppression of the hypothalamic–pituitary–ovarian axis to prevent premature ovulation using either a GnRH agonist or antagonist. The use of these agents is to promote multi-follicular development, resulting in increased oocyte yield compared to a natural, unstimulated cycle where typically a single follicle develops.

GnRH Agonist Protocols

Historically, early COS protocols did not benefit from ovulation suppression, and outcomes were quite poor compared to today's standards. Premature ovulation due to elevated estradiol (E2) levels was not uncommon, leading to reduced oocyte yields. The development of GnRH agonists allowed for greater control over the developing follicles and reduced the incidence of premature ovulation. It also allowed oocyte retrievals to be timed more conveniently! Because the agonists produce a flare of gonadotropins when initiated, the GnRH agonists were initiated in the mid-luteal phase of the cycle preceding stimulation. Pituitary suppression from continual exposure to GnRH analogues usually occurs after ~5–7 days of treatment, followed by menses and pituitary suppression. Gonadotropin administration could begin any time after the initiation of menstruation and, as long as the GnRH agonists were continued, premature ovulation would not occur. Stimulation of follicular development continues until lead follicles reach approximately 17 mm, at which time an hCG trigger is administered (mimicking the luteinizing hormone [LH] surge) for final oocyte maturation. This is commonly referred to as the GnRH agonist "long" protocol [8].

High responders have an increased risk of ovarian hyperstimulation syndrome (OHSS) with this protocol since hCG must be administered for final oocyte maturation. Additionally, poor responders may not respond as well due to profound pituitary suppression from the GnRH agonist and a need to overcome this with higher doses of exogenous gonadotropins. Additionally, due to the delay in treatment while waiting for the right time of the menstrual cycle to initiate the GnRH agonist and pituitary suppression, this protocol is rarely utilized for fertility preservation patients, especially cancer patients who cannot delay treatments (Fig. 1).

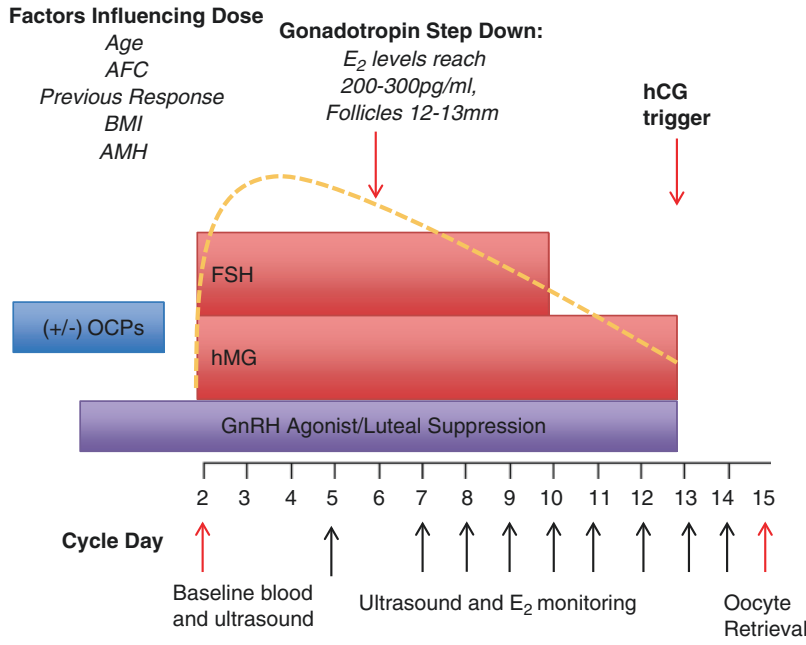

Fig. 1 GnRH agonist "long" protocol. Figure adapted from Huang and Rosenwaks, Assisted Reproductive Techniques. In: Rosenwaks Z and Wasserman P, *Human Fertility: Methods and Protocols*. Humana Press, Springer Science and Business Media, 2014

GnRH Antagonist Protocols

Since the introduction and improved tolerability of injectable GnRH antagonists, a more common protocol today involves aligning the initiation of gonadotropin administration with the beginning of the follicular phase (menstrual cycle day 2 or 3). A GnRH antagonist is then added to the regimen mid-cycle (either at a predetermined cycle day or when a specific E2 level or follicle size is reached) to suppress an intrinsic pituitary LH surge to prevent premature ovulation [8]. Common thresholds for initiation of the GnRH antagonist are follicular size >13 mm or estradiol level >300 pg/mL. Trigger for final oocyte maturation with hCG and/or GnRH agonist is then administered when at least two follicles reach 17–18 mm in size and is administered at ~35 h prior to scheduled oocyte retrieval.

The development of the GnRH antagonist protocol has many benefits for both infertility patients undergoing in vitro fertilization (IVF) as well as patients undergoing COS for fertility preservation. Unlike GnRH agonists, which cause an initial gonadotropin-stimulatory flare effect prior to pituitary suppression, GnRH antagonists cause an immediate pituitary down-regulation, and thus there is no lag time in efficacy. This shortens the total duration of injections for the patient and thus the time from initiation of treatment to oocyte retrieval date; hence, this is typically referred to as the "short" protocol [25].

An additional major benefit of the short protocol for patients undergoing COS for fertility preservation is that it allows the option of utilizing either a dual trigger with GnRH agonist plus lower-dose hCG or a GnRH agonist-only trigger for final oocyte maturation. This mitigates the risk of OHSS in this medically vulnerable population and provides a safer way to proceed with COS for patients necessitating medical treatment shortly after oocyte retrieval. Additionally, a quicker return to baseline allows for back-to-back stimulation cycles in patients with time to do so. The rationale for and benefits of using a GnRH agonist trigger for fertility preservation will be discussed in more detail later in this chapter (Fig. 2).

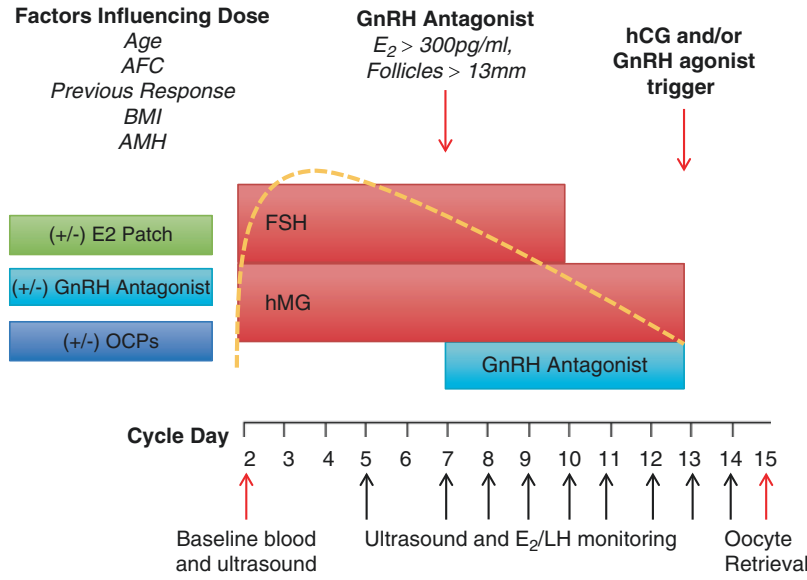

Fig. 2 GnRH antagonist "short" protocol. Figure adapted from Huang and Rosenwaks, Assisted Reproductive Techniques. In: Rosenwaks Z and Wasserman P, *Human Fertility: Methods and Protocols*. Humana Press, Springer Science and Business Media, 2014

Random-Start Protocols

Ovarian stimulation for fertility preservation patients allows for unique approaches that test the limits of our understanding of ovarian physiology. This is especially true when there is an urgent medical treatment planned that will affect future ovarian reserve and fertility, and the window of time to preserve fertility is shortened. Fortunately, evidence and experience over the past two decades have revealed that there is flexibility in the initiation of gonadotropins for ovarian stimulation when the goal is recruitment of follicles and mature oocytes, not pregnancy. It has been hypothesized that there are at least three major follicular recruitment waves throughout the menstrual cycle [3]. We believe there are not actual waves but continuous development of primordial follicles, allowing for "random-start" ovarian stimulation regardless of where the patient is in her menstrual cycle. This, in turn, limits any delay in initiating both the fertility preservation COS cycle itself as well as cancer treatment [5].

Random-start protocols initiate gonadotropin stimulation at any day of the current menstrual cycle, which could be late follicular (after dominant follicle recruitment) or even in the luteal phase. Aside from the timing of initiation, this protocol is similar to the short protocol described previously, with GnRH antagonist added based either on the presence of a follicle >13 mm (CRMI criteria) or laboratory evidence of an elevated estrogen level (>300 pg/mL in our center). Occasionally, these criteria are met earlier in the COS cycle than during a traditional protocol, and thus the patient takes the GnRH antagonist for a longer time in the COS cycle. In fact, in some centers, GnRH antagonists are not even used if the patient initiates gonadotropin stimulation during the luteal phase of her cycle as LH surges are rare in the presence of progesterone [6]. While this may save some money as GnRH antagonists are not inexpensive, the potential of a premature surge and lower than expected oocyte yield or cancellation of a cycle in a cancer patient's only shot at preserving her fertility does not make sense; thus, we advise all fertility preservation patients to utilize GnRH antagonists to prevent this from occurring. While it has also been shown that a random-start protocol may add 1–2 days of additional stimulation to the whole cycle compared to the traditional early follicular start, the ability to stimulate immediately and initiate chemotherapy treatment without delay clearly outweighs the small additional cost of 1–2 days of gonadotropins [21, 23].

There have been multiple case reports demonstrating that this type of random-start protocol—

initiating stimulation at a random time in the menstrual cycle—is not only feasible, but has similar success rates to conventional day 2 stimulation starts. Oocyte yield, maturity rates, and fertilization rates are comparable, demonstrating that oocytes retrieved during random-start cycles are equally competent to lead to a successful pregnancy when they are utilized in the future [5, 12, 16, 19, 31].

While the above outline may make it seem easy to initiate ovarian stimulation at any time and standardize the protocols across the board, there are many nuances to getting it "just right" in these patients. Gonadotropin dose must be individualized based on the patient's anticipated ovarian reserve (AFC and AMH if not on hormone suppression), age, body mass index (BMI), and any history of prior ovarian stimulation. This random-start protocol can be utilized both in patients with high ovarian reserve as well as those with low apparent reserve (low antral follicle count, low AMH). Additionally, in our center, we will not initiate gonadotropin stimulation in patients if they are late follicular. When a patient presents with a dominant follicle that is ≥15 mm and an estradiol level consistent with a dominant follicle, we administer a GnRH agonist trigger (leuprolide acetate, 4 mg) in order to ovulate the dominant follicle. This follicle may also be retrieved (this may be advisable in the case of patients with diminished ovarian reserve) and, if mature, cryopreserved, adding to the total oocyte yield. Gonadotropin stimulation is then initiated 2 days after retrieval in the induced "luteal phase" of the cycle. This protocol was developed by us based on historical data in patients undergoing in vitro maturation (IVM) cycles [26, 29]. In those studies, patients with a dominant follicle at the time of HCG administration (>15 mm) had poorer embryo quality and worse outcomes compared to patients whose trigger was performed prior to the development of a dominant follicle of 15 mm in size. This may indicate that decreasing serum gonadotropin levels seen with the selection of the dominant follicle with regression of the smaller follicles leads to atresia of these small follicles beyond the point of recovery. Triggering ovulation with 4 mg leuprolide acetate and waiting 2 days to start stimulation may allow for healthier small follicles not already atretic to be recruited with the subsequent gonadotropin stimulation. This method is still more rapid than conventional day 2 early follicular starts and prevents significant delays for the patient both in terms of fertility preservation and their cancer treatment.

While particularly useful for cancer patients, this random-start protocol can be utilized for any patient undergoing COS for fertility preservation, such as those needing to schedule around planned travel or work commitments. This protocol can also be used for oocyte donors, many of whom have intrauterine devices (IUD) in place. Their IUDs may remain in place throughout stimulation, and this has the added benefit, for instance, of enabling timing of their stimulation cycles with their oocyte recipients during a programmed cycle.

Recently, our group published a case report of a patient who underwent successful fertility preservation with cryopreservation of multiple embryos shortly after emergency termination of a pregnancy due to a diagnosis of breast cancer [22]. Stimulation was initiated 5 days after termination of a 5-week gestation pregnancy, at which time the serum beta-hCG level was 119.8 mIU/mL. Due to the half-life of hCG, it was anticipated that the levels would continue to fall to values that would not cause premature luteinization of the developing follicles during stimulation. HCG levels >30 mIU/ml with follicles >12 mm would be considered thresholds that cause premature luteinization and poor oocyte quality. This patient's COS cycle consisted of 11 days of stimulation, after which 29 oocytes were retrieved, of which 17 were fertilized and 10 were cryopreserved as blastocysts. This case highlights the feasibility of a random-start protocol even shortly after termination of pregnancy, when time is of the essence for patients with new cancer diagnoses who must start oncological treatment as soon as possible but also value their future fertility.

With random-start cycles, patients should be counseled that while there is additional flexibility for their COS cycle and the outcomes are equivalent after retrieval, the one major downside is an

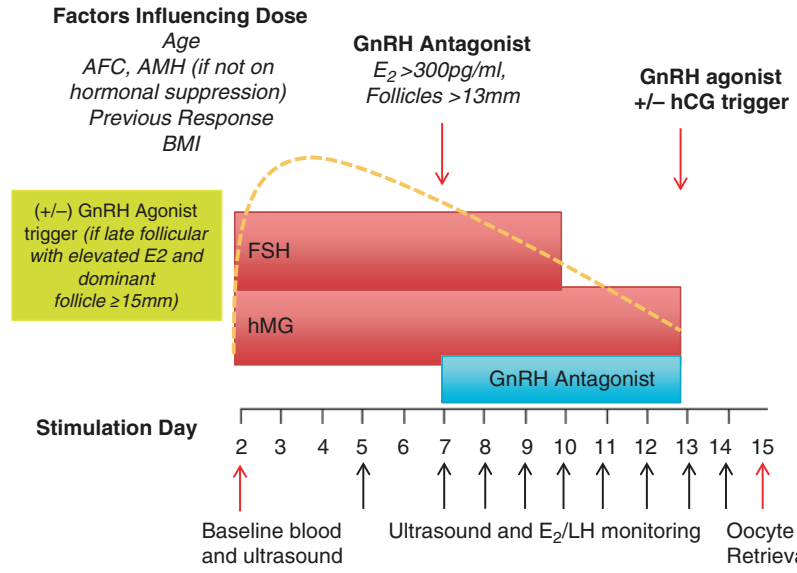

Fig. 3 "Random-start" protocol. Figure adapted from Huang and Rosenwaks, Assisted Reproductive Techniques. In: Rosenwaks Z and Wasserman P, *Human Fertility: Methods and Protocols*. Humana Press, Springer Science and Business Media, 2014

additional 1–2 days of medication prior to oocyte retrieval. Furthermore, patients should be counseled that if they do start stimulation during the late follicular or luteal phase, it is possible and likely that they will experience vaginal bleeding during their stimulation cycle prior to retrieval. While disconcerting to the patient, this will not affect oocyte development (Fig. 3).

Tamoxifen Protocols

While not commonly used today due to the superiority of other available protocols, it is notable to mention tamoxifen as a medication used for ovarian stimulation in breast cancer patients as well as any patient with an estrogen-sensitive disease [14, 17]. Tamoxifen is a non-steroidal triphenylethylene estrogen receptor modulator similar to clomiphene citrate and originally developed in the UK as a contraceptive. It was found to initially stimulate follicle growth and was also used as an ovulation induction agent in Europe. When tamoxifen was found to have suppressive effects on breast cancer, it became popularly used (and still is today) for neoadjuvant hormonal treatment in patients with estrogen (E2) receptor–positive breast cancers. Given these overlapping utilities—blocking the estrogen receptor with suppression of tumor cells requiring estrogen for growth and elevating FSH levels leading to follicle development—tamoxifen was suggested for COS in breast cancer patients desiring fertility preservation as well as patients who were disease-free after treatment and desiring pregnancy. Supraphysiological E2 levels seen from multifollicular recruitment were of less concern due to the blockade of the E2 receptor with tamoxifen. Using a protocol of 40–60 mg/day of tamoxifen, with the addition of gonadotropins and GnRH antagonist, tamoxifen was found to yield higher numbers of oocytes and embryos compared to the natural cycle [17]. At the time, natural-cycle, or unstimulated, IVF to retrieve typically a single follicle was the only option for many breast cancer patients desiring fertility preservation.

A later study by the same group compared tamoxifen IVF (Tam-IVF) to a combination of tamoxifen with gonadotropins (TamFSH-IVF) or to a combination of letrozole, a third-generation aromatase inhibitor, with gonadotropins (Letrozole-IVF). Tamoxifen was administered orally in a dose of 60 mg/day, either alone or with injectable gonadotropins based on protocol [18]. Letrozole was administered orally in a dose of 5 mg/day along with injectable gonadotropins. This study found a statistically significant increase in the number of embryos from both the

TamFSH-IVF and Letrozole-IVF protocols compared to Tam-IVF alone. However, letrozole is associated with lower peak estradiol levels and thus has gained relative popularity as a promising adjunct to gonadotropins for fertility preservation COS for many cancer patients.

Continuous Letrozole Protocol

The additional risks of ovarian stimulation in cancer patients and patients with certain chronic medical conditions include the theoretical risk of stimulation of estrogen-sensitive malignancies, as well as an increased risk of thromboembolic disease. In addition, any delay in treatment may be particularly detrimental to an individual's overall health if delaying essential cancer treatments will lead to a poorer outcome. Patients with breast cancer or other estrogen-sensitive malignancies, melanoma, endometriosis, and endometrial cancer, as well as BRCA gene mutation carriers and individuals with underlying prothrombotic chronic conditions, may be at particular risk by elevated estrogen levels [1, 2, 13]. For these patients, a COS protocol involving the potent aromatase inhibitor letrozole is recommended. Due to its mechanism of action of competitively preventing the final aromatization step of androgens to estrogens, letrozole results in the suppression of serum estradiol levels. Tempered estradiol levels have been observed even with multi-follicular growth and thus have major benefits for patients in whom significantly elevated estradiol levels would otherwise be worrisome.

Oral letrozole has been utilized for ovulation induction using a 5-day course of treatment similar to clomiphene citrate. By blocking estradiol production, endogenous gonadotropins are increased, leading to enhanced follicular recruitment. Letrozole is usually stopped once follicular recruitment is initiated, and the follicle will continue to develop independent of additional stimulation, usually after a 5-day course of treatment. In patients not attempting to achieve pregnancy where suppression of estradiol exposure is important, aromatase inhibitors may be continued until the end of stimulation (the point of final oocyte maturation with GnRH agonist or hCG trigger). We usually reinitiate the aromatase inhibitor (5 mg/day) in patients not attempting pregnancy the day following oocyte retrieval until menses starts to keep luteal estradiol levels suppressed. Since recruitment of follicles using an aromatase inhibitor alone is usually modest, the addition of gonadotropins to the aromatase inhibitors is common to improve response.

Letrozole protocols for estrogen-sensitive diseases have been described by us and others: the aromatase inhibitor is started in the early follicular phase to cause a rise in endogenous gonadotropins for 2 days, followed by co-administration of the aromatase inhibitor and gonadotropins (tailored to the individual patient based on BMI, AFC, and AMH) to augment the natural FSH rise. Both the aromatase inhibitor and gonadotropins are administered daily throughout the stimulation along with a GnRH antagonist until the day of trigger. The original description of this protocol utilized a standard dose of 5 mg/day; however, this protocol requires waiting for the initiation of letrozole until the early follicular phase. We evaluated a titrated or "sliding scale" approach to be initiated when the estradiol level reaches a predetermined threshold. The dose is then adjusted throughout the cycle based on estradiol levels in order to maintain levels within the physiological range (comparable to a natural menstrual cycle). Typically, we use a dose ranging from 2.5 to 7.5 mg/day or higher based on estradiol levels. In our practice (CRMI), the letrozole sliding scale is as follows: E2 < 150 pg/mL: no letrozole; E2 150–250 pg/mL: 2.5 mg letrozole; E2 251–350 pg/mL: 5 mg letrozole; E2 > 350 mg pg/mL: 7.5 mg letrozole. One very important difference between letrozole and standard gonadotropin cycles is the follicle size at which to trigger final maturation; trial and error has shown us that a mean diameter of 20 mm or greater is required to obtain mature MII oocytes. On the day of trigger, letrozole is withheld and may be restarted 1 day after oocyte retrieval. Letrozole is usually continued for about 2 weeks until menses starts, indicating low hormonal values. When looking at oocyte yield and quality (i.e., pregnancy rates), many studies have shown

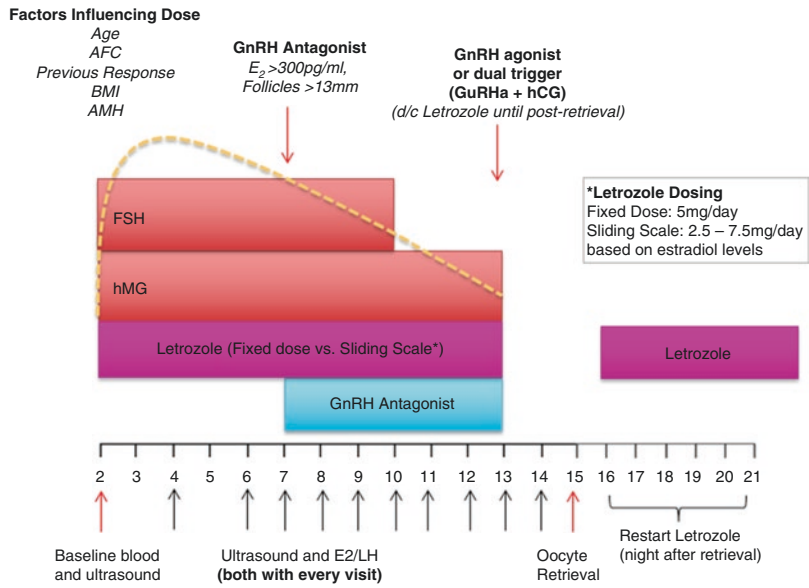

Fig. 4 Continuous letrozole protocol. Figure adapted from Huang and Rosenwaks, Assisted Reproductive Techniques. In: Rosenwaks Z and Wasserman P, *Human Fertility: Methods and Protocols*. Humana Press, Springer Science and Business Media, 2014

either comparable or increased numbers of oocytes and mature oocytes with letrozole plus gonadotropin protocols compared to standard gonadotropin-only protocols [10, 11, 20, 30].

An important difference with this protocol is that patients undergoing COS with continuous letrozole protocols must have *both* ultrasound and bloodwork performed each time they present for monitoring during their COS cycle. Bloodwork alone, as is typically performed occasionally during a COS cycle to assist with gonadotropin dosing adjustments, is useless in this scenario, as the serum estradiol levels do not correlate to follicular response and are thus not indicative of progress in a cycle. Dosing adjustments should be made on follicular response from ultrasound in combination with baseline characteristics used to determine dosing such as a patient's age, BMI, and baseline ovarian reserve. It has been suggested that continuous letrozole may lessen the duration or severity of OHSS if it does develop. However, despite suppressed serum estradiol levels, these patients are still at risk for developing OHSS if there is an extremely prolific follicular response and should still be monitored closely to ensure optimal health conditions prior to initiation of any oncological treatments. Using a GnRH agonist trigger only in appropriately selected patients will also significantly reduce the probability of this occurrence. A common practice today is to eliminate the use of hCG for trigger entirely, and to administer a pure GnRH agonist trigger to patients undergoing fertility preservation [1]. Studies have shown that not only is a pure GnRH agonist trigger feasible but, in some studies, GnRH agonist triggers were found to have significantly higher rates of mature oocytes and thus fertilized embryos available for cryopreservation [16, 21, 23]. The pure GnRH agonist trigger we typically employ is a single 4-mg injectable dose of leuprolide acetate, and subsequent oocyte retrieval occurs at the standard 35 h post-trigger (Fig. 4).

Back-to-Back Stimulation Cycles

After completing a stimulation cycle, patients undergoing fertility preservation with cancer or other medical diagnoses may find that they still have time prior to the need to start gonadotoxic therapy. In some cases, and with the approval of their oncologist, patients who are not fully satisfied with the outcome of their first cycle may choose to delay their therapy in order to increase their number of oocytes/embryos cryopreserved. In these settings, it is possible to perform back-to-back stimulation cycles or restart stimulation

approximately 4 days following oocyte retrieval. In patients considering back-to-back cycles, it is especially critical to utilize pure GnRH agonist triggers as the ovaries return to a relatively normal appearance much quicker than with hCG triggers. When this is performed, stimulation is considered a "luteal start" and follows the same protocol as the random-start protocol described above. The ovaries will be hyperstimulated from their baseline appearance, often with multiple corpora lutea visible on each ovary. The parameters to start stimulation follow the same guidelines as the random-start protocol, with bloodwork and ultrasound checked on the start day. Clinically, it is important to ensure that the patient feels medically well to proceed with the back-to-back stimulation and is not hyperstimulated with OHSS.

Trigger Selection: GnRH Agonists

Equally as important as determining the stimulation protocol in a COS cycle is selecting an appropriate trigger for final oocyte maturation. Traditionally, final oocyte maturation occurred by administration of an hCG trigger. It is well established that hCG triggers, due to their prolonged half-life, may contribute to the evolution of severe OHSS in patients at risk, a condition that is particularly worrisome in this medically vulnerable patient population. This is largely due to its long half-life and ability to induce vascular endothelial growth factor (VEGF), which increases vascular permeability. In the setting of hyperstimulation with COS, this increased vascular permeability contributes to third spacing of fluids and is clinically manifested as intravascular hypovolemia and increasing ascites. Both hCG and LH have been shown to induce VEGF; however, because of the longer half-life of hCG of approximately 24 hours, compared to a half-life of 2 h for LH, the risks associated with an hCG trigger are greater than with native LH induced by GnRH agonists [7, 9, 15, 28].

In some settings, where there is a concern that patients may not respond adequately to a GnRH agonist trigger and the cycle would be lost, combinations of GnRH agonist and a small dose of HCG (ranging from 1000 to 3300 IU depending on risk factors for OHSS and BMI) may be appropriate. This has the benefit of even further mitigating risks of OHSS, as well as decreasing undesirable side effects related to excessive hCG administration.

Conclusions

Determining the COS protocols for fertility preservation depends largely on the indication for fertility preservation and the subsequent available timeline. When urgent medical treatment creates a narrow timeline for COS, we are fortunate that studies have revealed excellent outcomes with immediate-start or random-start protocols. Avoiding further delay in oncological treatment is of immeasurable benefit. Studies in breast cancer patients have shown similar disease-free survival over 10 years in patients who have undergone ovarian stimulation and those who did not, revealing that there is likely little downside to the short delay in initiating certain neoadjuvant chemotherapies, for instance, in order to enable appropriate timing for a COS cycle [1]. The addition of aromatase inhibitors, specifically letrozole utilized in a continuous manner, has additional utility and benefit in this population. The use of GnRH agonists further mitigates risks associated with COS and enables patients interested in COS for fertility preservation to proceed with minimal risk to their overall health and medical treatment plans. While coordination of COS efforts must be a deliberate multidisciplinary effort among patients, their families, oncologists, surgeons, therapists, and reproductive endocrinologists, it is becoming increasingly evident that not only is this effort feasible, but there are many options to achieve ovarian stimulation and fertility preservation in a safe and efficacious manner with limited delays or negative implications on overall medical care.

In summary, there exist a plethora of protocols to choose from when helping patients attain their fertility preservation goals safely and efficiently in the setting of new or chronic diagnoses and the prospect of gonadotoxic therapy.

- While technically feasible, the older, more traditional GnRH agonist "long" protocol is less utilized in this population due to the longer length of stimulation, as well as the elimination of the option to use a GnRH agonist for the trigger.
- If the timing of presentation to a fertility specialist and plan to start stimulation align with the patient's menstrual cycle, a traditional day 2 GnRH antagonist "short" protocol may be followed.
- When the timing does not exactly synchronize with the beginning of menses, which is more common than not, a "random-start" protocol may be utilized: beginning stimulation either in the late follicular or luteal phase, with similar overall outcomes.
  - This method has the major benefit in this population of avoiding unnecessary delays in potentially life-saving medical treatment.
  - If a patient has a dominant follicle ≥15 mm in size, a GnRH agonist (leuprolide acetate) trigger can be administered, with stimulation started 2 days later.
- For patients at particular risk of hyperestrogenic states (breast cancer, estrogen-sensitive malignancies, BRCA mutations, prothrombotic conditions), a continuous letrozole protocol may be used that tempers the estrogen levels while allowing multi-follicular development and similar final outcomes.
  - It is important to remember that an ultrasound must be performed at each monitoring visit as estradiol levels will not be indicative of response to gonadotropin stimulation.
  - A fixed-dose letrozole (5 mg daily) or a sliding scale (2.5–7.5 mg daily as described above) letrozole protocol may be utilized based on provider's preference.
- GnRH agonists, particularly leuprolide acetate, should be utilized for trigger when medically possible and appropriate to minimize risks of OHSS. If it is necessary to use hCG, the lowest effective dose, often in the setting of a dual trigger with hCG + GnRH agonist, should be used.

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In Vitro Maturation of Oocytes

Michel De Vos

Introduction

Oocyte in vitro maturation (IVM) is a laboratory tool in reproductive medicine defined as the maturation in vitro of immature cumulus–oocyte complexes collected from antral follicles. This concept of IVM was first described by Pincus and Enzmann [49] and further developed by IVF pioneer Edwards [16]. Edwards, who was intrigued by IVM during his entire career [18], observed that immature oocytes can reach metaphase II (MII) spontaneously in vitro upon removal from their follicular environment [17]. However, it was not before the early 1990s when the first healthy live births in human were reported [4]. The first successful pregnancy and birth from IVM in an anovulatory patient with PCOS was reported in 1994 [70]. To allow spontaneous meiotic maturation in vitro, cumulus enclosed oocytes have typically been cultured in a basic tissue culture medium or in media formulated for blastocyst culture, supplemented with a protein source (autologous patient serum or serum albumin), FSH, and often hCG, until they reach metaphase II stage, 24–48 h after aspiration [64]. However, these IVM culture media have not been specially designed to support the metabolic needs of the cumulus and the oocyte during maturation, although the molecular signaling cascades leading to the progress of meiosis in spontaneous IVM differ from those occurring during in vivo oocyte maturation in the follicle. As a consequence, the developmental competence of oocytes matured in vitro is generally lower compared to the competence of their in vivo counterparts. Oocyte maturation in vivo is a complex process involving the cytoplasm and the nucleus and is regulated through various cascades of signaling molecules that play a central role in the interaction between the oocyte, cumulus cells, and mural granulosa cells [28]. Cyclic nucleotides, cAMP and cGMP, are the key molecules controlling mammalian oocyte meiosis. In vivo, the preovulatory gonadotropin surge leads to a transient spike in cAMP in the somatic compartment of the follicle, which is crucial for the subsequent developmental capacity of the oocyte. However, standard IVM systems have so far not been capable of mimicking this cAMP surge in vitro. This has resulted in reduced clinical outcomes following IVM compared to conventional controlled ovarian stimulation (COS) in the setting of assisted reproduction [29, 36, 74]. The lower potential of oocytes that matured in vitro has also limited its use in fertility preservation for cancer patients. Indeed, very few live births have been reported after cryopreservation of oocytes that had been matured in vitro. The first live birth after IVM and oocyte cryopreservation has been reported using

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the slow-freezing method at the germinal vesicle (GV) stage of oocytes retrieved from conventional ovarian stimulation cycles [72]. In a recent publication, five more pregnancies were reported, resulting in live births after vitrification at the MII-stage following IVM of immature oocytes collected from hCG-primed IVM cycles [8]. In these patients, oocyte cryopreservation was not related to oncofertility. Only two live births have been achieved so far following vitrification and warming of oocytes that had been matured in vitro in a cancer patient (Grynberg et al., in preparation; Segers et al., in preparation).

Definition

In vitro maturation (IVM) of oocytes is the maturation in vitro of immature cumulus-oocyte complexes collected from antral follicles [16]. Oocyte collection may be preceded by the administration of FSH to promote follicle growth. However, when gona dotrophins such as hCG or GnRH agonists are administered with the intention to trigger oocyte maturation in vivo, then this cannot be considered as IVM [12]. Because cumulus cell support is crucial for oocyte maturation in vitro, only maturation of cumulus-enclosed oocytes should be advocated; IVM does not include the incubation of denuded oocytes. The latter practice is sometimes applied to immature oocytes that are collected after conventional ovarian stimulation and ovulation trigger. This practice is referred to as "Rescue IVM" and generally yields oocytes with poor developmental potential [27].

Technical Aspects of IVM

Strictly speaking, IVM involves the aspiration of immature oocytes from antral follicles after minimal or no exogenous gonadotropin administration. Follicle aspiration is typically performed when antral follicles are small (less than 10 mm diameter); in women with ovulatory cycles, the selection of a single dominant follicle is avoided to prevent a negative impact on the potential of oocytes from subordinate follicles. After a baseline ultrasound scan to rule out the presence of cysts or other pathology, serial ultrasound scans are scheduled to assess the growth of the antral follicles. Using a standard IVM system, oocyte maturation rates in vitro are generally lower than maturation rates of oocytes retrieved in a conventional IVF program after administration of an ovulation trigger, suggesting that a considerable proportion of immature oocytes from small antral follicles are still meiotically incompetent and would have required more time within their follicular environment to accomplish physiological nuclear and cytoplasmic maturation. Higher oocyte maturation rates can be obtained when a bolus of hCG is administered, typically 36–38 hours before oocyte retrieval [5]. In these cases, meiotic resumption is initiated in vivo and a proportion of oocytes are found to have reached metaphase II at the time of oocyte retrieval – these oocytes have completed meiosis in vivo and can readily be inseminated. As such, the hCG-triggered IVM system may represent a semantical contradiction, but it is applied more often than the non-hCG-triggered system, where all oocytes are at GV stage at the time of egg collection. Nevertheless, there is ongoing debate as to the most efficient clinical and laboratory protocol for patients undergoing IVM [12] and there is currently no robust evidence suggesting any preference for the non-hCG-triggered IVM system above the hCG-triggered system or vice versa. Nevertheless, recent developments have shown significant improvements in oocyte maturation rates and embryological outcomes, most notably of oocytes from small antral follicles, when oocytes are retrieved in a non-hCG-triggered cycle, incubated in a biphasic IVM system involving a prematuration step of meiotic arrest and cytoplasmic capacitation, followed by a second maturation step [59].

IVM in Patients with Polycystic Ovary Syndrome

Historically, IVM has typically been advocated as a mild approach-assisted reproductive technology in patients with polycystic ovary syndrome,

the most common endocrinopathy with impact on reproductive function in young women. In contrast with conventional ovarian stimulation (COS) protocols, IVM involves aspiration of cumulus-oocyte complexes (COC) from antral follicles instead of pre-ovulatory follicles [12]; hence, the oocyte retrieval is preceded by a shorter course of gonadotropins, although the role of exogenous FSH has been controversial [20, 71], and ovarian stimulation, if any, requires less frequent hormonal and ultrasound monitoring. Because of the increased number of follicles capable of growth in response to gonadotropins, patients with polycystic ovary syndrome (PCOS) are more prone to hormonal side effects related to COS compared with women who do not have PCOS. Therefore, patients with PCOS have historically been the target patient population for IVM [7]. Moreover, compared to normal responders, patients with PCOS have a significantly increased risk to develop the ovarian hyperstimulation syndrome (OHSS), the most common iatrogenic complication of COS. However, the development of strategies to dramatically reduce the risk of OHSS, notably elective vitrification of embryos instead of fresh embryo transfer [13], and the use of a GnRH agonist trigger for final oocyte maturation in a GnRH antagonist protocol [38], has mitigated the need for IVM as a strategy to avoid OHSS. Therefore, the loss of an incentive to further develop alternative, safe methods of ART, and the obvious lack of interest from pharmaceutical companies to invest in strategies that encompass reduced gonadotropin consumption has been a major impediment to the progress of IVM.

Nevertheless, although the risk of OHSS has been reduced significantly [30], high responders to ovarian stimulation, such as the majority of women with PCOS, still suffer from hormonal side effects when they undergo COS; for these women, IVM may be an attractive alternative approach. Moreover, IVF treatment can be very stressful for women and their partners, and may lead to treatment termination before a successful pregnancy is achieved [24]. In view of this, and in spite of the existence of OHSS-free controlled ovarian stimulation protocols, a subset of patients is still keen to embrace IVM as an alternative simplified, low-burden ART. To which extent these patients would accept a lower chance of pregnancy is currently unknown. On the other hand, IVF treatment in general and gonadotropins in specific are not reimbursed by public health schemes in many countries. Because of the reduced cost of IVM for the patient compared to COS in these countries, for example, in Vietnam, financial incentives can also play a role.

The cornerstone of an efficient IVM program is proper patient selection — women with PCOS are the best candidates for IVM as they yield sufficiently high numbers of immature oocytes to make up for the inherently lower efficiency of IVM compared to standard IVF [21, 32, 63]. The serum concentration of AMH, a biomarker that correlates well with the severity of the PCOS phenotype [14], is a strong predictor of the number of immature oocytes that can be retrieved by follicle aspiration, and, by proxy of oocyte number, AMH correlates with the probability of pregnancy after IVM [32]. Nevertheless, an AMH cutoff at which level the efficiency of IVM surpasses that of COS has not been established. There may be a learning curve for the physician to perform egg collections from small antral follicles and closed-circuit needle flushing systems have been developed to avoid blood clots in the aspirated follicular fluid, although the optimal technique of immature oocyte collection from antral follicles requires further study [55]. Finally, we have recently established in a retrospective study encompassing 320 patients with PCOS according to the Rotterdam diagnostic criteria that infertile patients with a hyperandrogenic PCOS phenotype who undergo IVM achieve a higher cumulative live birth rate per IVM cycle compared to their normo-androgenic counterparts (Mackens et al., submitted). Although this may be related to the higher yield of cumulus-oocyte complexes in women with the full PCOS phenotype compared with the milder normo-androgenic phenotype, the exact mechanism remains to be understood. Nevertheless, future developments of more physiological IVM culture systems may appear to represent a valid alternative to hormone-driven IVF treatments in a broader patient population.

The Role of IVM in Fertility Preservation

Although cryopreservation of embryos or oocytes is currently the only well-established technique for fertility preservation [46], ovarian stimulation to obtain mature oocytes may be contraindicated in a subset of cancer patients. There may not be sufficient time available for ovarian stimulation when an urgent start of cancer treatment is required or increased levels of estradiol levels as observed in COS cycles may need to be avoided in patients with hormone receptor positive disease. For these patients, IVM may be considered a potential alternative, although estradiol levels in COS cycles can also be mitigated through the addition of an aromatase inhibitor during ovarian stimulation [53].

Since antral follicles are present in the ovaries of women of reproductive age irrespective of the menstrual cycle, immature oocytes can be readily obtained from these antral follicles at any time during the cycle. This makes IVM a highly suitable technique to be applied in emergency situations when the start of gonadotoxic treatment is imminent. In a prospective study in 248 breast cancer patients who were planned to have neoadjuvant chemotherapy, Grynberg et al. demonstrated that immature oocytes can be obtained from antral follicles in the follicular and luteal phase of the cycle [31]. In this largest series of cancer patients undergoing IVM for FP so far, with a mean age of 31.5 ± 0.3 years, a mean number of 6.4 ± 0.3 mature oocytes were cryopreserved after IVM, which was comparable to the number of mature oocytes available for cryopreservation after standard ovarian stimulation with letrozole co-treatment in patients with breast cancer who were scheduled to have adjuvant chemotherapy. Nevertheless, based on a recent retrospective study in cancer patients, the developmental potential of in vitro matured oocytes is still inferior to that of oocytes that matured in vivo [9]. Oocytes can also be retrieved from extracorporeal ovarian tissue from cancer patients [52], to be matured in vitro, be fertilized, and result in live births [51, 73]. Cryopreservation of ovarian cortex, followed by ovarian tissue transplantation when the patient has become infertile after cancer treatment, is still considered experimental, although more than 130 live births have been reported following ovarian cortex transplantation procedures with a live birth rate per patient of 25% [15]. Ovarian tissue cryopreservation is the only available method of fertility preservation in prepubertal girls and is also applied in postpubertal girls and young women when there is no time available for ovarian stimulation to harvest mature oocytes. After ovariectomy, during tissue processing in the lab (Fig. 1), small compacted oocyte–cumulus complexes can be retrieved from the laboratory dish (Fig. 2) or through direct aspiration from visible follicles. These oocytes are an additional source of gametes for cryopreservation (Fig. 3). So far, at least 15 publications reporting this approach in a total number of 240 patients have documented this combined strategy of fertility preservation [60]. Based on published series,

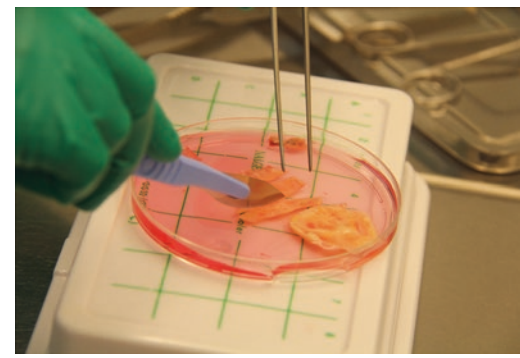

Fig. 1 Dissection of the ovarian cortex in Leibovitz L-15 medium

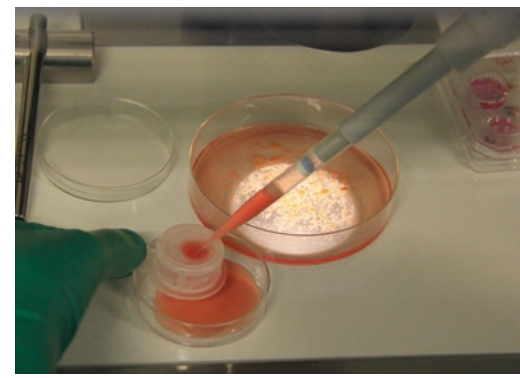

Fig. 2 To enhance identification of cumulus-oocyte complexes, the medium in the culture dish is filtered through a cell strainer

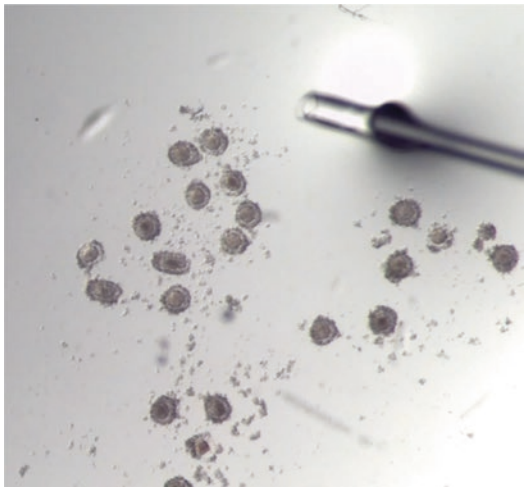

Fig. 3 Cumulus-oocyte complexes derived from small antral follicles without gonadotropin pretreatment

on average 14.7 oocytes-cumulus complexes were retrieved from extracorporeal ovarian tissue and available for IVM. It appears that the retrieval of oocyte–cumulus complexes ex vivo is independent of the phase of the menstrual cycle or the use of oral contraceptives. These complexes can be incubated in specifically designed IVM media and result in mature oocytes, with an overall maturation rate of 39% according to the literature. Although this approach has only resulted in a low number of reported live births so far, it holds promise for the future of the large number of patients who undergo ovarian tissue cryopreservation but who have a high risk of malignant cell reintroduction after ovarian tissue grafting, for example, patients with leukemia [57]. Nevertheless, although this combined approach is theoretically also feasible in prepubertal children, and although a proportion of immature oocytes from these children have reached full nuclear maturation in vitro, the true potential of this approach in prepubertal cancer patients is unknown. Although there is evidence from in vitro studies that ovaries from prepubertal girls harbor a large proportion of follicles with abnormal morphology, there is no information about the developmental capacity of immature oocytes derived from pre-antral and antral follicles in prepubertal ovaries [1]. Nevertheless, the report of the first live birth in a patient who had her ovarian tissue harvested and cryopreserved before menarche illustrates that oocytes embedded in prepubertal ovaries can be cryopreserved and that fertility can be restored in these patients [11].

Which Cancer Patients Could Benefit from IVM?

A currently important limitation of the transvaginal oocyte aspiration technique is the low recovery rate of oocyte cumulus complexes from small antral follicles. This recovery rate is around 50% [56] and does not seem to be influenced by the administration of hCG to the patient before oocyte collection. Oocyte recovery rates are typically lower compared to those of mature oocytes derived from large follicles in stimulated ovaries. As a consequence, and in parallel with the reduced maturation rate of oocytes in IVM systems, the yield of mature oocytes will be limited in women with a low number of antral follicles or low circulating levels of AMH. On a similar note, AMH levels may also correlate with the number of oocytes retrieved ex vivo from extracorporeal ovarian tissue, although robust data to confirm this correlation are currently lacking. In theory, virtually all patients who undergo ovarian biopsies or ovariectomy (except those who received chemotherapy before ovarian tissue harvesting) are suitable for the combined approach with IVM of oocytes retrieved ex vivo. Patients with borderline ovarian carcinoma may also be suitable candidates for this combined approach [37], and so are patients with a high risk of malignant invasion of the ovary. Restoration of fertility in this particular group of patients can only occur safely using isolated follicles embedded within an artificial ovary [43] or by in vitro follicle culture [67, 75]. However, since these approaches have not yet been implemented in the clinic, the collection of immature oocytes from ovariectomy specimens in these patients represents more realistic prospects of fertility restoration after cancer.

Breast cancer patients constitute the largest population of cancer patients who are referred for fertility preservation [25]. Not only do these patients have a high risk of cancer therapy-

induced ovarian insufficiency, there is also a general increase in breast cancer incidence in young women [40, 44]. Furthermore, among all cancer types, breast cancer patients have the lowest prospective probability of ever becoming pregnant after cancer for multiple potential reasons [48]. These reasons include the perceived risk of pregnancy after breast cancer, the recommendation of long-term adjuvant hormonal therapy in hormone receptor-positive patients, and the increased likelihood of a hereditary predisposition to cancer in these patients. Indeed, patients who carry a deleterious mutation in a breast cancer predisposing gene may request pre-implantation genetic testing of embryos, which may negatively affect the chance of pregnancy if no transferrable embryos are obtained. Furthermore, a significant proportion of patients with breast cancer will need urgent neoadjuvant chemotherapy before breast surgery. In this particular subset of patients, the available time for fertility preservation is significantly reduced, and ovarian stimulation followed by oocyte collection and vitrification may not always be possible. Therefore, there is a strong incentive to advocate the development of IVM as an emergency cryopreservation tool in this population.

Clinical Cases: IVM in Oncofertility

Patient 1

After diagnosis of stage IV Hodgkin's lymphoma at the age of 23 years, requiring urgent intensive chemotherapeutic treatment, the patient was referred to the oncofertility clinic. Because of the lack of time for ovarian stimulation, unilateral ovariectomy combined with IVM of oocytes retrieved ex vivo was considered the best option. In total, 26 ovarian cortex pieces were cryopreserved using slow freezing. Twenty-two cumulus-oocyte complexes (COC) were identified during tissue processing in the lab, and these COC were incubated in IVM media for 30 hours, resulting in seven mature oocytes to be vitrified (maturation rate: 32%). Following completion of chemotherapy and after being in remission for almost 3 years, the patient returned to the fertility clinic with a desire to conceive. Chemotherapeutic treatment had induced premature menopause: circulating follicle stimulating hormone (FSH) was 100 IU/l and anti-Müllerian hormone (AMH) level was <0.03 ng/ml. Seven oocytes were warmed and six survived the process of warming. Four oocytes were inseminated using ICSI resulting in two top quality cleavage stage embryos: one was transferred, resulting in pregnancy, and the other embryo was vitrified. The pregnancy was physiological and uneventful, resulting in the live birth of a male infant at term.

Patient 2

A nulliparous woman was diagnosed with a T2N0M0 hormone receptor (ER and PR)-positive breast cancer at the age of 34 years. Because of the urgency to start chemotherapy and in view of the hormone receptor-positive status, the patient was counseled to undergo ovarian tissue cryopreservation combined with IVM of oocytes retrieved ex vivo. The patient declined the alternative option to stimulate the ovaries using gonadotropins supplemented with aromatase inhibitor letrozole. There were in total 14 ovarian cortex pieces available for cryopreservation. Eight COC were identified in the culture dish after tissue processing and in vitro maturation was performed for 28 hours, resulting in three MII oocytes (maturation rate: 38%). The oocytes were inseminated with partner's sperm using ICSI, which resulted in three good quality cleavage stage embryos that were cryopreserved. Genetic testing of breast cancer predisposition genes was performed but resulted negative. After five years, the patient returned to the clinic with a desire to conceive. Consent for pregnancy was given by her oncologist, and therapy with goserelin and tamoxifen had been suspended 5 months before. The patient was amenorrhoeic. Basal hormonal evaluation

showed a serum FSH of 37 IU/l. The decision was made to transfer an embryo generated using IVM of oocytes retrieved ex vivo in a HRT cycle. This resulted in a viable intrauterine pregnancy, leading to the birth of a healthy female neonate. The patient has still two embryos cryopreserved as well as ovarian cortical strips.

How to Improve Pregnancy Rates Following IVM?

Improving in Vitro Culture Techniques

The nuclear maturation through meiosis I and II is a prerequisite for successful oocyte maturation. Cytoplasmic maturation is equally important and includes relocation of organelles, synthesis and modification of proteins and mRNAs, and regulation of biochemical processes that support subsequent fertilization and embryonic development [26]. Regulation of oocyte maturation in vivo involves complex signaling pathways that occur in the microenvironment of the maturing oocyte. The oocyte and cumulus cells communicate through gap junctions [6] that allow passage of regulatory molecules and growth factors. The oocyte is in meiotic arrest until meiotic progress is triggered. In vivo, maturation is triggered by the endogenous LH surge and mediated by growth factors, such as epidermal growth factor (EGF) family members amphiregulin, epiregulin, and beta-cellulin [47]. In vitro, oocyte maturation occurs spontaneously when the oocyte is removed from the follicular environment that inhibits meiotic progression [17]. When immature oocytes are removed from small antral follicles, meiotic resumption will occur precociously, that is, before completion of cytoplasmic maturation. Therefore, the timing of resumption of meiosis is important in oocyte maturation. To solve this problem for in vitro maturation systems, some authors suggest delaying spontaneous nuclear maturation while promoting development of the cytoplasm at the same time [26]. The intracellular messenger molecule cAMP plays a significant role in the regulation of mammalian oocyte maturation [19]. High levels of cAMP and cAMP analogs prevent meiotic resumption [10]. Spontaneous oocyte maturation in vitro can be inhibited or delayed by increasing the cAMP level within the cumulus-oocyte complex environment by adding any of the following substances to the media: (i) cAMP analogues such as dibutyryl cAMP, (ii) activators of adenylate cyclase, such as FSH, forskolin, or invasive adenylate cyclase, and (iii) phosphodiesterase (PDE) inhibitors, such as the non-specific inhibitor IBMX, the PDE type 4-specific inhibitor rolipram, or the PDE type 3-specific inhibitors milrinone, cilostamide, or Org9935 [10]. These agents delay germinal vesicle breakdown and simultaneously increase the extent and prolong the duration of oocyte-CC gap-junctional communication during the meiotic resumption phase [10, 68, 69], which in turn extends the exchange of regulatory factors and metabolites between the oocyte and the cumulus cells [42].

Although IVM research has not yet revolutionized IVM systems in the clinical setting, improved IVM systems are being developed. Lessons have been learnt from experiments in animal models, where the addition of oocyte growth factors, such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), can result in substantially higher numbers of blastocysts [32]. An alternative approach to enhance oocyte potential during maturation in vitro is based on modulation of cyclic adenosine 3′,5′-monophosphate (cAMP). Studies of systems involving cAMP modulators (IBMX and Forskolin) in the human model have revealed serious practical hurdles by the unexpected interaction with heparin (which is used routinely during egg collection to prevent the formation of blood clots in follicle aspirates) [77]. A recent study has shown that the introduction of a prematuration culture (PMC) system that blocks meiosis using C-type natriuretic peptide (CNP) has the potential to narrow down the efficiency gap between IVM and conventional ART (Fig. 2) [58]. Oocyte maturation in vitro after PMC incubation appeared

to result in increased blastocyst rates, which is likely to be related to maintenance of cumulus-oocyte transzonal projections (TZP) that can sustain the dynamic changes in oocyte chromatin remodeling.

Safety of IVM

There have been concerns with the possible interference of IVM with epigenetic reprogramming in the oocyte [34]. Nevertheless, although epigenetic changes can occur in animal embryos cultured under suboptimal conditions [62], normal DNA methylation levels were found at differently methylated regions in human oocytes after IVM [41]. Previous studies have shown abnormal methylation in oocytes after "rescue" IVM, but the "rescued" immature oocytes used in these studies were derived from conventional IVF cycles and had failed to complete meiosis after an ovulation trigger. Furthermore, the occurrence of aneuploidy and other chromosomal abnormalities in human embryos after IVM appears not different to standard IVF, which underscores the safety of IVM as a reproductive technique [66].

Up to 16% of early pregnancies in women with PCOS undergoing COS may be complicated by moderate or severe OHSS [39]; IVM eliminates this risk [74], ensuring a less hazardous and more patient-friendly experience for many women with PCOS who undergo ART to conceive. Nevertheless, early studies reporting the results from IVM demonstrated a significantly increased risk of miscarriage [3]; however, with the introduction of a freeze-all approach in IVM cycles, the rate of miscarriage appeared similar compared to conventional stimulation and ART [74]. The recent review of obstetric outcomes from Belgium demonstrated that women undergoing IVM were almost twice as likely to develop hypertensive disorders of pregnancy, in comparison to women with PCOS undergoing conventional IVF, although it was acknowledged that patients who underwent IVM had a more severe PCOS phenotype. The rates of preterm birth and the birth weights of the children were no different [45].

Singletons born after IVM and COS had a similar birthweight standard deviation score (SDS) (0.51 ± 0.94 after IVM vs. 0.33 ± 1.05 after COS, P = 0.19). Preterm birth rate (32–36.9 weeks) and early preterm birth rate (<32 weeks) were also similar in both groups. Although it has been established that IVF treatment is associated with an increased incidence of congenital malformations in the offspring [33], there was no increased risk of congenital malformations in neonates conceived using IVM. The findings in our series of IVM offspring in Belgium confirm those of a previous study from Italy, although more than 80% of patients enrolled in an IVM program in that study did not have PCOS [22] and a large subset of the children in the latter study were issued from an oocyte matured in vivo after hCG triggering.

There is a potential association with increased risks of metabolic disorder in children conceived using ART [35]. Consequently, longer term follow-up studies of children born from IVM are essential, although, so far, the limited data demonstrates the safety of IVM is comparable to standard IVF [2, 76]. Although there has been a potential concern of possible epigenetic influences of IVM culture, only a few small studies have undertaken epigenetic assessments of any potential influence of IVM on the offspring, suggesting that IVM does not interfere with genomic imprinting establishment in human oocytes [41], and the epigenetic stability of DNA methylation patterns has previously been demonstrated in chorionic villus and cord blood samples from children born from IVM [50]. Although the imprinting patterns did not differ from children born from conventional ART treatment, the small sample sizes preclude the generalizability of the results.

With regard to early childhood development, a French study of children born from IVM treatment at 2 years of age had similar heights and weight to children born from traditional ICSI, although girls were significantly heavier in IVM group [23]. A further German follow-up study of 69 children born from IVM provided reassuring data to the early childhood development of children born form IVM [54], and similar reassuring

reports on the neurodevelopment of 2-year-old children born form IVM have been published from Finland [65] and Taiwan [61], the results of longer-term follow-up studies of IVM offspring is eagerly awaited. Indeed, the impact of potential epigenetic alterations and long-term heritable changes should become an important focus of research of long-term health in the growing population of ART children in general and IVM children in specific. Nevertheless, there are currently no available safety data in offspring of IVM in the setting of fertility preservation. Hence, follow-up programs of children born to women who had IVM in the context of fertility preservation are warranted.

Take Home Message

Oocyte IVM requires further scrutiny as an additional tool in modern ART practice in general and in the setting of oncofertility in specific. IVM requires no major modifications in the ART laboratory. Immature oocytes can be obtained from antral follicles in the follicular and luteal phase of the cycle when there is not enough time to stimulate the ovaries and harvest mature oocytes. Oocytes can be retrieved from extracorporeal ovarian tissue, matured in vitro, fertilized, and result in live births. The option to combine IVM with other fertility preservation methods may increase hope of delayed childbearing to young cancer patients who undergo ovarian cortex cryopreservation before gonadotoxic treatment. Nevertheless, because of the complexity of physiological oocyte maturation, the IVM systems that are currently available need further improvement.

Conflicts of Interest M.D.V. reports honoraria for lectures in the last 2 years from MSD, Gedeon Richter and Ferring, outside the submitted work, as well as grant support from MSD.

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Ovarian Tissue Cryopreservation and Transplantation: Scientific and Clinical Implications

Sherman Silber and Sierra Goldsmith

Introduction

The developed world is in the midst of a widespread infertility epidemic. Economies in Japan, the USA, southern Europe, and even China are threatened by a decreasing population of young people having to support an increasing population of elderly and retirees [1]. Infertility clinics are popping up throughout the world in huge numbers because of a worldwide decline in fertility as women age and become less fertile [2]. In her teen years, a woman has a 0.2% chance of being infertile, and by her early 20s, it is up to 2%. By her early 30s, it is up to 20% [2, 3]. Many modern women today do not consider having children until their mid-30s, by which time nearly 20% are infertile, simply due to the age-related decline in the number and quality of their oocytes. This is clearly demonstrated by the high pregnancy rate using donor oocytes from young women placed into the uterus of older women [2–12].

As important for reproductive medicine as is aging of the population and the subsequent worldwide epidemic of infertility is the high incidence of cancer in girls and young women, curable in the majority of cases at the cost of rendering them sterile. Almost 6% of women of reproductive age are cancer survivors. They will eventually have been sterilized by their chemotherapy or radiation [13–21].

Until recently, oocyte freezing had very poor to no success, and thus ovary tissue slow freezing was the only cryopreservation method we could rely upon [22–24]. More commonly now, vitrification is used instead of slow freeze for oocyte cryopreservation [25–34]. However, many programs do not have follow-up results with oocyte freezing especially in cancer patients undergoing sterilizing chemotherapy and radiation. Furthermore, it may require several cycles of ovarian stimulation to obtain enough oocytes to give women some level of comfort, because even with fresh oocytes, there is only a 5% pregnancy rate per egg [35].

As an alternative strategy for cancer patients, ovarian tissue freezing has benefits over egg freezing. Freezing ovarian tissue obviates the need to delay treatment for a stimulation cycle. Furthermore, transplanting ovarian tissue not only restores fertility but also restores endocrine function. Finally, with the Anderson IVM method, we can obtain many MII oocytes from the ovary tissue at the same time we freeze it, without the need for any ovarian stimulation at all.

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Ovarian Cortex Cryopreservation and Transplantation

Cryopreservation and transplantation of ovarian tissue has a long history in animal studies and in early human studies. In 1960, Parrott and colleagues showed that ovarian tissue could be successfully frozen and autografted in mice, and similar studies by Gunasena and colleagues 37 years later verified live births of mice after autologous transplantation of cryopreserved mouse ovaries, originally shown in rats in 1954 [36–45]. Others have shown that mice have a normal reproductive life span after autografts of fresh tissue [46]. Researchers in the 1990s showed that in both mice and sheep, frozen ovarian tissue could be successfully thawed and autotransplanted leading to normal ovarian function and live births [22, 47]. We reported in 2004 the first live birth from fresh human ovarian tissue transplanted between identical twins discordant for premature ovarian failure [37] (Fig. 1a–d). Donnez and colleagues reported what is deemed to be the first human live birth from orthotropic transplantation of frozen human ovarian tissue in 2004, with another successful live birth achieved by Meirow in 2005 [48, 49].

Our large series of 11 fresh ovary transplants resulted in 14 pregnancies and 11 healthy babies, and a remarkably consistent return of menstrual cycling and normal day 3 follicle stimulating hormone (FSH) concentrations by 4 to 5 months in all patients, which gave hope that a large series of cryopreserved transplants might also provide

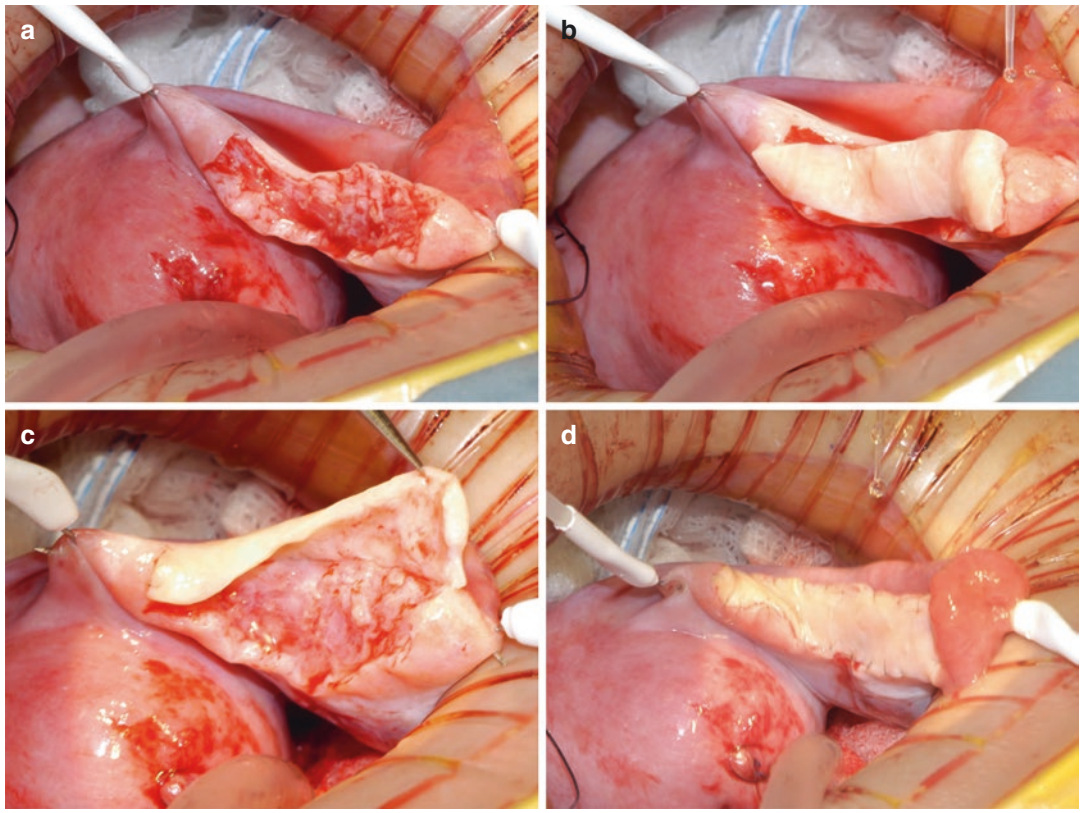


Fig. 1 Steps in the procedure of ovarian transplantation between MZ twin sisters: (**a**) preparation of donor ovarian cortex by dissection in a Petri dish on ice; (**b**) preparation of recipient ovarian medulla; (**c**) attaching donor cortical tissue to recipient ovarian medulla; (**d**) attaching thawed donor cortical tissue for retransplant to the recipient medulla [37]

robust results [39, 50–52]. In fact, the use of similar surgical techniques with cryopreserved ovarian tissue for patients with cancer led to 16 healthy babies from 13 cryopreserved transplants. Our unusual series of fresh and frozen ovary transplants allowed us to evaluate the effect, if any of cryopreservation versus the transplant itself. In fact, cryopreservation had no significant impact on ovarian reserve, but over-recruitment of primordial follicles did (Figs. 2 and 3). The finding that as FSH decreases and ovulation resumes, with AMH initially rising to high levels followed by a return to very low levels, indicated that a massive over-recruitment of primordial follicles led to a subsequent depletion in the ovarian reserve. Interestingly, despite low AMH levels, the grafts nonetheless sustained ovarian function for long periods of time (Figs. 4 and 5).

It is clear that as ovarian reserve goes down the rate of follicle recruitment also goes down. If a child or young adult undergoes unilateral oophorectomy, she does not undergo menopause more than 1 year earlier than if she had two ovaries [53]. Therefore these small strips of ovary tissue will last for a long time despite depletion of follicles caused by early over-recruitment. It is the return of ovarian cortex pressure after healing that prevents further over-recruitment of primordial follicles.

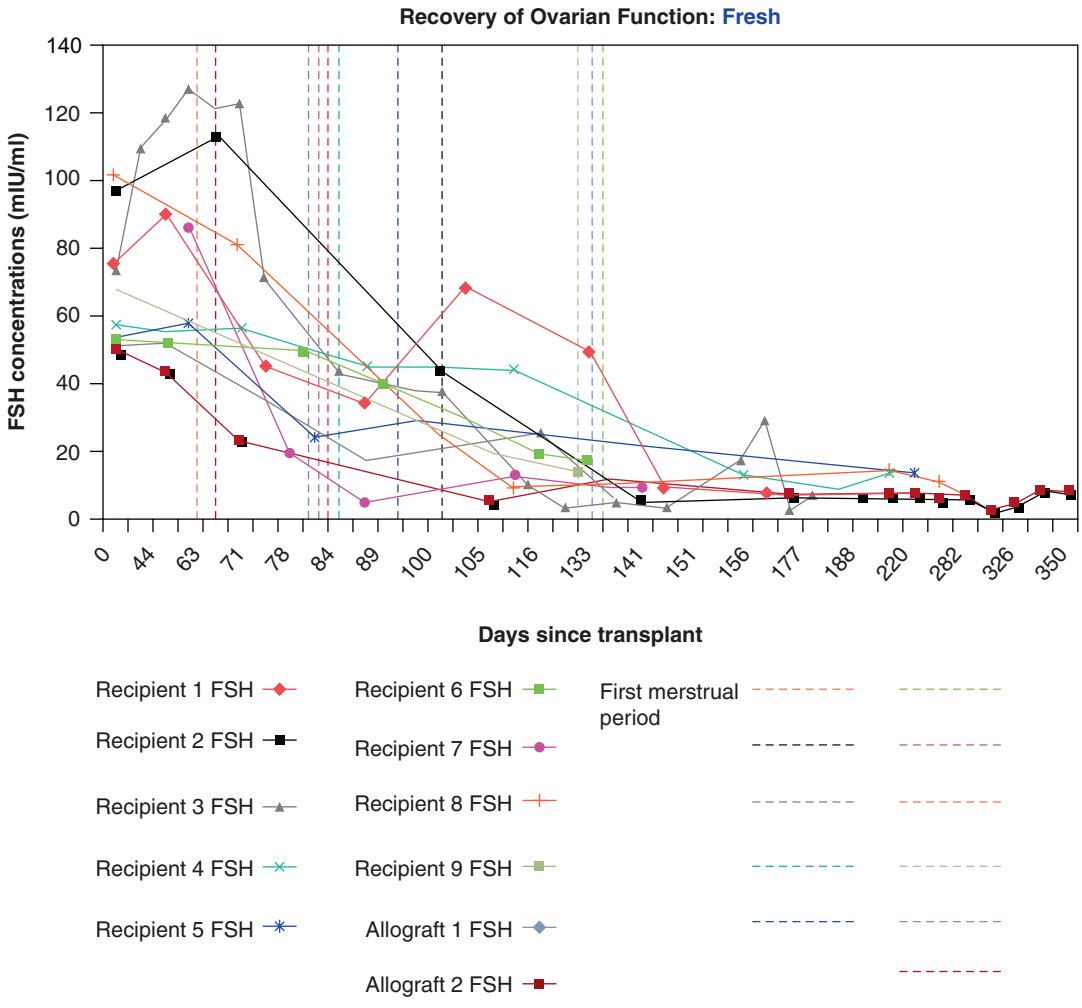


Fig. 2 Serum FSH returns to normal consistently by 4.5 months after fresh transplant [39]

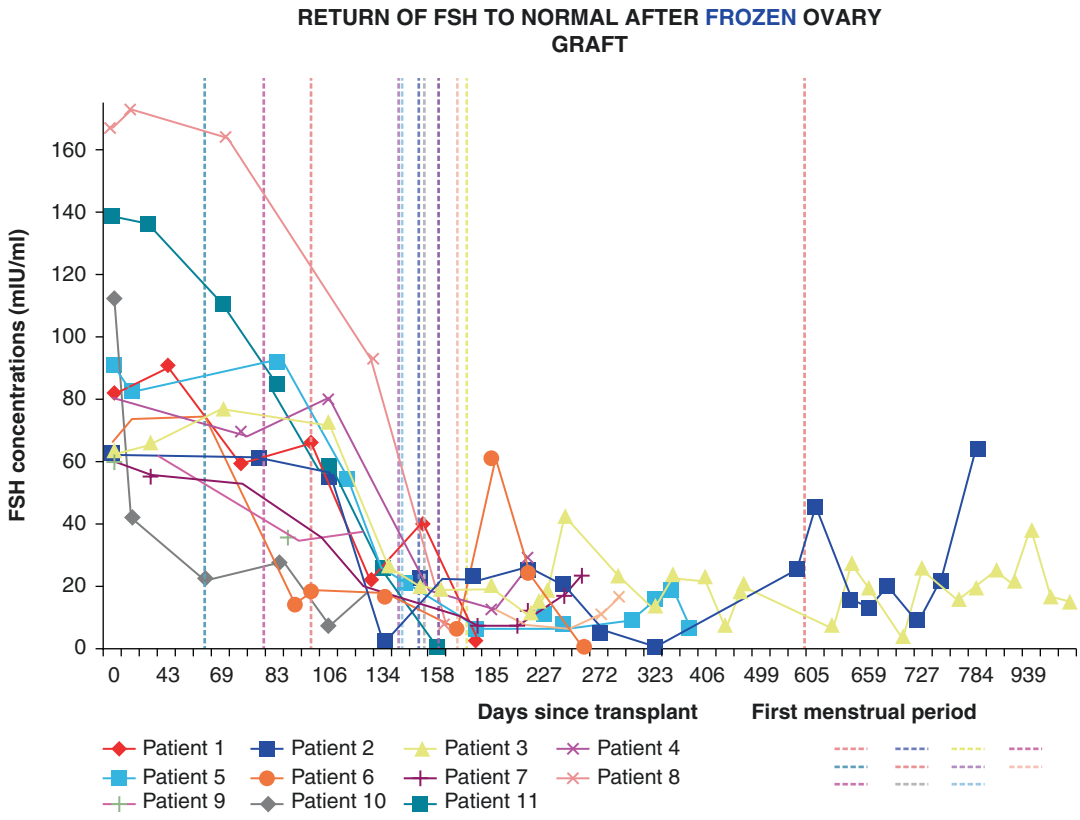
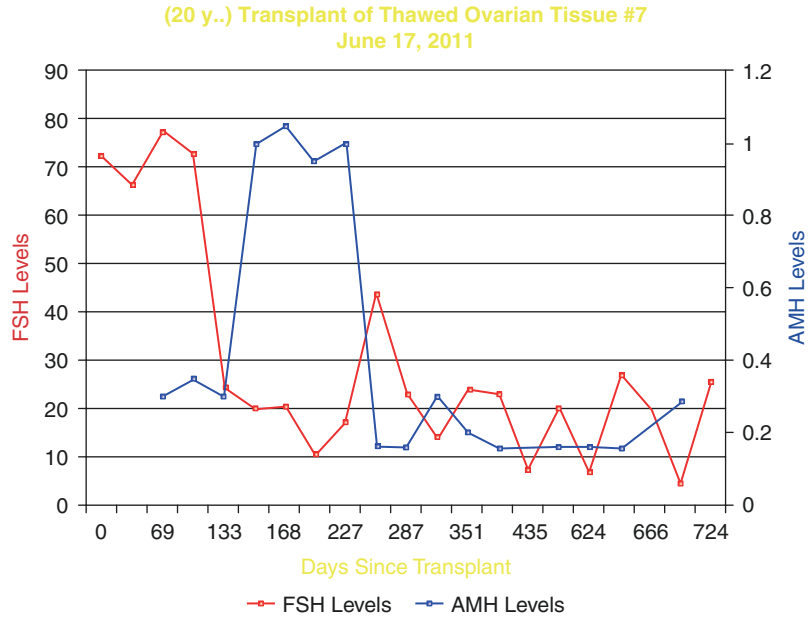


Fig. 3 Similar to fresh transplant, the FSH returns to normal about 5 months after frozen transplant [39]

Fig. 4 As the FSH returns to normal, the AMH rises very high and then goes down to very low levels



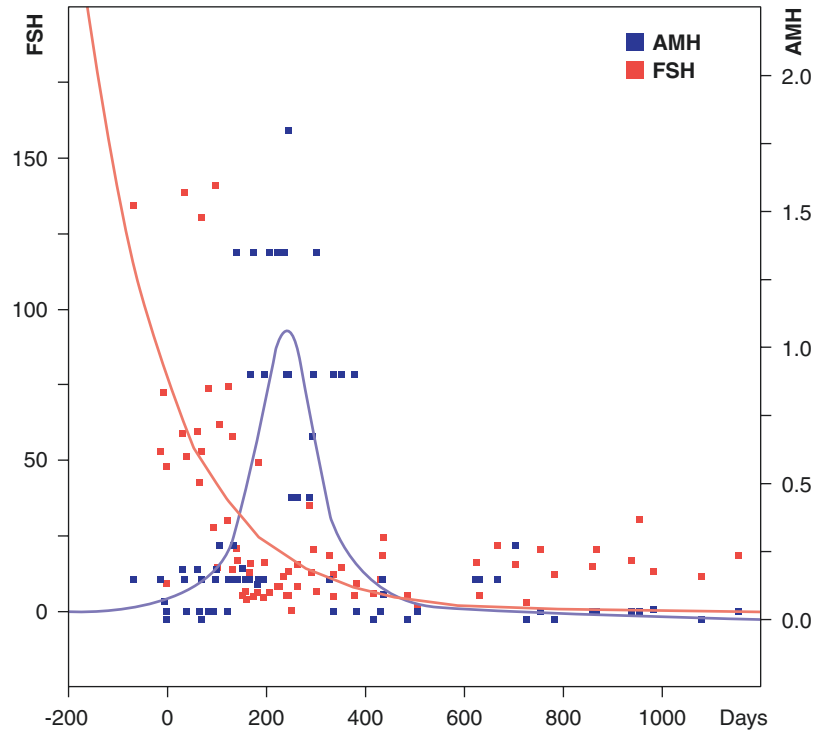


Fig. 5 Composite dot graph summarizing the return of FSH to normal and the rise of AMH and subsequent decline after frozen transplant [38]

Unilateral oophorectomy does not negatively affect fertility and does not hasten the onset of menopause; this would support partial or complete oophorectomy and ovarian tissue cryopreservation to expand the reproductive life span of normal women who wish to delay childbearing but do not want to lose their current reproductive potential. Thus, we felt comfortable in undertaking a series of fresh ovary transplants, which led the way toward improving our ovarian freezing transplantation methods [54–56]. Also, we feel confident that removing an ovary will not harm long-term fertility. Contrarily, it follows that transplanting the removed ovary could extend a woman's reproductive life span.

Several techniques have been described for transplantation of the ovarian cortex [37, 43, 48–50]. In mice, Parrot used sliced little pieces of ovarian cortex. Others prepared peritoneum near the ovary but then switched to a technique similar to that described for fresh ovarian tissue [37]. Ovarian cortical slices can also be transplanted under the surface of the cortex in the nonfunctional ovary [49]. All these techniques have resulted in babies, and there is no consensus on which is best.

The key to successful transplantation of frozen ovarian tissue is to consider it as though it were a skin graft (Fig. 6a, b). Microhematoma formation under the graft is avoided by microbipolar cautery pulsatile irrigation and micropressure stitches of 9–0 nylon. Constant pulsatile irrigation with heparinized saline prevents adhesions, improving chances of spontaneous pregnancy with no need for IVF, difficult in these cases due to a reduced ovarian reserve yielding few oocytes after ovarian hyperstimulation. The transplant is best if orthotropic and adhesions are minimized [57, 58] (Fig. 1a–d).

Initially, there were only a few case reports, some very recently, of successful cryopreserved ovary transplantation but no unified single series [58–66]. However, it appears now that there is a worldwide live birth rate of over 30–70%, with more than 180 babies, and long-term function of the transplant has been observed despite very low AMH. Robust results are seen in series from St.

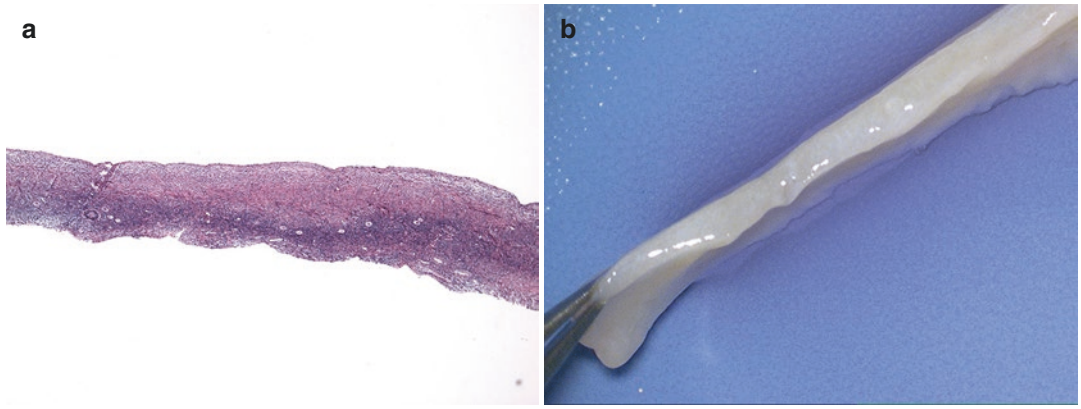


Fig. 6 (**a**, **b**) All of the resting follicles are located in the outer 1 mm of the fibrous ovarian cortex

Louis, Brussels, Paris, Spain, Denmark, Israel, Japan, Italy, Germany, Australia, and Russia [67, 68]. Cryopreserved ovarian tissue grafts with the slow-freezing method as well as with vitrification are functional for more than 5 years, and many spontaneous pregnancies have been reported with no need for in vitro fertilization or other ancillary treatment. Most pregnancies were achieved without the need for in vitro fertilization and resulted instead from regular intercourse with no other treatment.

With this in mind, the remarkable long-term hormonal and ovulatory function of these cortical grafts despite extremely low AMH after the initial primordial follicle over-recruitment emphasizes the compensatory relationship of a low remaining ovarian reserve to a slower recruitment rate of primordial follicles [63].

The most common benefit of ovarian transplantation was previously thought to be the preservation of fertility and future endocrine function in young women undergoing cancer treatment. However, in the absence of pelvic irradiation for cancer treatment, why not use ovarian tissue cryopreservation in otherwise healthy women who wish to preserve their fertility for nonmedical reasons? With vitrification methods, there is no difference in the viability or integrity of cryopreserved ovarian tissue compared with fresh ovarian tissue [56]. Furthermore, with cryopreserved ovarian tissue transplantation, hormonal function is restored in addition to fertility [63–67].

Ovarian Cryopreservation Techniques

In the past, all of the frozen ovary cases transplanted back to the patient have utilized the slow freeze approach [22–24, 53]. However, we now use vitrification exclusively for cryopreservation in humans because of the results of in vitro viability analysis in humans, as well as in vivo transplant studies in the bovine and human [55, 56]. Three of our successful eight pregnancies were from vitrified ovarian tissue. Five were from ovarian tissue that was frozen long ago (as early as 1996) with slow freeze.

The high viability (92%) of oocytes in control (fresh) and vitrified specimens indicates virtually no damage to the eggs from ovarian tissue vitrification [56]. Overall, 2301 oocytes were examined from 16 specimens. There was no significant difference between fresh and vitrified tissue, but the viability of slow freeze-cryopreserved tissue was less than one half that of vitrified tissue or controls (42%) ($P < 0.01$) (Fig. 7a, b). Transmission electron microscopy also has been used to analyze ovarian tissue that had been either cryopreserved by slow freezing or vitrified by ultra-rapid freezing, showing vitrification to be superior [54]. Standard H&E histology showed no difference between prefreeze ovarian tissue and post vitrification ovarian tissue. Finally, quantitative histologic study of primordial follicles in the bovine after vitrification and trans-

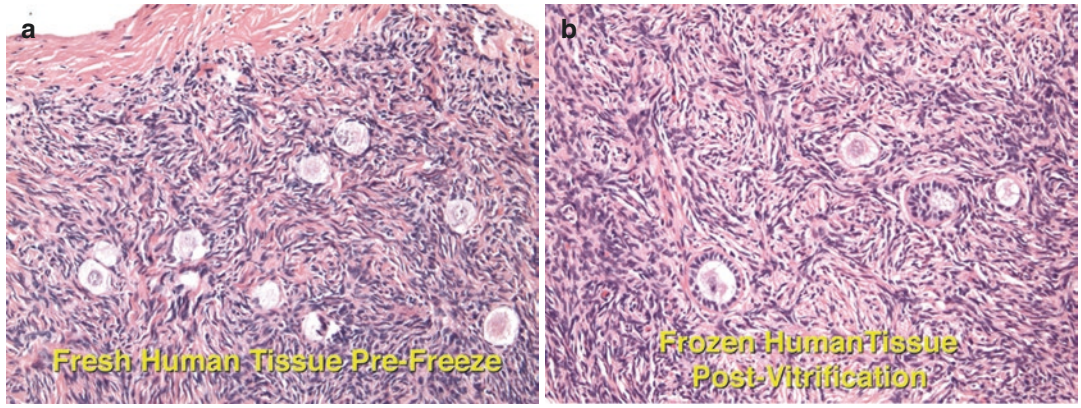


Fig. 7 (**a**, **b**) There is no discernible difference between fresh ovarian tissue and vitrified [66]

plantation back to the cow 2 months later remarkably showed no follicle loss [55]. Nonetheless, for clinical use, slow freeze gives pregnancy results as good as vitrification. The only advantage of vitrification, and why we prefer it, is the ease of use.

Using the vitrification technique, cortex tissue of each ovary is cut into slices 10 mm by 10 mm Å ~ 1 mm. Ovarian tissues are initially equilibrated in 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) in handling medium (HM: HEPES-buffered TCM-199 solution supplemented with 20% serum for 25 min, followed by a second equilibration in 20% EG and 20% DMSO with 0.5 mol/l sucrose for 15 min). Ovarian tissues are then placed in a minimum volume of solution onto a thin metal strip (Cryotissue: Kitazato BioPharma, Japan) and submerged directly into sterile liquid nitrogen [55], after which the strip is inserted into a protective container and placed into a liquid nitrogen storage tank (Fig. 8).

For thaw, the protective cover is removed and the Cryotissue metal strip is immersed directly into 40 ml of 37 °C HM solution supplemented with 1.0 mol/1 sucrose for 1 min. Then, ovary tissues are transferred into 40 ml of 0.5 mol/1 sucrose HM solution for 5 min at room temperature and washed twice in HM solution for 10 min before viability analysis or transplantation. No ice crystal formation occurs during any of these vitrification procedures [55, 56].

Fig. 8 Thin slices of ovarian cortical tissue preserve all of the resting follicles

Clinical Benefit of Ovarian Tissue Freezing: Cancer, Ovarian Reserve, and Long-Term Function

The most common benefit of ovarian transplant is not the unusual case of fresh grafting in identical twins but rather to protect the fertility and future endocrine function of young women undergoing treatment for cancer or other diseases that result in ovotoxicity. Since 1996, we have frozen ovary tissue for over 100 young women with cancer, or at risk for POF, of whom 16 had spare frozen tissue subjected to detailed viability testing before cryopreservation and after thaw.

None of our cases who were cured of cancer have had any tumor cells in their ovary. There

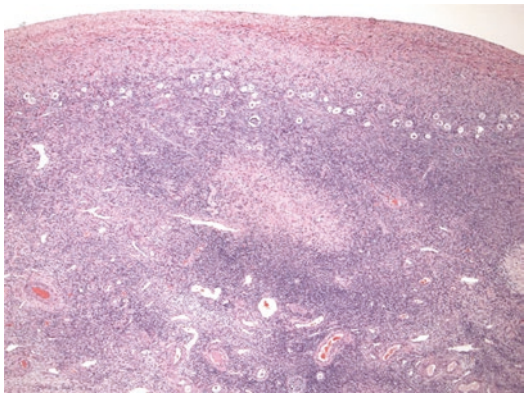


Fig. 9 No metastasis in the ovary

have been no cases reported of transmission of cancer via transplant of frozen ovarian cortex [67–69] (Fig. 9). The reason for the remarkable absence of ovarian metastasis might possibly be due to the fibrous avascular nature of the ovarian cortex [70]. The reason why fetal ovarian tubules (which in the male become seminiferous tubules) invade the fibrous cortex and become follicles is that the dense fibrous tissue of the cortex (which in the fetal and adult testis is just tunica albuginea) is needed to suppress the resting follicles from developing all at once prematurely by forming primordial follicles. Primordial follicles arrest the fetal oocytes from continuing meiosis to completion and subsequent apoptosis. The dense fibrous tissue of the ovarian cortex not only controls follicle development but also represents a relatively inhospitable location for cancer cells.

The return of FSH to normal at 4–5 months indicates that this is the period of time required for primordial follicles, once recruited, to develop on to the antral and ovulatory stage. The concomitant rise of AMH to well over normal levels followed by a drop to very low levels indicates a massive over recruitment of follicles and subsequent depletion. This is substantiated by the current report of Winkler-Crepaz et al. transplanting human ovarian tissue into SCID mice, demonstrating no ischemic apoptosis of follicles, but rather a massive over-recruitment [36]. However, these transplanted slices of ovarian cortex continue to function normally for many years because of a decreased rate of primordial follicle recruitment that occurs when there is decreased ovarian reserve [71].

Obtaining Mature Eggs from Ovary Tissue with no Stimulation

More recently, thanks as of yet unpublished work by Claus Andersen's group in Denmark, we can collect many GVs from the dissection of the ovarian cortex. They are abundantly found in the media when you do the ovarian cortex dissection. There have been many papers showing the "impossibility" of culturing MII oocytes from the primordial to even secondary follicles in ovarian tissue. That is due to the "locking" of the primordial follicles by pressure-induced rotation of the egg's nucleus. In addition, decades of attempting IVM with retrieved oocytes or unstimulated oocytes have failed. But GV oocytes derived by dissecting the cortex from cryopreservation are a different story (Fig. 10).

In fact in most animal IVF, there is no hyperstimulation. The ovaries are simply removed, and GVs are obtained from the removed ovary and cultured. Many MII oocytes are thus obtained, and many healthy calves and pups born from IVF with these in vitro matured GV oocytes. So Andersen raised the question, "Why can't we do the same with humans?". Obviously with infertile humans, we are not going to do an oophorectomy to obtain large numbers of GV oocytes. But for patients requiring ovary tissue freeze, this

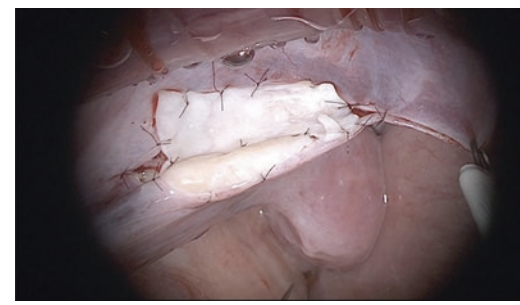


Fig. 10 Ovary transplant

would be a great benefit, to collect her GVs and mature them to MII and then freeze them.

We have now begun to employ Andersen's technique and are amazed by how successful this IVM is. Of course, if the patient has already had preliminary chemotherapy, we find no GVs to culture. We then just have to rely on the much more sturdy and resistant primordial follicles, which are successfully activated by transplantation with huge success. But if the patient has not yet had any chemotherapy at all, we do not need ovarian stimulation to get mature eggs to freeze. We only need IVM of GVs derived from the ovary tissue dissection prior to the freeze.

Mechanism of Ovarian Follicle Recruitment and Ovarian Longevity

To review, we have found that after ovary cortex transplant, it requires 4–5 months for primordial follicles to reach the ovulatory, gonadotropin-sensitive stage of development in vivo. At this time post-op, the FSH comes down to normal or near normal, and the AMH climbs every high, only to return to low levels 4–8 months later. Nonetheless the transplanted thawed cortex continues to function for many years even with a low AMH.

These findings are consistent with tissue pressure being the regulator of primordial follicle recruitment and indeed ovarian longevity. This key role of tissue pressure has been supported by many other experiments. Number one, Hayashi has shown that if you incubate stem cell-derived oocytes in high incubation pressure, they skip the primordial stage and develop automatically into meiotic competent oocytes that can make healthy offspring.

However if you incubate them at high pressure, they are arrested past like in primordial follicles. Furthermore, under high pressure, these arrested follicles undergo nuclear rotation. Under normal atmospheric pressure, the nuclei stop rotating, and the follicles began recruitment and development.

There is also a natural experiment in pregnant woman that supports the role of pressure in regulating ovarian resting follicle recruitment and hence ovarian longevity. Teramoto and others has shown in huge population studies that AMH does not decline, as it normally should decline with time, during pregnancy. From mid-trimester pregnancy to 4–5 months after delivery, the AMH remains flat. The increased abdominal pressure of pregnancy turns off follicle recruitment, and 4 months later in mid-pregnancy, this results in a lower antral follicle number and hence lower AMH. This low AMH persists through the second half of pregnancy and then for 4–5 months after pregnancy when the resting follicles are recruited right after delivery and then reach the antral phase 4–5 months later. Therefore grand multiparas, who are always pregnant, tend to have later menopause and retain their fertility for a long time. During pregnancy, the ovaries "go to sleep."

Leukemia and Concerns for Transmission of Cancer from Frozen Ovarian Tissue

There are three cases reported from our US series, the first in 2013, all three published in 2018, of leukemia patients who now have five babies from their three ovary transplants and no transmission of leukemic cells from the thawed, transplanted tissue. There actually have been no cases observed yet of transplanting cancer cells along with ovarian cortex; for leukemia cases, the key is to let them first go into remission with initial chemotherapy, before they eventually relapse and need a bone marrow transplant [53]. There will be no viable transplantable cancer cells then in the frozen cortical tissue. The chemotherapy that puts them in remission will destroy all the developing follicles and put her in menopause. But it will not destroy all of the more resistant primordial follicles with rotating nuclei. So years later you can transplant that frozen tissue back without any transmission of cancer, and with full ovarian function and spontaneous pregnancy after 5 months.

Is it "Experimental" or Accepted Treatment: Insurance Coverage

Ovary cryopreservation has finally been determined not to be experimental. Although we have the only series in the USA of frozen ovary transplants, with an 81% spontaneous pregnancy live baby rate, there was no reference from this American Society of this American study. The ASRM in fact only referred to one leukemia case from Israel and never referred to over three leukemia cases with five babies from our center in the USA. There was no transmission of cancer in any of these cases and all were successful [72, 73].

Cryopreservation and Transplantation of Ovarian Tissue: Results from One Center in the USA

A series of 108 cases of ovary tissue cryopreservation initiated in 1997 gave us an opportunity to assess its efficiency for young women about to undergo sterilizing cancer treatment [38, 74]. Successful fresh and cryopreserved ovarian cortex transplants in humans were first published in 2004 and 2005, as case reports, and many other case reports have subsequently followed [37, 48–53, 66, 69–72, 75–78]. The first human applications were preceded by a long history of animal experimentation. As far back as 1954, Deanesly showed in rats, and in 1960, Parrott showed in mice, that ovarian tissue could be successfully frozen and autografted resulting in live births [44, 79]. Candy et al. showed these mice had a normal reproductive life span [46]. Interest in human applications began after Gosden's report of successful pregnancies in sheep in 1994 [22]. Interest in cryopreserved ovarian cortical transplantation is rapidly growing, but systematic reports have been published from only a few centers [75]. Despite this great interest, there is a paucity of consistent series (none from the USA) reported from one center in which the expectation of success rate for this procedure can be gleaned [80–82].

The primary impetus for this procedure has been to cryopreserve ovarian tissue before sterilizing cancer treatment, with the objective of transplanting the tissue back after cancer has been cured, thus allowing patients to preserve their fertility. It is also possible that grafts taken from young women with cancer could be used in the future to delay their menopause [13, 17, 19, 52, 83]. This latter possibility of preserving not only fertility but even hormonal function against the natural decline caused by aging has even been speculated as a possible indication for young healthy women as well [25, 53, 84, 85]. Most published research in this field consists of case reports of cryopreserved transplants only, because oncologists refer very few cases, and there has been a fear of reintroducing cancer cells, which has only recently been dispelled [80–84]. Thus far there have been no cases reported of transmission of cancer either in our series or elsewhere in the world. A worldwide survey of 37 babies born from cryopreserved transplants still could not establish a clear success rate [52]. Here, we report a single series (though small) of cryopreserved transplants from one center, carried out with the same technique and assessed uniformly over follow-up. This is the only series we are aware of to be reported from the USA.

Over a period from 1997 to 2017 (20 years), 108 females between age 6 and 35 years were referred for possible ovary tissue freezing for fertility preservation. Ninety-two (85%) of these women underwent unilateral oophorectomy and cryopreservation either by slow freeze or vitrification. Sixty-six were for cancer, 5 for threatened premature ovarian failure (ovary tissue of discordant identical twin that was cryopreserved for her sister), 9 for social reasons, and 12 for a variety of conditions including Turner's syndrome, multiple sclerosis, endometriosis, aplastic anemia, a daughter born with no ovary, or massive bilateral ovarian teratoma. Hodgkin's disease accounted for 20 of the cancer cases (30%), breast cancer 13 (20%), leukemia 7 (11%), and non-Hodgkin's lymphoma 5 (8%). The rest of the cancer cases (21) were a wide variety of less common cancers such as Ewing's sarcoma, embryonal sarcoma of the liver, colon cancer, calf sarcoma, spinal cord tumor, dys-

germinoma, medulloblastoma, rhabdomyosarcoma, stomach cancer, carcinoid tumor, and brain cancer. Social reasons included not being ready to have children and wishing to have more children perhaps at a later date. Those who chose ovary freezing for social reasons did so before oocyte freezing was widely accepted, or because they were just too occupied in their hectic life to find time for three cycles of ovarian stimulation and oocyte retrieval and did not want to go through hormonal stimulation. Nineteen of the cancer patients underwent slow freeze prior to September 2007, and 47 subsequent cases underwent vitrification of their ovarian tissue. Six of the 66 (10%) cancer patients have died, and 54 either underwent transplantation or are prepared eventually to undergo transplantation. All patients were counseled in detail with the advice that the transplant might not ever be performed or might not function. All underwent IRB consent.

Thirteen of the 92 ovary freeze cases (14%) have come back to have their ovary tissue thawed and transplanted back. Of those 13, the 4 most recent cases had been cryopreserved by vitrification, and the other 9 had been frozen by slow freeze. Ten were cancer survivors, and three were POF patients who had frozen ovary tissue from an identical twin sister. The nine slow freeze cases had their ovary tissue frozen before 2007, and so naturally they composed the majority (nine) of the cases of cryopreserved tissue thaw and that were transplanted. The other four cases of frozen ovary transplant had their tissue cryopreserved after 2007, and so these were vitrification cases. In addition to these cryopreserved ovary tissue transplant cases reported here, there have been 11 fresh transplants either between identical twins and allografts that have already been reported, for a total of a large series of 24 ovary transplants that have been performed at one center with one technique [38]. All patients were menopausal for 3–20 years prior to the transplant. Three of the nine women undergoing transplant of their frozen tissue had leukemia, but their tissue was cryopreserved when they were in remission prior to their bone marrow transplant [38]. Assessment of multiple fragments by histology and immunochemistry by oncology and pathology departments revealed no tumor cells.

Clinical Cases

All 13 cases had return of ovarian function from 4 to 5 months after transplantation, as determined by return of FSH to normal levels, and regular menstrual cycling, similar to what was previously reported with fresh ovary tissue transplantation. At the same time that FSH returned to normal or near normal levels, the AMH rose to high levels and then fell to very low levels after 4 more months.

Eight of the 13 grafts were still functioning from 62 to 96 months after surgery (5–8 years). The other five grafts ceased functioning from 22 to 51 months (2–4 years). The longest functioning graft from slow freeze was 96 months and is still functioning. The longest functioning graft from vitrification was 62 months and is still functioning. The oldest female at the age of freeze was 31 years, and the oldest at the age of transplant was 39 years. All recipients were between 19 and 31 years of age at the time of freeze for a median age of 24 (Tables 1, 2, and 3).

Nine of the 13 transplants resulted in spontaneous pregnancy and delivery of at least one live healthy baby (69%). In one case, four singletons thus far have resulted from one transplant, and in another case, two singletons and two spontaneous twins have resulted (no patients underwent IVF). There have thus been a total of 16 live, healthy babies from spontaneous pregnancy in these 13 cases. There has only been one miscarriage (10%) (Tables 1, 2, and 3).

Eleven babies have resulted from nine cases of slow freeze ovarian tissue, and two from four cases of vitrified ovarian tissue. Thus, 9 of the 13 cases resulted in at least one live birth spontaneous pregnancy (69%), two patients so far with four babies. All of the four vitrified tissue cases are still functioning, and four of the nine slow freeze cases are still functioning, which just means that the earliest cases were all slow freeze. All of the offspring are normal and healthy.

Three of the transplants were leukemia cases, for which their oncologist gave approval. Two of the three resulted in spontaneous pregnancy and delivery of five healthy babies.

(Tables 1, 2, and 3). There has been no recurrence of leukemia and in fact no recurrence in any of the cancer cases. These were among the first cases of success for leukemia patients having babies from transplanting their frozen tissue although the first such case (from Israel) was published in 2017 [81]. The first leukemia case in this series was frozen in 1997 and transplanted back in October 2013. Her baby was born in November 2017. The second leukemia case was frozen in 2006 and transplanted back April 2013, and her first of four babies was born in May 2015.

Table 1 Overall results and age

Dale of transplant	Age at transplantation	Age at freeze	Diagnosis	Pregnant	Live birth or ongoing	Time until pregnancy (days)	Miscarriages	Duration of ovarian function (months)
3/607	26	24	POF	Yes	Female	174		23(ended)
1/13/09	31	20	Hodgkins	Yes	Male	272		29(ended)
6/9/09	29	24	POF	Yes		276	1	19(ended)
6/17/11	33	20	Hodgkins	No				38 (ended)
10/12/12	33	31	MS	Yes	Female	481		67(ended)
3/29/13	32	25	POE	Yes	Female	243		26 (ended)
4/5/13	33	30	Brain cancer	Yes	Male	665		61 (still functioning)
4/12/13	25	18	Leukemia	Yes	Male	502		61 (still functioning)
				Yes	Female	998		
				Yes	Female	1578		
10/1/13	29	28	Synovial sarcoma	No				56 (still functioning)
10/7/13	39	24	Leukemia	Yes	Female	1287		56 (still functioning)
7/21/15	28	25	Leukemia	No				34 (still functioning)
8/5/15	32	21	Hodgkins	Yes	Female	343		33 (still functioning)
9/18/14	36	20	Hodgkins	Yes	Female	473		44 (still functioning)
				Yes	Female	908		
					Female			
Totals	13 cases	13 babies	10 pregnant (77%)		1 miscarriage		10 females	4 vitrification
							3 males	9 slow freeze

Table 2 Overall results vitrified versus slow freeze

Pregnancy after frozen autografts									
Vitrified slow freeze	Duration of ovarian function (months)	Date of transitant	Date of first menstruation post OT	Diagnosis	Miscarriage	Baby born	Ongoing	Girl	Boy
Slow freeze	23(ended)	3/6/07	9/19/08	POF		1		1	
Slow freeze	29(ended)	1/13/09	6/7/09	Hodgkins		1			1
Slow freeze	19 (ended)	6/9/09	11/28/09	POF	1	0			
Slow freeze	38 (ended)	6/17/11	11/15/11	Hodgkins		0			
Vitrified	67(ended)	10/12/12	3/2/13	MS		1		1	
Slow freeze	26 (ended)	3/29/13	4/5/13	POF		1		1	
Vitrified	61 (still functioning)	4/5/13	12/27/13	Brain cancer		1			1
Slow freeze	61 (still functioning)	4/12/13	1/1/14	Leukemia		3		2	1
Vitrified	56 (still functioning)	10/1/13	12/19/13	Synovial sarcoma		0			
Slow freeze	56 (still functioning)	10/7/13	3/6/14	Leukemia		1		1	
Vitrified	34 (still functioning)	7/21/15	11/15/15	Leukemia		0			
Slow freeze	33 (still functioning)	8/5/15	10/28/15	Hodgkins		1		1	
Slow freeze	44 (still functioning)	9/18/14	2/2/15	Hodgkins		3		3	
Totals	13 cases	13 babies	10 pregnant (77%)	1 miscarriage	10 females		4 vitrification		
					3 males		9 slow freeze		

Table 3 Leukemia cases

Ovary issue freeze transplants leukemia								
Date of transplant	Age at transplantation	Age at freeze	Diagnosis	Pregnant	Live birth	Time until pregnancy (days)	Miscarriages	Duration of ovarian function (months)
4/12/13	25	18	Myeloproliferative(blood disorder)	Yes	Yes	502		56 (still functioning)
				Yes	Yes	998		
				Yes	Yes	1578		
10/7/13	39	24	Acute lymphocytic leukemia	Yes	Yes	1287		50 (still functioning)
7/21/15	28	25	Acute myeloid leukemia	No				29 (still functioning)
Totals	3 cases	4 babies	4 pregnancies	0 miscarriage			Average age (30 years old)	
			2 became pregnant (67%)					

Take-Home Messages

Primordial follicle arrest is the key to saving the oocyte from disappearing after the fetal initiation of meiosis and the continuation all the way through meiosis with subsequent apoptosis [7, 24, 38–40, 64, 76, 79, 86–90]. It is also the key to the cautious gradual release every month of oocytes in the adult to develop over 4 months into gonadotropin-sensitive antral and Graafian follicles, sparing the resting oocytes from sudden total depletion [91]. After this initial massive depletion of resting follicles is halted, the ovarian transplant then proceeds to function surprisingly for many years quite well despite a very low AMH and a low remaining number of follicles. That is because as the ovarian reserve goes down, the rate of primordial follicle recruitment in a compensatory way also goes down. Huge population studies have indicated that unilateral oophorectomy does not cause much of an earlier menopause [62]. However, this assumption has been contradicted by a recent IVF study [92]. Nonetheless, this recent IVF study does not affect the finding of reduced primordial follicle recruitment in the face of reduced ovarian reserve. The less the number of remaining oocytes, the better the primordial follicles are able to maintain their locking mechanism and, as a result, limit the number of resting follicles allowed to activate and hence maintain follicle reserve [38–40, 53, 55, 56, 60, 89, 90, 93].

The impression this series gives is the robustness of this procedure. Our high success rates are most likely aided by having tissue only from younger women with no prior history of infertility. This is a relatively small series compared to the impressive experience of the Belgian, Israeli, Spanish, and Danish centers. Nonetheless, our live baby rate for these otherwise sterile cancer survivors and the obvious effectiveness of standard slow freeze as well as vitrification (despite a previously demonstrated high oocyte loss compared to vitrification) for ovarian tissue cryopreservation testify to its robustness and simplicity. Furthermore this is still the only series and indeed the only cases of ovarian cryopreservation and transplant in the USA.

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Techniques for Ovarian Tissue Transplantation

Jacques Donnez, Luciana Cacciottola, and Marie-Madeleine Dolmans

Introduction

In recent decades, anticancer treatments have become increasingly effective, yielding significantly improved survival rates in cancer patients. Nevertheless, young women of reproductive age are at high risk of experiencing chemo/radiotherapy-induced premature ovarian insufficiency and subsequent infertility [1]. Ovarian tissue cryopreservation and transplantation are the only alternatives for prepubertal girls and patients who require immediate chemotherapy. It has gained popularity over the past decade thanks to its success in restoring not only patient fertility in more than 40% of subjects [1, 2] but also ovarian endocrine function in over 95% of cases [1, 3]. Since the first reported live birth in 2004 [4] and the second in 2005 [5], the number of babies born has shown a logarithmic increase, reaching more than 200 by 2020 [6]. Several ovarian tissue transplantation techniques have been developed and described in the literature, with different outcomes and success rates. The aim of the present chapter is to illustrate and summarize all surgical procedures for ovarian tissue transplantation that have been published to date, also providing information on their use and effectiveness.

Orthotopic Ovarian Tissue Transplantation

Orthotopic transplantation involves grafting ovarian cortical fragments to the exposed medulla of the denuded ovary or a specially created peritoneal site [7].

The majority of orthotopic transplantations are carried out by minimally invasive surgery (by laparoscopy in our group; by minilaparotomy in Meirow's, Andersen's, and Silber's groups). The choice of grafting site and decision to graft in one or more locations depend on whether or not the patient previously underwent complete unilateral or bilateral oophorectomy [7]. Indeed, if at least one of the ovaries is present, ovarian tissue may be transplanted both to the ovary after decortication and to a newly created peritoneal window. On the other hand, if no ovaries remain, the only alternative for orthotopic transplantation is use of a peritoneal window [7, 8].

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There are a number of variations in surgical techniques between leading teams in ovarian tissue cryopreservation and transplantation worldwide.

Several variations have been introduced since Donnez's first successful orthotopic transplantation was a landmark in the history of ovarian tissue transplantation [8].

If at Least One Ovary Is Present

The procedure starts with decortication of the ovary. A large piece of ovarian cortex is removed by means of scissors to have access to the medulla and its vascular network (Fig. 1a). In line with microsurgical techniques, ovarian cortical pieces are then fixed with 7/0 or 8/0 propylene stitches, or simply placed on the medulla and fixed with Interceed® (Johnson & Johnson) (Fig. 1b). Fibrin glue (Tissucol®, Baxter) is used to fix the edges (and only the edges) of Interceed® (Fig. 1c).

If both Ovaries Are Absent

A peritoneal window may be created in two steps to induce angiogenesis before the grafting procedure, as in the case published in 2004 [4], or in one step [7]. The incision for this peritoneal window is made on the anterior leaf of the broad ligament in an area where a vascular network is visible (retroperitoneal vessels) (Fig. 2a). The fragments are placed in the window (Fig. 2b) and subsequently covered with Interceed®, the edges of which are fixed with fibrin glue (Fig. 2c). The first live birth by this technique was reported in 2012 [7]. The reimplantation of frozen-thawed

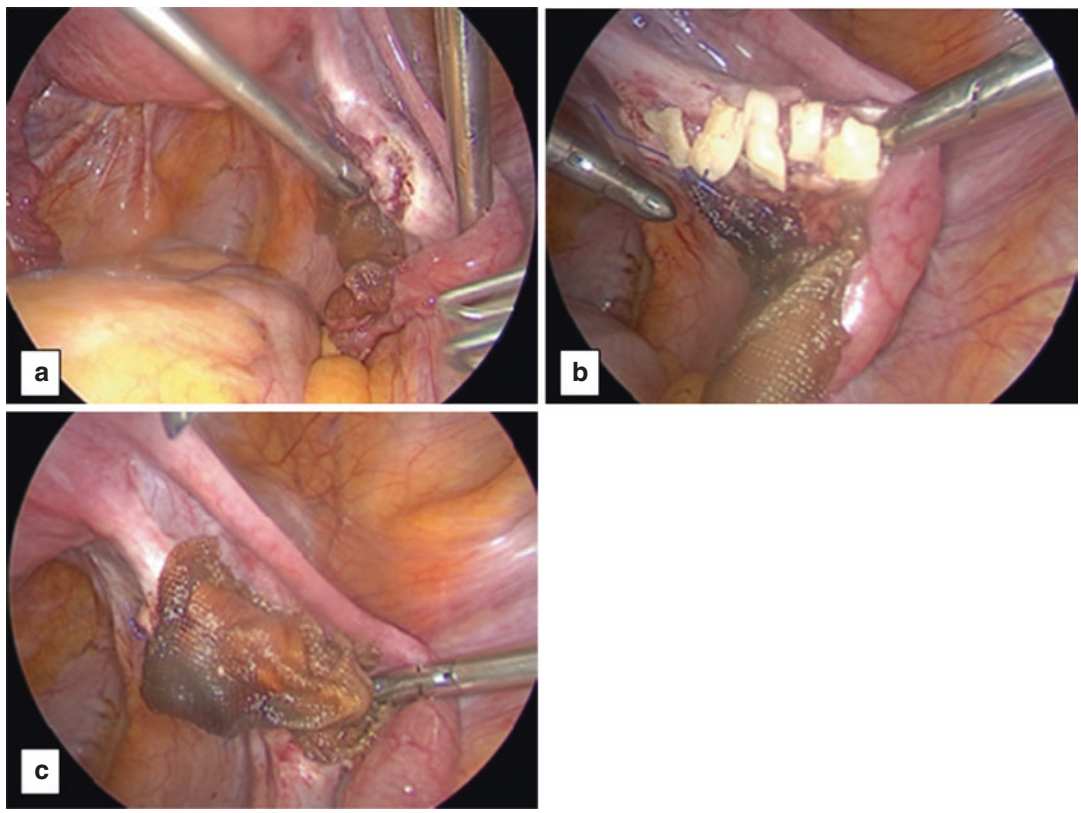


Fig. 1 Donnez's technique: (**a**) image of a decorticated ovary showing the medulla and its vascular network; (**b**) ovarian cortical pieces placed in the denuded ovary without stitches and (**c**) subsequently covered and fixed with Interceed® or fibrin glue

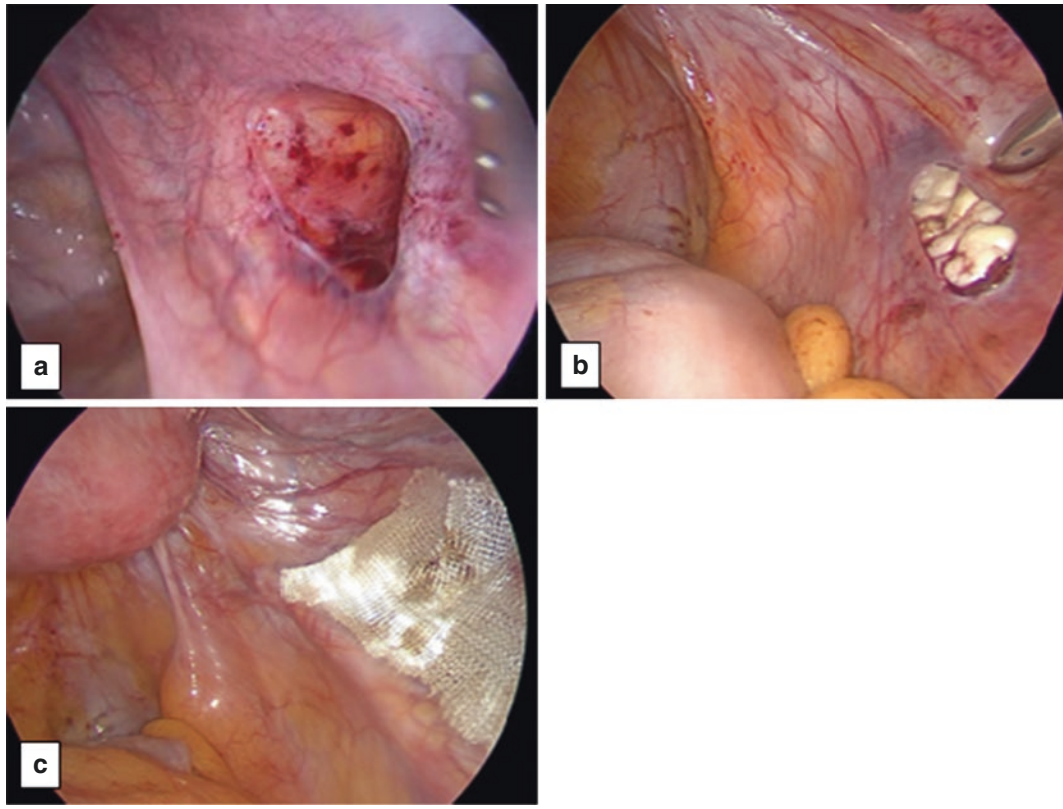


Fig. 2 Donnez's technique: (**a**) creation of a peritoneal window by means of scissors. (**b**) Ovarian cortex fragments placed with the cortical surface facing the abdominal cavity and (**c**) subsequentially covered and fixed with Interceed® or fibrin glue

tissue was performed in 2009 (Fig. 3). Restoration of ovarian function began at 20 weeks and was achieved 24 weeks after transplantation. Mild stimulation was performed: two embryos were obtained and transferred. The patient was pregnant and delivered a healthy baby [7]. A second healthy baby was born 2 years later. As in this woman both ovaries were completely removed, this case therefore provided definitive proof that pregnancy can occur from transplantation of frozen-thawed ovarian cortex [7].

Combined Technique

A third option for patients with one or two ovaries still in place is grafting the tissue to both orthotopic sites simultaneously (if there is enough ovarian tissue), namely, to the denuded ovary and the peritoneal window [9] (Fig. 4). To perform this type of transplantation, it is of utmost importance to cautiously choose the amount of tissue for transplantation, anticipating the potential need of further reimplantation to the same patient. It is recommended to thaw and graft only one third of the cryopreserved tissue in each patient.

Strategies to Improve Transplantation Outcomes

Early post-transplantation hypoxia remains an issue because of its negative impact on follicle survival, with follicle loss of >50% often observed during the first few days post-grafting [10, 11], leading to massive follicular activation

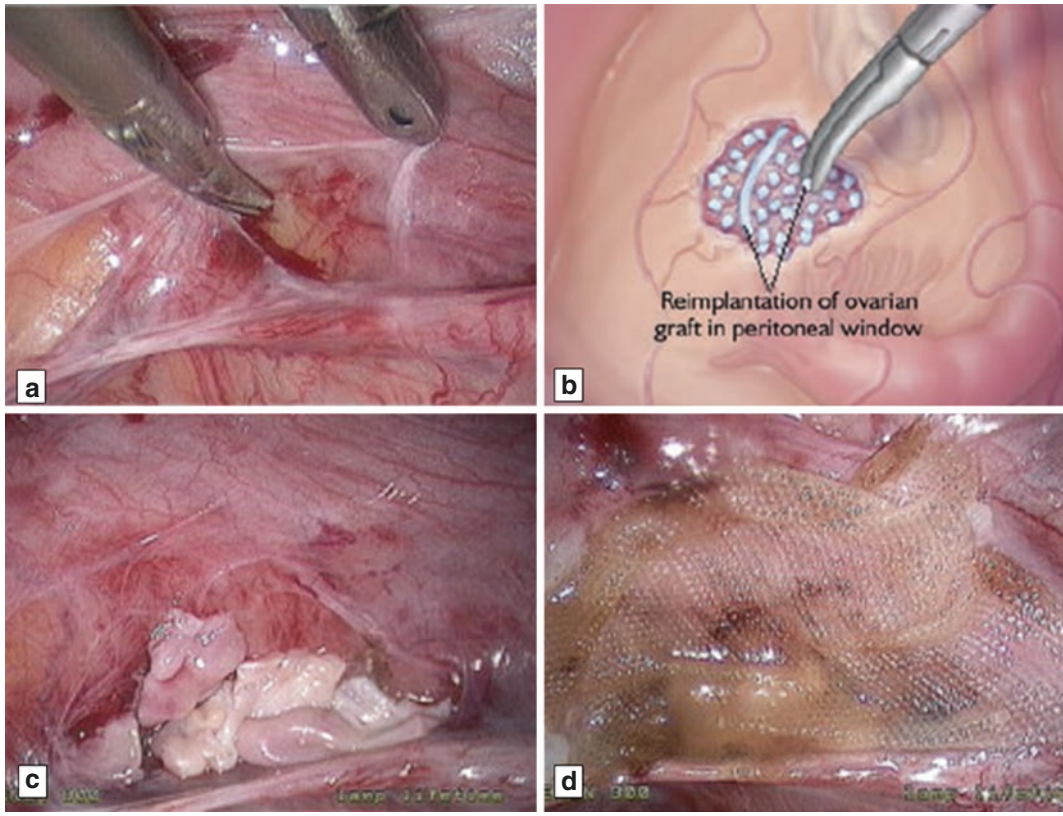


Fig. 3 These figures, published in Fertility and Sterility in 2012, are from the first case which provided definitive proof that pregnancy can occur from transplantation of frozen-thawed ovarian cortex, as the patient had undergone previously a bilateral oophorectomy. Surgical procedure. (**a**) Creation of the peritoneal window in the anterior leaf of the broad ligament. (**b**, **c**) Cryopreserved thawed ovarian fragments are placed in the peritoneal window and then covered by Interceed® (**d**)

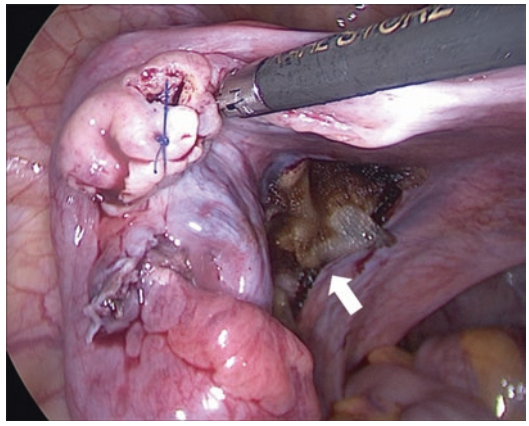


Fig. 4 Combined technique: simultaneous reimplantation of ovarian cortical fragments fixed by means of stitches to the previously denuded ovary and to a peritoneal window (white arrow) (adapted from *Donnez et al. Minerva Gynecol 2018)*

and "burnout" as described for the first time by Dolmans et al. [12]. Reoxygenation of ovarian tissue indeed occurs progressively, reaching stable levels 7–10 days after transplantation [13]. Moreover, oxidative stress by reactive oxygen species release was found to occur even later and end around 18 days after transplantation, when ovarian tissue metabolic activity is also reestablished [14].

Increasing vascularization in grafted tissue is crucial, and efforts are being made to improve follicle survival rates with a view to increasing the efficiency of ovarian tissue transplantation. One approach involves enhancement of graft revascularization by delivering both angiogenic and antiapoptotic factors [1, 6], while another seeks to boost neovascularization using adipose

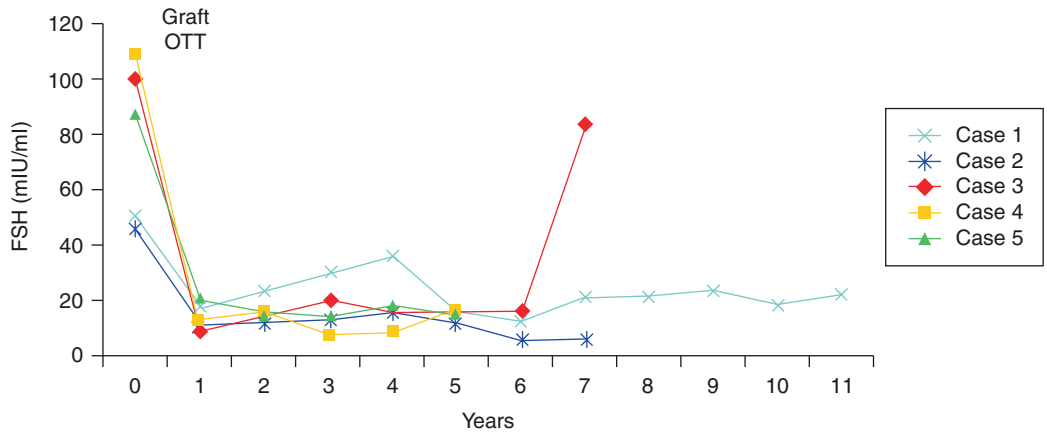
tissue-derived stem cells in an experimental model, introducing a novel two-step transplantation procedure [15]. Using this approach, we very recently demonstrated higher rates of oxygenation and vascularization of ovarian tissue in the early post-grafting period, ultimately leading to lower apoptosis and increased follicle survival [16].

After orthotopic reimplantation of frozen-thawed ovarian cortical fragments, ovarian endocrine activity is reinstated in nearly all cases (95%) [1, 2] (Fig. 5). Even though it is difficult to determine the life span of grafted tissue, the mean duration of ovarian function after transplantation is 4 to 5 years, but it can persist for up to 7 years [17] (Fig. 5). Duration of function in the grafts depends on a number of factors, such as age at cryopreservation, follicle density, and quality of grafter tissue, among others. Indeed, women giving birth after ovarian tissue transplantation were significantly younger at the time of cryopreservation compared to those who fail to conceive despite a pregnancy wish [3].

In our series of 22 women undergoing ovarian tissue reimplantation, the live birth rate was 41% (9 of 22) [1, 2]. One of our patients delivered three times, making her one of the two patients in the world to achieve three pregnancies from a single ovarian tissue reimplantation procedure [18]. In a recent paper, reporting the results of the Dolmans, Meirow, and Silber teams, the pregnancy rates and the live birth rates were respectively 50% and 41% [2, 3].

Silber's Technique (Fig. 6a,b,c)

The technique described by Silber in 2005 [19] was used for transplantation of fresh tissue. The transplantation was carried out by minilaparotomy through a 3.5-cm incision above the pubis [19, 20]. Remaining medulla from ovarian sections to be grafted should be removed before reimplantation. The cortex of each streak ovary is resected under magnification, exposing the entire raw surface of the medulla (Fig. 5a) [21]. Hemostasis should be meticulously controlled inside the medulla using microbipolar forceps and continuous irrigation with heparin-treated saline in order to prevent the formation of a hematoma beneath the graft. At the same time, care should be taken to avoid impairing revascularization by minimizing cauterization. A section of ovarian cortex is laid over the raw medulla of



Case 1: second orthotopic transplantation at 5 years (still functioning after 11y)
Case 2: 3 pregnancies and deliveries (still functioning after 7y)
Case 3: second orthotopic transplantation at 2 years; 1 pregnancy (egg donation) and delivery
Case 4: 2 pregnancies and deliveries (still functioning after 5 years)
Case 5: graft still functioning after 5 years

Fig. 5 Long-term duration of ovarian function after orthotopic transplantation in a series of five women having undergone ovarian tissue cryopreservation at the age of <22 years old (adapted from *Donnez and Dolmans, JARG 2015*)

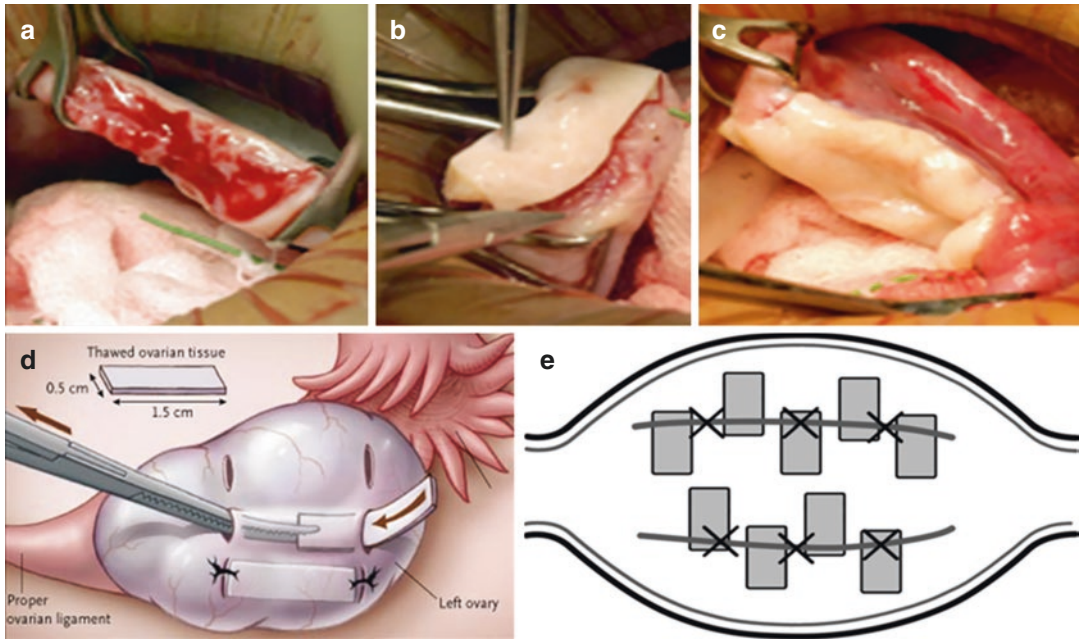


Fig. 6 Silber's technique: transplantation by minilaparotomy. Cortex of each streak ovary is resected under magnification, exposing the entire raw surface of the medulla (**a**). A section of ovarian cortex is laid over the raw medulla of each ovary (**b**) and sutured to the medulla with 9/0 nylon interrupted stitches (adapted from Silber et al.) (**c**) [21]. Meirow's technique: three pairs of transverse incisions made in the ovary through the tunica albuginea. With blunt dissection, cavities formed beneath the cortex for each strip. Each piece of thawed ovarian tissue gently placed in the cavities, and the incisions were closed with 4/0 Vicryl sutures (adapted from Meirow et al.) (**d**). Andersen's technique: schematic representation of how the cortical strips were transplanted to the remaining postmenopausal ovary. Two incisions were made on each side of the ovary, where the strips were positioned next to one another with the cortical side facing away from the ovary (adapted from *Andersen et al., Hum Reprod 2008)* [22] (**e**)

each ovary [5, 21] and sutured the medulla with 9/0 nylon interrupted stitches.

Meirow's Technique (Fig. 6d)

Meirow's transplantation procedure was first described in 2005 [5] and involves three pairs of 5 mm transverse incisions in the ovary through the tunica albuginea. Blunt dissection is used to create cavities beneath the cortex for each of the strips of thawed ovarian tissue (1.5×0.5 cm in area and 0.1–0.2 cm in thickness), which are gently placed in the cavities. The incisions are closed with 4/0 Vicryl sutures.

Andersen's Technique (Fig. 6e)

Andersen's transplantation procedure is performed as a combined laparoscopy/minilaparotomy and was first described in 2008 [22]. Ovarian cortical tissue fragments are transplanted into subcortical pockets in the remaining follicle-depleted ovary in all patients. The ovary is mobilized laparoscopically and made accessible through a 5-cm abdominal incision. Longitudinal incisions are made in the ovarian cortex to create two pockets, one on each side of the ovary, and the fragments are aligned next to one another in the pockets.

Heterotopic Ovarian Tissue Transplantation

Common sites for heterotopic transplantation are the abdominal wall, forearm, and rectus muscle, among others. According to Kim [23], heterotopic transplantation may offer some advantages: (1) it avoids invasive abdominal surgery; (2) follicular development can be effortlessly monitored, and oocytes can be easily retrieved; (3) the technology is cost-effective when repeated transplantation are required; (4) it is feasible even in case of severe pelvic adhesions that preclude orthotopic transplantation; and (5) the transplanted tissue can easily be removed and/or replaced if necessary.

According to Gook's technique [24, 25], all transplantations were performed via a laparoscopic procedure with each suture introduced to the abdominal cavity through a 10 mm port and a single suture transplanted at each site. For abdominal transplantation, the abdominal port was withdrawn slightly, and using atraumatic graspers at the port entry position, a lateral channel was made just under peritoneum. The jaws of graspers opened just at peritoneal membrane and the membrane cut. The graspers then pushed through opening and suture with tissue collected by grasper. The graspers then retracted back along channel, and once under peritoneal membrane, grasper jaws opened and the suture left in position (visualized just under peritoneal membrane) (Fig. 7). In the first case described in 2010, multiple cycles and episodic follicular development from both lateral pelvic and abdominal graft sites were observed, but only three oocytes were retrieved and two embryos were transferred with no pregnancy [24], demonstrating that heterotopic sites are not optimal for follicular development. Two years after the initial grafting procedure [25], additional slices of ovarian tissue were grafted into the right and left anterior abdominal walls by a small incision. Subsequent ovarian stimulation and transabdominal pickup were performed: two mature oocytes were retrieved, which were fertilized, and two embryos were transferred, resulting in the first pregnancy after. It should be noticed that according to the

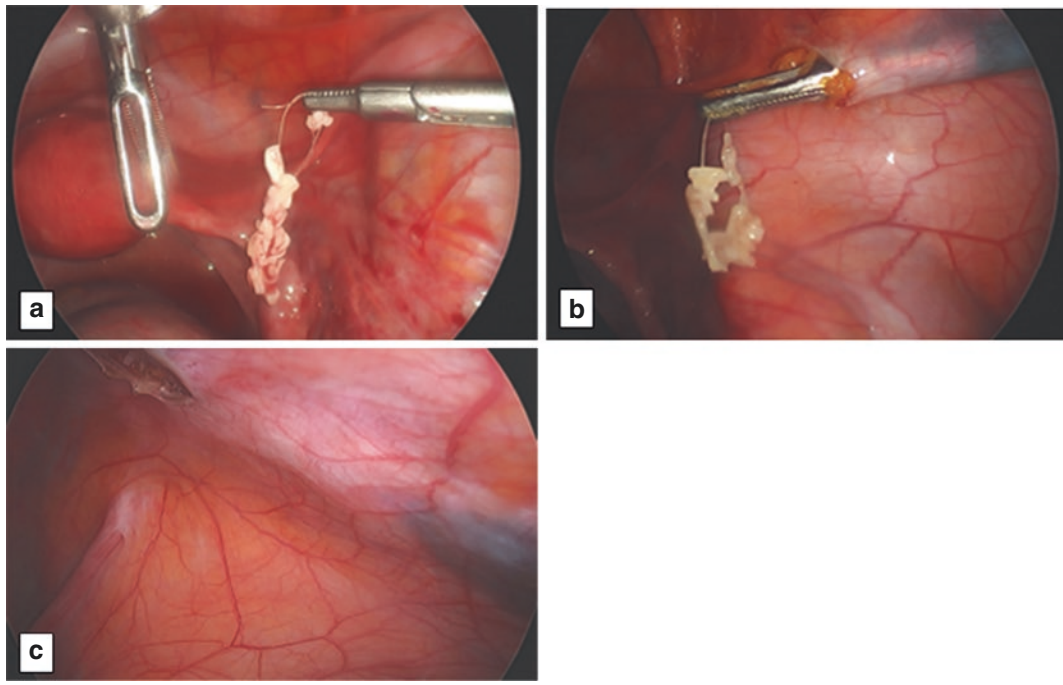


Fig. 7 Gook's technique: ovarian cortex fragments are stitched together (**a**), then grabbed by laparoscopic forceps (**b**) and placed in the lateral channel made under peritoneum using atraumatic graspers (**c**)

published figures in the case report [25], the grafts were placed just beneath the peritoneum.

Whole Ovary Transplantation

Whole ovary transplantation requires specialized expertise with microsurgical methods for vascular reanastomosis, as the vascular pedicles of the grafted tissue must be attached to the ovarian vessels in situ, thus increasing operative and postoperative risks. Only one pregnancy and live birth has been reported using a whole ovary transplantation approach with fresh tissue between monozygotic twins discordant for premature ovarian insufficiency [26].

Allotransplantation of Fresh Human Ovarian Tissue

Between Homozygotic Twins

The first case was reported by Silber in [19]. The donor underwent laparoscopic removal of her left ovary under general anesthesia. The ovarian cortical tissue was trimmed to thickness of 1–2 mm by excising medullary tissue. Meanwhile, the recipient underwent a minilaparotomy through a 3.5 mm incision above the pubis. The cortex of each streak ovary was resected under magnification, exposing the entire raw surface of the medulla. Continuous care was taken to avoid impairing revascularization by minimizing the amount of cautery. A section approximately one third of the donor ovarian cortex was laid over the raw medulla of each ovary in the recipient and sutured onto the medulla with the use of 9.0 nylon interrupted stitches.

Between Genetically Different Sisters

Donnez et al. [27] demonstrated that ovarian function and fertility may also be restored after allografting of ovarian cortex between genetically different sisters, reporting the first live birth after this procedure (Fig. 8) [1, 28]. In this case, a large biopsy was taken from left ovary of the donor sister, taking care not to remove medullary tissue, and divided in two parts measuring 2 × 1 cm (Fig. 9a). Each part was immediately sutured to the decorticated recipient ovaries. The ovarian pieces were thus sutured to the recipient ovarian medulla as soon as they were recovered. No medium or ice was used. The time interval between cortex removal and the start of suturing was <1 min, and both sutures were achieved within 30 min of the fragment being excised. The edges of the cortical frag-

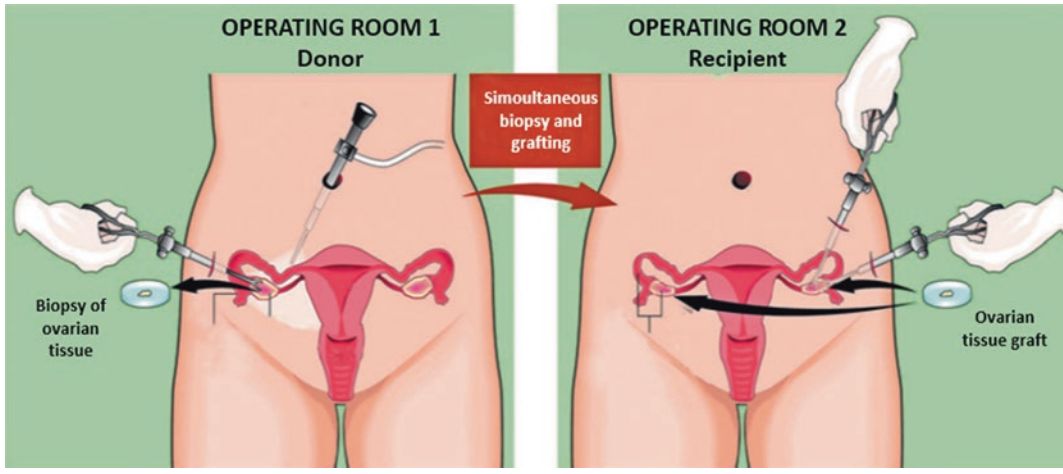


Fig. 8 Illustration of two simultaneous surgeries in two contiguous operating rooms. Two genetically different sisters were operated on: ovarian tissue was laparoscopically removed from the donor's ovary and immediately grafted to the recipient's ovarian medulla (adapted from *Donnez and Dolmans, NEJM 2017*) [1]

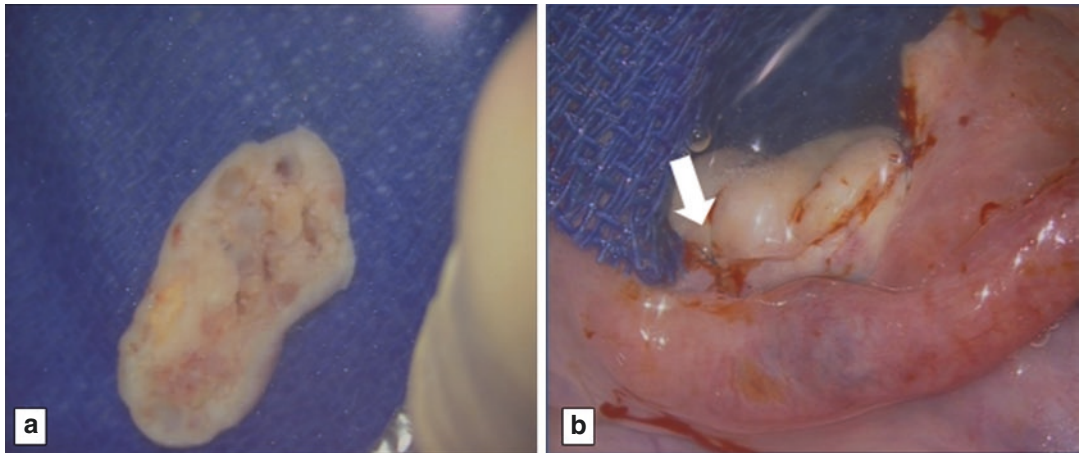


Fig. 9 Allograft of ovarian cortex between genetically different twins: (**a**) a large biopsy was taken from the left ovary of the donor sister and (**b**) immediately sutured (white arrow) to the decorticated recipient ovary

ments were sutured to the decorticated edges (Fig. 9b), so that contact between the donor cortex and receiver medulla was optimal. Several months later a laparoscopy was carried out to control the tubal patency and to correct it in case of stenosis, and the ovary showed development of follicles and corpus luteum (Fig. 10). Sixteen months after transplantation, stimulation was initiated with recombinant FSH and GRH antagonist. Two embryos were obtained from three mature oocytes; one embryo was transplanted, and the patient, pregnant afterwards, delivered a healthy baby. It was proved by genetic testing that the baby born was indeed conceived from an oocyte originated from donor ovarian tissue. Allografting of human ovarian tissue thus has the potential to restore not only ovarian activity but also natural fertility.

The recipient had received bone marrow from her human leukocyte antigen (HLA)-compatible sister. Transplantation of organs like kidneys has already been performed between HLA-compatible sisters who have previously undergone bone marrow transplantation (BMT), with one sister acting as donor to the other. Indeed, Hamawi et al. [29] reported cases of kidney transplantation after BMT, the donor being the BMT donor in all cases. The patients did not receive immunosuppressive treatment, and there was no sign of rejection, proving that immunosuppression is not required for BMT recipients who receive a kidney from their bone marrow donor.

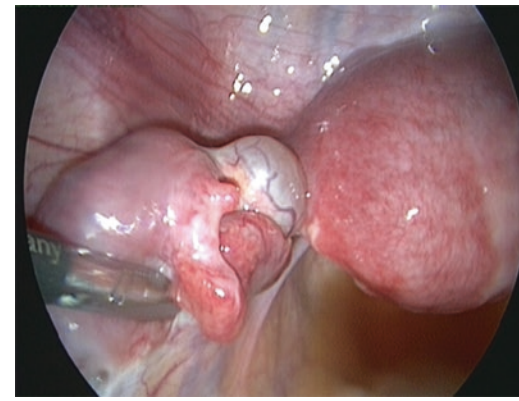


Fig. 10 Ovarian activity restoration, with follicles and corpus luteum development, several months after the allograft between genetically different sisters

In our case, HLA group analysis revealed complete chimerism (HLA compatibility) between the two sisters [28]. It was therefore proposed that ovarian tissue be grafted from the sister who had already donated bone marrow to the recipient sister with premature ovarian insufficiency.

Conclusions

Numerous surgical techniques have been reported and are currently being used for frozen-thawed ovarian tissue reimplantation, but successful

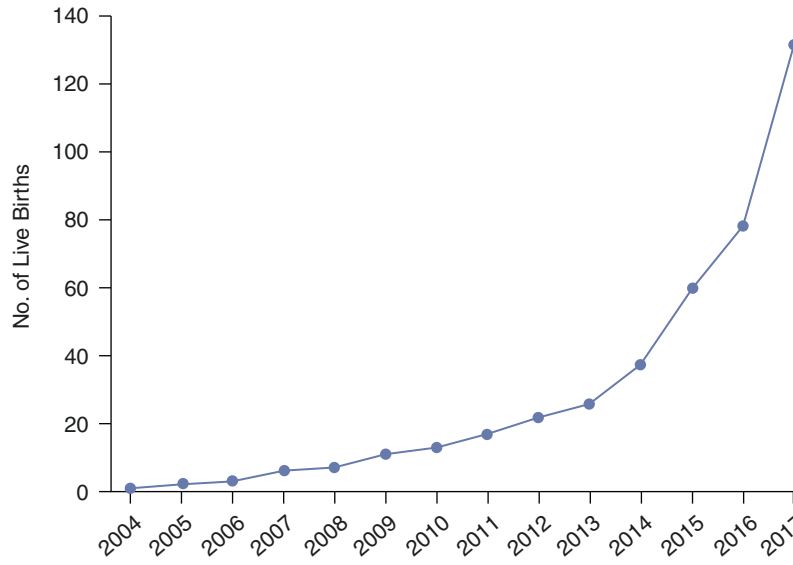


Fig. 11 Success rate of orthotopic ovarian tissue transplantation. Since 2004, when the first pregnancy was reported, the number of live births has reached more than 130 showing an exponential increase (adapted from *Donnez and Dolmans NEJM 2017*)

approaches all adhere to the same fundamental microsurgical principles: (1) finding a well-vascularized orthotopic transplantation site and (2) attaching the ovarian tissue by means of stitches or Interceed®, whose edges are fixed with fibrin glue. Orthotopic reimplantation has proved to be the most effective technique in terms of ovarian activity resumption of all reported cases, with fertility restoration in over 40% of patients in our series and exponential success rates in terms of pregnancies (Fig. 11), as published in the NEJM [1].

Practical Clinical Tips

Orthotopic transplantation is carried out by minimally invasive surgery, by grafting either to the ovary after decortication or to a newly created peritoneal window if both ovaries are absent.

Orthotopic ovarian tissue transplantation has proved to be highly effective, restoring endocrine activity in 95% of cases and fertility in 40% of cases.

Heterotopic transplantation can be performed to the abdominal wall, forearm, or rectus muscle, with the advantage of being less invasive and allowing easy monitoring of follicle development and oocyte retrieval. Its disadvantage is that it is less effective than orthotopic transplantation and does not allow natural conception.

Ovarian function and fertility may also be restored after allografting of ovarian cortex between genetically different sisters, without requiring immunosuppression if they are HLA compatible.

Take-Home Message

Ovarian tissue transplantation has proved to be successful for both endocrine function and fertility restoration. As the ovarian tissue is grafted without vascular anastomosis, choosing a well-vascularized site is the first step to addressing hypoxic injury occurring after transplantation and therefore ensuring the success of the procedure itself. Of all techniques developed to reimplant ovarian cortical strips, the most effective involve orthotopic sites, grafting either to previously decorticated ovaries or to newly created peritoneal windows.

Key Reading

Orthotopic transplantation of frozen-thawed ovarian cortical strips restores fertility in more than 40% of cases, but efforts should be intensified to improve the process of graft revascularization [14–16].

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Assessing Safety in Ovarian Tissue Transplantation

Marie-Madeleine Dolmans
and Rossella Masciangelo

Introduction

Thanks to marked improvements in cancer treatments over recent years, there is a growing population of young women whose fertility potential is impaired as a result of lifesaving gonadotoxic therapy, causing premature ovarian insufficiency. As statistics continue to rise, it is vital that we give these women a chance to preserve their fertility. However, patients who require immediate oncological treatment and indeed prepubertal girls are not in a position to undergo ovarian stimulation to produce oocytes for freezing. Hence, their only option is cryopreservation of ovarian tissue prior to treatment for future reimplantation. Fragments of tissue can be swiftly and easily retrieved, frozen, and stored until the patient is declared free of disease, before being grafted back to the pelvic cavity when she is ready to conceive [1]. This procedure restores ovarian function over 95% of cases, and it is often maintained for 5–7 years. The younger the patient at the time of biopsy, the larger the ovarian reserve, so the higher the chances of conception. To date, over 130 live births have been obtained using this approach.

Unfortunately, not all cancers are the same in terms of risk to patients, and there is great concern about the possible presence of malignant cells in frozen ovarian tissue. It is essential that we rule out any potential tissue contamination to avoid reimplanting malignant cells that could cause recurrence of the primary disease. To do so, we must identify and examine the risks posed by different cancers but also investigate ways of eliminating cancer cells from frozen-thawed tissue in case of high-risk malignancies. In this chapter, we will explore the different indications for ovarian tissue cryopreservation and reimplantation and assess the risk involved according to cancer type: high risk, moderate risk, and low risk (Table 1).

Hematological Malignancies

Leukemia

Leukemia is one of the most common cancers in children and adolescents [3], and also one of the most curable, with survival rates exceeding 80%. However, it requires prompt and aggressive

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Table 1 Risk of ovarian metastasis according to cancer type

High risk (>10%)	Moderate risk (2–10%)	Low risk (0–2%)
Leukemia	Breast cancer, advanced stage	Breast cancer, early stage
Neuroblastoma	Colorectal cancer	Squamous cell carcinoma of the cervix
Burkitt lymphoma	Adenocarcinoma of the cervix	Hodgkin lymphoma
	Non-Hodgkin lymphoma	Rhabdomyosarcoma
	Ewing sarcoma	Soft tissue sarcoma
	Ovarian cancer	
	Borderline ovarian tumor	

From *Dolmans and Masciangelo, Minerva gynecologica, [2]*

chemotherapy with alkylating agents and often leaves patients infertile, making them prime candidates for ovarian tissue cryopreservation. Because leukemia is blood-borne, malignant cells reside in the bloodstream, so the threat of ovarian involvement is very real when tissue is reimplanted. It is therefore vital to discern any minimal disseminated disease prior to grafting.

Histology and immunohistochemistry usually constitute the first port of call but may fail to detect malignant cells in frozen-thawed ovarian tissue specimens. Polymerase chain reaction (PCR) analysis yields a more accurate picture. Indeed, the BCR-ABL gene was detected by RT-qPCR in the ovarian tissue of a chronic myeloid leukemia (CML) patient, thereby avoiding potentially unsafe transplantation of the tissue back to this subject [4].

Our team [5] also investigated the presence of leukemic cells in ovarian tissue frozen during the active phase of the disease, both from CML and acute lymphoblastic leukemia (ALL) patients, first by PCR and then by xenografting to immunodeficient mice. PCR revealed contaminated ovarian tissue issuing from 70% of ALL patients and 33% of CML patients. After xenografting the tissue, none of the CML-grafted mice developed the disease, while almost 50% of those grafted with ALL tissue were invaded (Fig. 1).

Rosendahl and Greve analyzed ovarian tissue from leukemia patients in complete remission [6, 7]. RT-qPCR identified malignant cells in ovarian tissue in two out of four patients with known molecular markers, but these results were not confirmed by subsequent xenografting, as no leukemic cells were detected by RT-qPCR after 20 weeks of grafting [6]. It therefore appears that ovarian tissue from leukemia patients in complete remission does not contain viable malignant cells able to transmit the disease, but specimens taken during the active disease phase should not be used for transplantation.

However, this does not mean that those at risk of ovarian metastasis should give up hope of ever giving birth. Indeed, there are two existing options for these leukemia patients to use their cryopreserved ovarian tissue. The most straightforward is to check very carefully for the absence of malignant cells in a particular patient. If all the safety tests prove negative, a multidisciplinary decision may be taken, along with informed patient consent, to cautiously graft some fragments back. This approach was adopted by the Israeli team, who reported the first live birth after tissue reimplantation in an acute myeloid leukemia patient [8]. In this instance, the tissue was frozen when the patient was in complete remission and prior to hematopoietic stem cell transplantation. Three fragments of frozen-thawed ovarian tissue were analyzed by light microscopy, cytogenetic analysis, next-generation sequencing, and xenotransplantation, and none were found to contain leukemic cells. Transplantation was then performed, allowing the patient to conceive twice, once after IVF and once spontaneously.

If the safety tests reveal a risk, the second option is isolating and purging individual follicles that can subsequently be grafted back to patients inside a scaffold (artificial ovary) [9]. To this end, ovarian tissue fragments undergo a process of enzymatic digestion, and isolated follicles are effectively washed several times [10]. In a previous study, suspensions of these follicles,

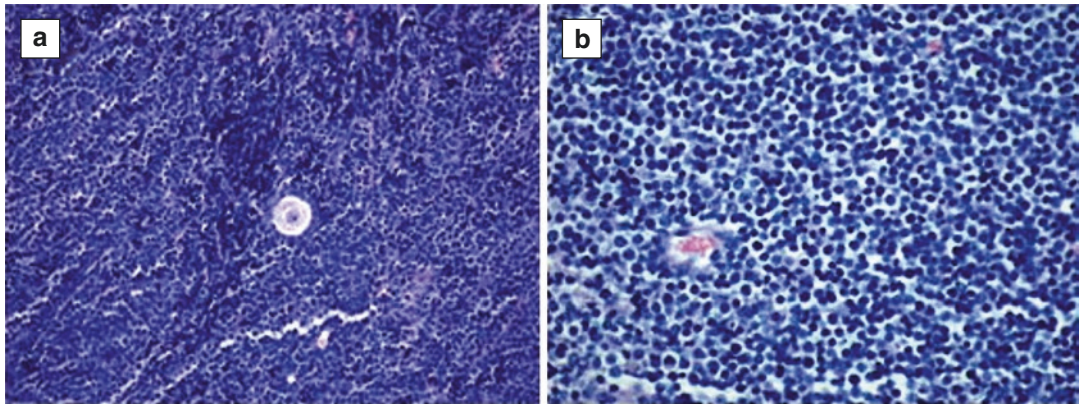

Fig. 1 (**a**) Ovarian follicle surrounded exclusively by lymphocytes; normal ovarian stroma is no longer present. Magnification ×100. (**b**) Massive lymphocytic invasion in an ovarian xenograft. Histological abnormalities were identified as malignant and attributed to leukemic invasion. Magnification ×200. Adapted from *Dolmans, Blood, [5]*

enzymatically digested from the ovarian tissue of 12 leukemia patients, did not show the presence of disease [11]. We must nevertheless tread very carefully; autotransplantation of ovarian tissue should be discouraged in leukemia patients if there is any sign of risk, but grafting of isolated follicles inside a specially created matrix may well prove feasible in the future.

Lymphoma

Hodgkin's (HL) and non-Hodgkin's lymphoma (NHL) are also frequent indications for ovarian tissue cryopreservation and transplantation, but while HL is in the low-risk category, NHL appears to carry a greater risk (moderate risk). A number of teams have attempted to evaluate the safety of autotransplantation in HL patients, and none have detected any malignant cells in their cryopreserved ovarian tissue, even in patients with advanced stage disease [4, 12–16], with the exception of one case report that identified malignant cells in the ovary of a patient with stage III HL [17]. However, there are no reports in the literature on HL recurrence after grafting, so we can conclude that the overall risk of ovarian metastases in case of HL may be considered low and autotransplantation safe.

Concerning NHL, no ovarian involvement was found by histology in the few studies conducted [13, 4]. Kim's group performed xenografting experiments with ovarian tissue from five NHL patients and was unable to prove disease recurrence [13], but Meirow reported one case of a solid pelvic mass in a 33-year-old patient affected by high-grade B-cell NHL, in whom cryopreservation was not carried out [4]. Dolmans et al. also detected malignant cells in the cryopreserved ovarian tissue of two NHL patients by histology and anti-CD20 immunohistochemistry [12] (Fig. 2). Hence, caution is required in case of NHL, especially when patients present in the leukemic phase of the disease, often associated with cystic ovarian masses [4].

Gynecological Malignancies

Breast Cancer

Breast cancer is the most commonly encountered malignancy in women [18]. Although ovarian metastases were found in almost 25% of breast cancer patients at autopsy evaluation [19], two teams were unable to locate any malignant cells in the cryopreserved ovarian tissue of breast cancer patients by immunohistochemical detection methods [20, 21].

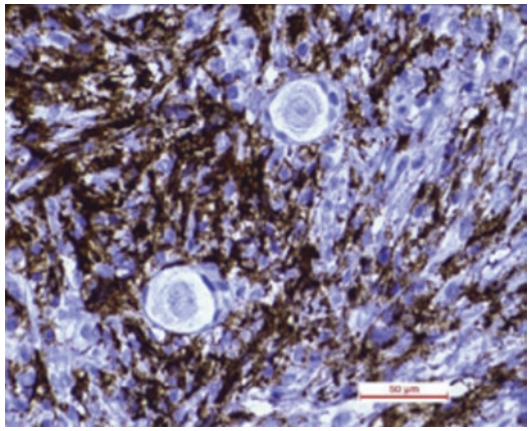

Fig. 2 Ovarian fragments from a non-Hodgkin's lymphoma (NHL) patient. Massive invasion by NHL cells in the cortex is shown by anti-CD20 immunohistochemistry. Adapted from *Dolmans, F&S, [12]*

Nevertheless, the suitability of breast cancer patients for frozen-thawed ovarian tissue transplantation depends on the stage of the primary disease. Reimplantation appears to be safe in women with low-stage breast cancer [22], while there is a risk in case of advanced stage breast cancer [23]. In a previous study by our group [23], qPCR detected mammaglobin 2 (MGB2) gene expression in 4 frozen-thawed ovarian tissue fragments and 1 graft out of 13 patients, even though histology and immunohistochemistry for epithelial membrane antigen (EMA), HER-2/neu, and gross cystic disease fluid protein-15 (GCD-FP15), as well as xenografting to immunodeficient mice, revealed no malignant cell contamination. These results confirm the potential risk of finding malignant cells in cryopreserved ovarian tissue in advanced stage breast cancer patients [23]. However, it is noteworthy that expression levels of specific breast cancer genes in the ovary are highly variable [24]. Mammaglobin 1 (MGB1) and GCD-FP15 have good positive predictive value to identify breast cancer cells in the ovarian cortex, while MGB2 detection shows greater specificity in the ovarian medulla. In the same study, MGB1 was detected in three out of five ovarian cortex biopsies from early-stage breast cancer subjects, but none with advanced stage disease, and none of the mice grafted with ovarian tissue expressing the marker showed any cancer recurrence. These authors therefore concluded that systematic analysis of the tissue should be encouraged using both immunohistochemistry and molecular tools prior to grafting, irrespective of disease stage.

Ovarian Cancer

Ovarian cancer is fifth on the list in terms of female mortality, and, while most cases occur in postmenopausal women, more than 10% of diagnoses are made in patients of childbearing age [25]. Cryopreservation of ovarian tissue from the unaffected contralateral ovary can be proposed to these patients, but safety concerns are well founded. Indeed, ovarian tumors originate from the same organ that will be cryopreserved and reimplanted, increasing the chances of preserving malignant cells. Grafting tissue back to ovarian cancer patients may therefore be somewhat problematic, as there is always a risk of reintroducing the primary disease along with the transplanted tissue.

Four cases of cryopreservation and subsequent reimplantation have been reported in ovarian cancer patients in the literature [26–29]. Pregnancies and live births were obtained in three patients after grafting, while in the fourth, hormone activity never resumed. In one case, a relapse occurred; the patient had previously had a granulosa cell tumor for which she underwent oophorectomy and prophylactic removal of the contralateral ovary, followed by ovarian tissue cryopreservation. Nine years later, her ovarian tissue was reimplanted, and, after low-dose hormone stimulation and IVF, two embryos were obtained and transferred, leading to the birth of healthy twins. However, during elective cesarean section, tumor dissemination was detected in diaphragmatic and peritoneal tissue, but not in the graft sites [29]. This relapse either could have been directly related to the transplanted ovarian tissue or could have been the consequence of microscopic peritoneal disease that responded to the pregnancy-induced hormone environment.

Minimal disseminated disease in ovarian cancer patients was investigated by Lotz's team by xenografting ovarian tissue from ten patients with ovarian tumors for 24 weeks and then performing histology and pan-cytokeratin analysis by immunohistochemistry [30]. These authors failed to detect any malignant cell contamination. In Kristensen's report, a 23-year-old patient diagnosed with stage 1C ovarian mucinous cystadenocarcinoma underwent tissue cryopreservation for fertility preservation and subsequent heterotopic transplantation to restore fertility 9 years later. After achieving a successful twin pregnancy by IVF, the grafted tissue was laparoscopically removed for safety reasons and refrozen. Histological evaluation revealed that a small number of growing follicles ($n = 5$) were indeed able to maintain normal hormone production and support ovarian function. This group also suggested that removing and refreezing grafted ovarian tissue could be a new way of managing cancer patients at risk of malignant cell recurrence [27].

Borderline Ovarian Tumors

Among ovarian tumors, borderline ovarian tumors (BOTs) account for 10–20% of cases. They are characterized by low malignant potential and mostly affect women of reproductive age, making them optimal candidates for ovarian tissue cryopreservation and transplantation. Masciangelo et al. evaluated the safety of reimplantation of cryopreserved ovarian tissue from 11 BOT patients [31]. Hematoxylin and eosin staining, immunohistochemistry for mucin1 (MUC1) and cytokeratin 7 (CK7), RT-qPCR for the CK7 gene, and xenografting were performed in order to investigate the presence of BOT cells in ovarian tissue. BOT cells were detected in the cryopreserved ovarian tissue and graft of 1 out of 11 patients (9.1%), proving their ability to survive after transplantation (Fig. 3a–f). For this reason, it is essential to conduct preimplantation analysis of cryopreserved ovarian tissue from BOT patients prior to its transplantation, as the risk of reintroducing BOT cells able to survive after grafting exists. It is not possible to exclude development of new BOT cells from grafted ovarian tissue, so patients affected by ovarian cancer or BOTs who wish to conceive by ovarian tissue transplantation could undergo laparoscopic removal of the grafted tissue after pregnancy.

Cervical Carcinoma

Reports on ovarian tissue transplantation after cervical carcinoma have been published by the team of Kim [32–34]. They autografted ovarian tissue from three patients with cervical carcinoma and encountered one pelvic relapse [34].

It is noteworthy that cervical cancer treatment might also involve hysterectomy, so patients requiring hysterectomy must be made aware that restoration of fertility through transplantation is not sufficient to achieve a pregnancy and that surrogacy will be needed. It is also worth mentioning that no pregnancy has ever been achieved after ovarian tissue reimplantation in patients diagnosed with cervical cancer, as confirmed in the latest European series [35].

Bone and Soft Tissue Sarcoma

Sarcomas are a heterogeneous group of tumors that develop from connective tissue. They represent more than 20% of solid tumors in the pediatric population and are the third most common cancer after leukemia and central nervous system tumors. More than 50 types of sarcoma have been identified, but the most frequent forms among infants are rhabdomyosarcoma (RMS) and Ewing sarcoma (EWS). Both RMS and EWS are often diagnosed when already disseminated (20% and 40%, respectively), involving the lungs, lymph nodes, and bone marrow. Standard therapies include surgery, chemotherapy, and radiotherapy, yielding high survival rates but impairing ovarian function and future fertility. Ovarian involvement has been reported in certain types of sarcoma, like EWS and osteosarcoma, but it is rare in RMS [36].

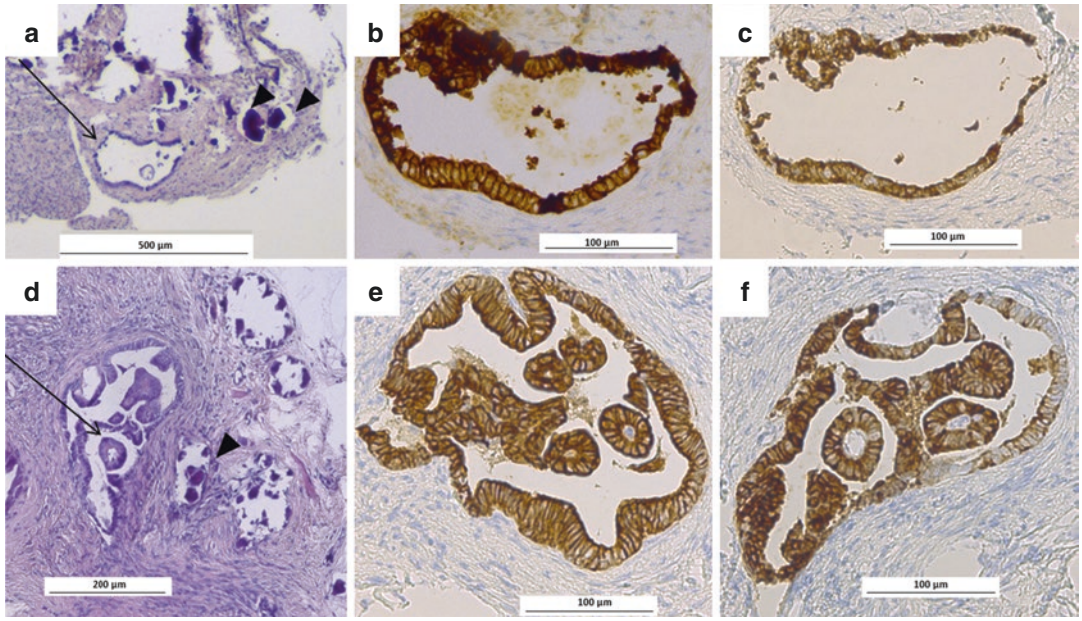

Fig. 3 Frozen-thawed human ovarian tissue and a human ovarian tissue xenograft from one BOT patient after 5 months of grafting to an immunodeficient mouse. (**a**) Histological aspect of frozen-thawed ovarian tissue: a BOT lesion can be seen (black arrow) surrounded by several psammoma bodies (arrowheads). (**b**) Positive immunohistochemical results for CK7 in ovarian tissue. (**c**) Positive immunohistochemical results for MUC1 in ovarian tissue. (**d**) Histology of the xenograft: a BOT lesion can be seen containing papillary projections (black arrow) and surrounded by psammoma bodies (arrowhead). (**e**) Positive immunohistochemical results for CK7. (**f**) Positive immunohistochemical results for MUC1. From Masciangelo et al., Human Reproduction [31]

Three teams have investigated the presence of malignant cells in cryopreserved ovarian tissue from sarcoma patients. Abir analyzed frozen-thawed ovarian tissue from eight EWS patients using anti-CD99 immunohistochemistry and, in five cases, RT-qPCR for the EWS-FLI1 fusion gene. Immunohistochemistry failed to detect any malignant cells, but the EWS-FLI1 fusion gene was identified in one patient, confirming the presence of malignant cells [37]. Greve's team evaluated cryopreserved ovarian tissue from 16 patients (9 EWS, 4 osteosarcomas, 2 synovial sarcomas, 1 chondrosarcoma) by xenografting, histology, and RT-PCR for the EWS-FLI1 fusion gene. They were unable to find any sign of malignant cell contamination in the ovarian tissue, and transplantation to immunodeficient mice did not induce disease recurrence [6]. Finally, Dolmans analyzed cryopreserved ovarian tissue from 26 sarcoma patients (14 EWS, 12 soft tissue sarcomas) after performing genetic characterization of the primary tumor by immunohistochemistry, fluorescence in situ hybridization, and RT-qPCR in order to identify tumoral markers to be tested on the tissue. None of the samples revealed the presence of any malignant cells. Reimplantation in sarcoma patients can therefore be considered safe, with the exception of EWS patients, in whom caution should be exercised.

Central and Peripheral Nervous System Tumors

Neuroblastoma is the most common extracranial solid tumor in infants, with high metastatic potential. Several cases of ovarian involvement in metastatic neuroblastoma have been reported, and this is the childhood cancer that most frequently spreads to the ovaries, with more than 60% of metastatic neuroblastomas showing circulating malignant cells at the time of diagno-

sis [38–40]. Grèze conducted a study in which healthy ovarian tissue (n = 20) was in vitro-contaminated with human neuroblastoma cell lines and subsequently tested for minimal disseminated disease detection by RT-qPCR for tyrosine hydroxylase (TH), paired-like homeobox 2B (PHOX2B), and doublecortin (DCX) genes. These authors found PHOX2B to be a reliable marker for neuroblastoma cells within ovarian tissue, while TH and DCX were also detected in uncontaminated ovarian tissue, leading to false-positive results, so they cannot be used for this purpose [39]. However, to date, no studies have been published on minimal disseminated disease evaluation of ovarian tissue from patients diagnosed with neuroblastoma.

Conclusion

In conclusion, the pathology that carries the highest risk of reseeding malignant cells upon transplantation is leukemia. Ovarian tissue transplantation is therefore not recommended in these patients. Nevertheless, cryopreservation of ovarian tissue at the time of disease remission, combined with thorough tissue evaluation using sensitive techniques, did allow safe and successful tissue reimplantation in a patient with acute myeloid leukemia. Regarding all other pathologies, even those considered to be low risk, safety cannot be guaranteed, so meticulous tissue examination and informed consent are essential before proceeding with ovarian tissue transplantation.

Practical Clinical Tips

Leukemia is at highest risk of malignant cell contamination, and the absence of malignant cells should be checked carefully in these patients. If all the safety tests prove negative, some fragments can be cautiously grafted back, while in case the tests reveal a risk, individual follicles can be isolated, purged, and subsequently grafted back to patients inside a scaffold (artificial ovary) [9].

Caution is required in case of non-Hodgkin's lymphoma, and histology and anti-CD20 immunohistochemistry are recommended. Similarly, systematic analysis should be encouraged in ovarian tissue of breast cancer patients, using both immunohistochemistry and molecular tools prior to grafting, irrespective of disease stage.

Concerning ovarian cancer and BOT patients, it is not possible to exclude development of new malignant cells from grafted ovarian tissue; therefore, removing and refreezing the grafted tissue could be a new way of managing these patients at risk of recurrence [27].

The overall risk of ovarian metastases in Hodgkin lymphoma and soft tissue and bone sarcomas, with the exception of Ewing sarcoma, may be considered low and autotransplantation safe.

Take-Home Message

Ovarian tissue transplantation allows for restoration in cancer patients, but a multidisciplinary approach, thorough preimplantation evaluation, and informed consent are necessary prior to transplantation. Leukemia carries the highest risk, and transplantation is not recommended, but performing cryopreservation of ovarian tissue at the time of disease remission, combined with thorough tissue evaluation using sensitive techniques, may give a chance of safe reimplantation in patients with acute leukemia. Regarding all other pathologies, even those considered to be at low risk, safety cannot be guaranteed, so meticulous tissue examination is essential before proceeding with ovarian tissue transplantation.

Key Reading

- Transplantation of ovarian tissue allows for restoration of fertility in young cancer patients, but tissue examination and informed consent are essential before proceeding with reimplantation.

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Fertility Preservation in Breast Cancer Patients

Loris Marin, Volkan Turan, and Kutluk Oktay

Introduction

Breast cancer is the most common malignancy in women as it currently represents 15.2% of all new cancer cases in the United States with 268,600 estimated new cases in 2019 [1]. Under 45 years of age, the percentage of new cases is 10.3% as it is the most common of all in women of childbearing age. Due to the increasingly advanced diagnostic and therapeutic techniques, the mortality rate remains low in recent years. For stages I and II, the 5-year survival is estimated to be respectively 95% and 85–70% (depending on whether they are IIA and IIB), while in advanced stages, survival rates are between 18 and 52% (for stages IIIA and B) [1].

Because the number of young cancer survivors is increasing and as women tend to have children in later reproductive ages, increasing attention has been paid to chemotherapy-related ovarian toxicity [2]. Most women with breast cancer are likely to undergo neoadjuvant or adjuvant chemotherapy that may result in premature ovarian failure and infertility [3–5].

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According to international guidelines all over the world, cancer patients should receive information about the side effect of cancer treatments on fertility before treatment, and they should be referred to a reproductive specialist to discuss the risk of ovarian damage and the current available fertility preservation options [6–8]. However, according to some reports, less than half of cancer specialists in the United States and in Europe refer young cancer patients to fertility preservation/reproductive medicine specialists [9–11]. Given the age of the women at the time of the diagnosis and the available therapeutic procedures, the treatment of breast cancer has a greater impact on female reproduction [12, 13].

Ovarian dysfunction after chemotherapy is difficult to predict as many factors play a role including age, the initial ovarian reserve, and the type and dose of chemotherapy regimen utilized [4, 14]. Cyclophosphamide, a commonly used chemotherapy drug in the treatment of breast cancer, is a highly gonadotoxic agent and causes human primordial follicle apoptosis through induction of double-strand breaks in DNA [4, 15, 16]. Anthracyclines are also commonly used in young women with breast cancer. This drug may cause ovarian injury both through direct effect on primordial follicles via the induction of DNA double-strand breaks [16–18] and through ovarian vascular damage [18]. Taxanes (docetaxel, paclitaxel) are commonly used in combination with anthracyclines and cyclophosphamide in

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women with breast cancer. However, there is paucity of data regarding their effects on ovaries. Nevertheless, because taxanes, in general, do not induce DNA damage, they are less likely to affect primordial follicle reserve.

Women with estrogen-sensitive breast cancer also receive hormonal therapy. These include tamoxifen and aromatase inhibitors, and they may be used for 10 years to lifetime. Action mechanisms of hormone therapy are mainly two: to block the proliferative actions of estrogen by binding to the estrogen receptors (i.e., tamoxifen and fulvestrant) or to inhibit estrogen production itself as aromatase inhibitors (i.e., letrozole, anastrozole, and exemestane) [19]. Though these agents are not gonadotoxic, women would have to delay their childbearing to completion of these treatments because they have teratogenic effects.

Fertility Preservation Techniques

The most established techniques for preservation of female fertility are embryo and oocyte cryopreservation [20]. Oocyte cryopreservation is recommended when a partner is not available or by patient preference. Both techniques require controlled ovarian hyperstimulation; hence, they are available only if there is at least a 2-week time interval before cancer treatment [8, 21]. The choice to cryopreserve oocytes or embryos is related to personal choices as well as social and cultural preferences [22]. Moreover, the choice may be restricted by law in certain countries. For example, in Japan embryo preservation is only allowed for married partners [22] while embryo preservation is never permitted in Italy [21].

As it has been previously reported [23], there are multiple major follicle recruitment waves during a normal menstrual cycle, creating multiple windows of opportunity for multiple follicle recruitment via ovarian stimulation. Based on this physiological evidence, a random-start controlled ovarian stimulation was first developed by Oktay et al. for women who do not have time to wait for menstrual period for conventional controlled ovarian hyperstimulation [24–27]. If there is a time constraint or if the patient is referred before puberty period for childhood cancers, ovarian tissue cryopreservation is another strong alternative for fertility preservation. Since the performance of first successful ovarian transplantation procedure by Oktay and Karlikaya [28], more than 152 children have been born worldwide after this technique.

Another fertility preservation technique that does not require controlled ovarian stimulation is immature oocytes retrieval. These oocytes can be retrieved during ovarian tissue harvesting for ovarian tissue [29]. Immature oocytes may either be directly cryopreserved or undergo in vitro maturation until they reach MII oocyte stage before cryopreservation. Although in vitro maturation is still considered experimental, live births have been reported with a success rate of 10–15 per embryo transfer [30].

Utility of GnRh Analogs for Fertility Preservation

The use of gonadotropin-releasing hormone analogs (GnRHa) to reduce ovarian damage caused by gonadotoxic chemotherapy agents has been widely studied though the quality of the studies varies [31]. However, primordial follicles do not express gonadotropin-releasing hormone (GnRH) receptors which means that follicles making up the ovarian reserve are hormonally insensitive [32]. The efficacy of GnRHa for prevention of chemotherapy-induced gonadal damage is controversial in the studies [33]. There is a lack of well-designed prospective randomized controlled trials that have assessed the role of GnRHa administration for fertility preservation. In the majority of the studies, the main outcome was the resumption of menstruation in different follow-up periods which was not associated with intact ovarian function. In some studies, resumption of the menstruation was not well defined, and any bleeding was considered as menstruation. Menstruation is not a reliable surrogate for fertility because women with severely diminished ovarian reserve may still continue to menstruate [34]. Furthermore, the use of less reliable ovarian reserve markers such as the untimed FSH

Table 1 Assessment of established and controversial fertility preservation options for premenopausal women with breast cancer

	Overview	Advantages	Disadvantages
Embryo cryopreservation	Most common method for fertility preservation in post-pubertal women with available sperm and when there is time for ovarian stimulation before starting gonadotoxic therapy	The most established method	Requires time for ovarian stimulation as well as sperm
Mature oocyte cryopreservation	Increasingly common and successful	No need for sperm and some ethical issues avoided	Requires time for ovarian stimulation
Ovarian tissue cryopreservation and transplantation	Increasingly successful and no longer experimental in the United States and some other countries	Minimum treatment delay No sperm or ovarian stimulation required Can be performed pre- or post-pubertal Restores endocrine function and natural fertility	At least two surgical procedures required Limited graft longevity Theoretical malignant cell reintroduction risk in leukemias
GnRH agonist suppression (not recommended)	Despite the suggestion as a gonadal protective agent, its mechanism of action and benefit, if any, are unproven. We do not recommend GnRHa for fertility preservation	Non-surgical	Preponderance of evidence shows that it does not preserve fertility Worsens quality of life through severe hot flashes and possible permanent bone loss with long-term use

was another limitation of the studies. Studies that utilized AMH levels as an outcome measure did not find that ovarian suppression by GnRHa is beneficial in preserving ovarian reserve against chemotherapy [35].

A recent meta-analysis limited to premenopausal women with early-stage breast cancer only claimed that GnRHa can preserve fertility [36]. The meta-analysis was limited to five randomized trials, accounting for less than half of all randomized controlled trials when considering other randomized trials investigating the effectiveness of GnRHa use for fertility preservation in other malignancy types. Given that the mechanism of ovarian damage by gonadotoxic agents is similar regardless of the underlying malignancy type, a diagnosis-based meta-analysis does not seem plausible to assess the efficacy of GnRHa. Elgindy et al. [37] conducted a meta-analysis with all prospective studies regardless of malignancy type and found that GnRHa administration during chemotherapy does not appear to protect the ovaries from gonadal toxicity. Therefore, other established fertility preservation methods should be recommended for those patients. GnRHa may be used in women who are at risk for severe chemotherapy-induced thrombocytopenia and/or anemia to prevent menorrhagia but not for fertility preservation. A more detailed and periodically updated version of the discussion can be found elsewhere [8, 38] (Table 1).

Ovarian Stimulation in Women with Breast Cancer

Conventional ovarian stimulation protocols can lead up to a 10–12 fold increase in peak serum estradiol levels, which can induce breast cancer cell proliferation [39]. Therefore, natural cycle

IVF was offered at first to protect these patients from the potential deleterious effects of increased estrogen levels. Later, Oktay et al. used tamoxifen, a competitive estrogen receptor blocker, or letrozole, an aromatase inhibitor, for ovarian stimulation which resulted in increased number of embryo recovery per patient compared to natural cycle IVF [40]. Similar numbers of embryos were obtained with the letrozole-gonadotropin protocol in women with breast cancer when compared with conventional stimulation protocols used in infertile women [41, 42]. Furthermore, no significant increase in short- and long-term recurrence risk has been observed in patients with breast cancer undergoing ovarian stimulation with letrozole plus gonadotropin [43, 44]. Moreover, the same protocol was used consecutively in women who were referred before or just after breast surgery; thereby, higher number of embryos was obtained until chemotherapy without any increase in recurrence rate after a mean follow-up interval of 58.5 ± 13.6 months [23]. Recently, we compared the cycle outcomes of women with cancer who underwent ovarian stimulation using either letrozole combined with gonadotropins (breast cancer, $n = 118$) or gonadotropin alone (other cancer types, $n = 24$) for fertility preservation to see whether letrozole has any impact on fertility preservation cycle outcomes. Women with cancer were divided into two groups: hormone-dependent cancer and other cancers. We found that after adjusting for age, body mass index, and *BRCA* status, letrozole appeared to enhance response to ovarian stimulation [24].

Letrozole is initiated 5 mg per day on cycle day 2 followed by gonadotropins (150–450 IU per day) on cycle day 4 and both continued throughout the stimulation. To prevent a premature luteinizing hormone (LH) surge, GnRH antagonist 0.25 mg per day is initiated when the lead follicle size reaches ≥12 mm mean diameter, and this dose is continued until the trigger day. When at least two follicles reached at least >20 mm in diameter, oocyte maturation is triggered with either human chorionic gonadotropin (hCG) or leuprolide acetate [25]. Unlike conventional protocols, because of earlier antral fluid space formation, follicle size for trigger needs to be about 20–21 mm to avoid retrieving higher number of immature oocytes [41]. Mature oocytes were fertilized by intracytoplasmic sperm injection, and embryos were frozen mostly at blastocyst stage. Estradiol (E2) level is measured 3 days after oocyte retrieval if hCG is used for trigger. If the E2 level is >250 pg/mL, letrozole is continued for approximately 3–6 days until the E2 levels decrease to <50 pg/mL. Therefore, the use of GnRHa for trigger is more common to reduce the risk of estrogen exposure and ovarian hyperstimulation due to its shorter half-life. Though our first choice is letrozole, if women with breast cancer are already receiving tamoxifen therapy or if they cannot tolerate aromatase inhibitors, we then perform ovarian stimulation with tamoxifen, in addition to gonadotropins.

The impact of cancer diagnosis on response to ovarian stimulation for fertility preservation was evaluated for the possible effects of cancer in human metabolism, and it was shown that cancer itself was not associated with reduced response to ovarian stimulation. When the analysis was limited to women with breast cancer diagnosis, no difference was found in controlled ovarian hyperstimulation cycle outcomes between women with cancer and age-matched women without cancer [26]. However, there is still a paucity of data providing the pregnancy and fertility preservation outcomes. The largest study regarding the pregnancy outcomes of women with breast cancer was performed by Oktay et al. [27]. In this study, out of 141 women, only a quarter of those who underwent ovarian stimulation with letrozole plus FSH for fertility preservation have returned for embryo transfer, and fertility was preserved in more than half of the women. Despite the limited sample size, those were the first data on newborns from women with breast cancer who used their frozen embryos generated after ovarian stimulation with letrozole. Furthermore, no minor or major fetal malformations or developmental abnormalities were observed after a mean follow-up of 3 years.

In a setting of a late referral, random-start ovarian stimulation can be initiated regardless of the menstrual cycle day with similar success rates

in comparison with conventional ovarian stimulation protocols [45]. This approached is based on the fact that there are multiple follicular recruitment waves at any day of the cycle. The existing data demonstrated favorable pregnancy rates after late follicular or luteal start ovarian stimulation [46]. Furthermore, no increased risk for minor or major fetal malformations or developmental abnormalities has been reported in random-start stimulation cycles compared with conventional stimulation cycles [47]. The protocols used in breast cancer patients are shown in Fig. 1.

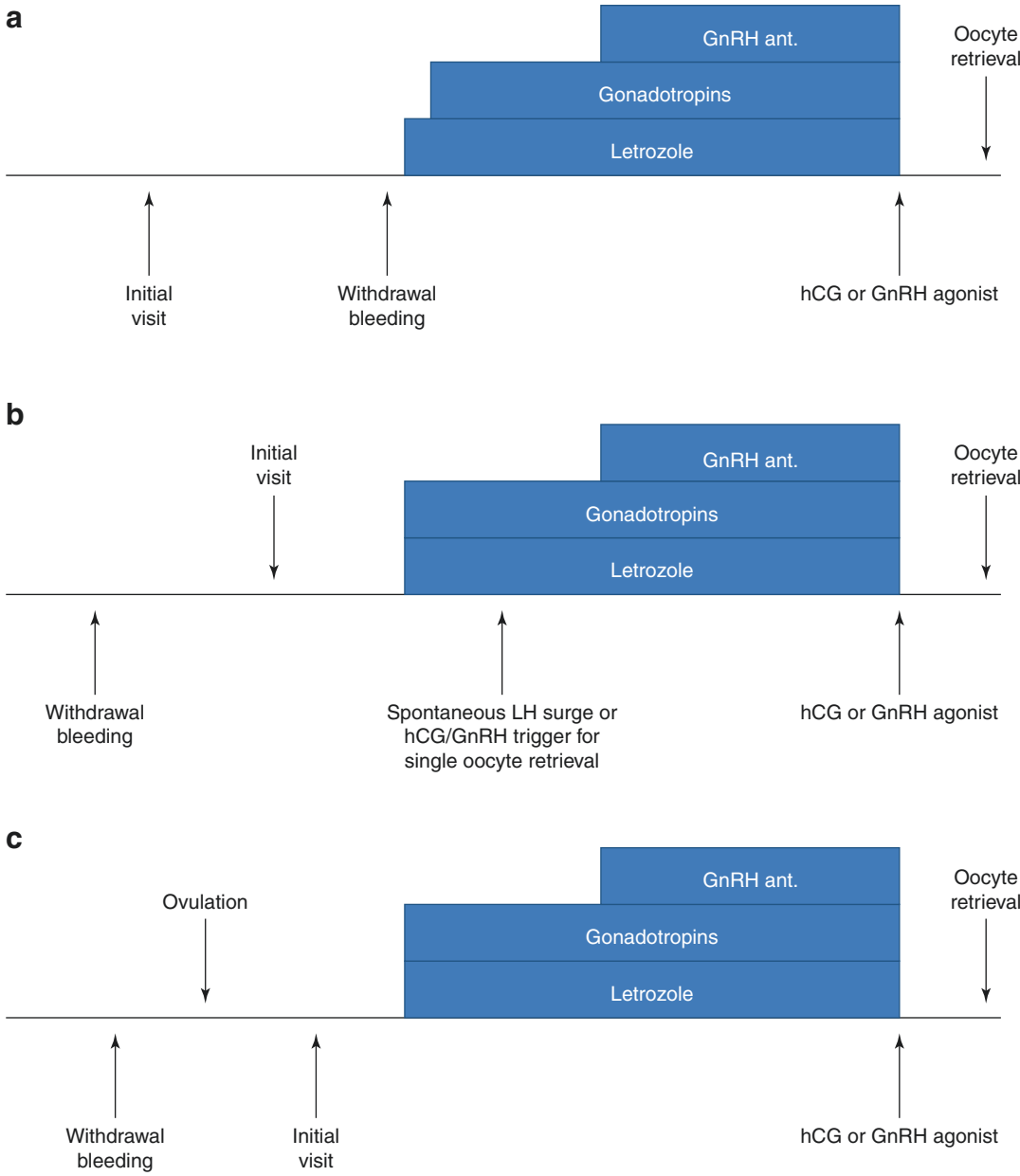

Fig. 1 Follicular and random-start letrozole antagonist ovarian stimulation protocols for breast cancer patients undergoing fertility preservation, as developed by Oktay et al. Conventional (**a**), random-start COH in the late follicular (**b**), and luteal phase (**c**)

In vitro maturation (IVM) is another option which can be used as a complementary strategy to improve the mature oocyte yield of breast cancer patients undergoing ovarian stimulation for fertility preservation. Following IVM, we showed that there is a 45% increase in mature oocyte yield in women with breast cancer who underwent ovarian stimulation for fertility preservation. Although data for pregnancy rate are limited, immature oocytes retrieved during oocyte/embryo cryopreservation cycles should be in vitro matured to improve the potential for future fertility [48].

Ovarian Tissue Cryopreservation in Breast Cancer Patients

Ovarian tissue cryopreservation and autotransplantation technique were introduced as a strategy to prevent fertility loss and premature ovarian insufficiency after gonadotoxic treatments. Although autologous ovarian transplantation of cryopreserved tissue has gone through remarkable evolution in the last 20 years and most recent live birth rates are exceeding 35%, it is still considered experimental by numerous international scientific societies [49, 50]. However multiple societies including the ASRM have moved forward to remove the procedure from the experimental category [51]. In prepubertal girls and in women who need to start cancer treatment urgently, ovarian tissue cryopreservation may be the only feasible technique for fertility preservation. Furthermore, other advantages include no need for a partner, and this approach is the only technique that guarantees ovarian endocrine function restoration and spontaneous conception [49]. There are several techniques for ovarian harvesting and transplantation such as laparotomic, laparoscopic, or robotic surgery [52–56]. The transplantation of ovarian tissue can be performed inside (orthotopic site) or outside the pelvis (heterotopic transplantation) [56]. The latter transplantation type is preferred when a close monitoring of the graft is needed or when a removal is planned after oocyte retrieval; however, in vitro fertilization and embryo transfer are the only options to conceive with this technique [49, 57]. The first embryo obtained after heterotopic transplantation of frozen-thawed ovarian fragments was reported in 2004 by Oktay et al. [58]. Ovarian cortical pieces were grafted subcutaneously to the upper forearm in a breast cancer patient 6 years after ovarian tissue cryopreservation, resulting in reversal of menopause [58, 59]. In the literature there are reported cases of spontaneous pregnancies after heterotopic ovarian transplantation [60–62]. First orthotopic successful frozen-thawed homologous ovarian tissue transplantation was performed in 1999 by Oktay and Karlikaya and reported in the year 2000 [28] with the restoration of endocrine ovarian function. Oktay et al. also reported the first oocyte retrievals and embryo development from frozen-thawed and transplanted ovarian tissue [63, 64]. Today, more than 152 pregnancies have been reported after ovarian tissue cryopreservation and orthotopic autotransplantation [65]. One of the concerns with this technique is the possibility of cancer cell reintroduction with transplantation. However, for most common indications for ovarian tissue cryopreservation, such as lymphomas and non-metastatic breast cancer, this is unlikely [66]. Another concern is regarding ovarian tissue transplantation in women carrying deleterious gene mutations associated with ovarian cancer [67]. For *BRCA* mutation carriers, the cumulative average risk of developing ovarian cancer reaches 39% for *BRCA1* mutations and 10% for *BRCA2* mutations [68]. However, if the ovarian tissue is cryopreserved at an early age when the risk of cancer is low and early removal is contemplated soon after achieving pregnancy goals, ovarian cryopreservation and transplantation may be considered in these women.

Fertility Preservation in Women with Deleterious *BRCA* Mutations

The cause of 5–10% of breast cancer is genetic, suggesting that an increased lifetime cancer risk is due to an inherited germline mutation in an autosomal dominant way. When the breast cancer is due to genetic causes, the median age of cancer

development is earlier than those with sporadic tumors. *BRCA1/2* (breast cancer susceptibility genes 1 and 2) are DNA double-strand break repair genes, mutations of which increase the breast, ovarian, and other cancer susceptibility. Although these germline mutations are inherited in a heterozygous fashion with an intact allele still functioning, subsequent impairment of the function of the intact allele with time results in a higher risk of predisposition to breast and ovarian cancers. In fact, carriers of mutations in *BRCA1/2* have 65–80% lifetime risk of developing breast cancer and up to 20–45% risk of developing ovarian cancer. The incidence of deleterious *BRCA* mutation is 1:300–500 in the general population. In some ethnic populations, such as those of the Ashkenazi background, the prevalence of *BRCA* mutations is as high as 1:40 [31].

BRCA 1 and *2* genes are involved in repairing DNA double-strand breaks. Impaired DNA repair may promote carcinogenesis due to accumulation of DNA double-strand breaks. Women with *BRCA1* and *BRCA2* mutations are more likely to develop breast and ovarian cancers which often occurs before menopause. In addition to increased cancer risks, recently, a growing body of laboratory and clinical evidence indicates the role for *BRCA* dysfunction in accelerated ovarian aging. The initial observation of the impact of *BRCA* mutations on ovarian aging first arose from our study showing that *BRCA*1 mutation-positive women produced lower numbers of oocytes (7.4 [95% CI, 3.1–17.7] v 12.4 [95% CI, 10.8–14.2]; $P = 0.025$) [69]. Later, clinical studies investigating the role of *BRCA* mutations in ovarian aging evaluated serum anti-Müllerian hormone (AMH) levels, menopausal age, primordial follicle density, as well as the number of oocytes retrieved after controlled ovarian hyperstimulation (COH) [70]. Before the evaluation of these studies, it should be kept in mind that women with most severe *BRCA* dysfunction would either develop breast, ovarian, or other cancers or undergo risk reducing salpingo-oophorectomy. This would result in the elimination of most of women with more severely impaired DNA repair mechanisms and may lead selection bias particularly in retrospectively designed studies. When the majority of the studies with larger sample size and/or which are prospective were evaluated, it was demonstrated that ovarian reserve is diminished in women with *BRCA* mutations, especially in those who are affected. Moreover, ovarian damage in those women may be more severe after chemotherapy due to defective DNA double-strand breaks repair [71]. Recently we showed that breast cancer patients with BRCA mutations had lower AMH levels after chemotherapy at the end of 24-month follow-up in comparison with breast cancer patients without BRCA mutation [72]. Therefore, women carrying *BRCA* mutations may need to be specially counselled for fertility preservation even if seeking fertility preservation before any diagnosis of malignancy. Women with *BRCA* mutations who pursue fertility preservation can be recommended to undergo prenatal genetic diagnosis in order to avoid 50% risk of transmitting the genetic abnormality to their children if sufficient oocytes are collected [73]. If ovarian tissue cryopreservation is the only option for fertility preservation, removal of ovarian tissue after obtaining sufficient number of embryos is crucial to avoid future risk of ovarian cancer. Moreover, possible low ovarian reserve in *BRCA*-mutated women might decrease the success of ovarian tissue cryopreservation.

It can be questioned whether COH protocols with letrozole supplementation for fertility preservation is safe in breast cancer women with *BRCA* mutations. We observed that after a mean follow-up of 5 years, *BRCA* gene mutation status in women undergoing COH for fertility preservation did not affect survival outcomes when compared with controls [44]. Further research should focus on the pregnancy outcomes of this special population because of limited data available.

Safety of Pregnancy in Breast Cancer Survivors

The number of breast cancer survivors who attempt a pregnancy is still low due to multiple reasons.

First, most women face premature ovarian insufficiency after gonadotoxic treatments, and

they do not have a chance to become pregnant [17, 33]. On the other hand, if women do not experience menopause, evidence over the last decade has shown that pregnancy in breast cancer survivors is not a bad prognostic factor for recurrence or death, irrespectively of the hormone receptor status of the tumor [74]. Moreover, some studies evidenced a survival advantage among women who conceived after oncological treatments [75]. In 1994, it was reported the "healthy mother effect" [75], in which survivors who did not conceive had 41-fold increased risk of dying earlier. More recent studies showed no significance differences in survival rates between breast cancer survivors who achieved a pregnancy and who did not [76, 77]. The European School of Oncology (ESO) and the European Society for Medical Oncology (ESMO) support pregnancy and breastfeeding after breast cancer and reported that pregnancy after survival is safe and feasible for women at low risk for recurrence [78].

The safe time interval between cancer treatments and pregnancy is not clear. This time interval should consider both the cancer relapse risk and the washout period following cancer therapy (i.e., a minimum of 3–6 months following the last administered dose). According to the expert opinion, this period should be individualized according to the woman's age, her ovarian reserve, her previous treatments, and her individual risk of relapse [79]. If the breast cancer survivor had a hormone-sensitive tumor, adjuvant hormone therapy should also be taken into account for the safety and feasibility of a temporary interruption of endocrine therapy to allow pregnancy [3].

In breast cancer survivors with *BRCA* gene mutations, there are limited data available regarding the safety of a pregnancy. For this reason, it is difficult to counsel *BRCA*-mutated survivors on the safety of conception even if a Hereditary Breast Cancer Clinical Study Group showed no difference in survival rates between *BRCA*-mutated cancer survivors who achieved a pregnancy and who remained nulligravida, and it is biologically unlikely to expect a different prognostic effect of pregnancy between women with and without *BRCA* mutations.

Definitions

Breast cancer: Breast cancer is a disease in which cells in the breast grow out of control. The most common types of breast cancer include invasive ductal carcinoma and invasive lobular carcinoma. Moreover, breast cancers are further classified according to the receptor status. Over 70% of all breast cancers express nuclear estrogen receptor α, and hence their growth can be stimulated by high estrogen levels.

***BRCA* mutation:** *BRCA1/2* (breast cancer susceptibility genes 1 and 2) are double-strand DNA break repair genes, and specific germline mutations in either of these genes result in the susceptibility of breast, ovarian, and other cancer types. Women with mutations in the *BRCA1/2* carry a 65–80% of developing breast cancer and up to 20–45% risk of developing ovarian cancer.

Gonadotoxic treatments: Treatments for malignancy and other medical disorders that can permanently impair reproductive function. These treatments include chemotherapy, radiation, and radical surgery. The impact of these treatments on gonadal function depends on the class of the treatment, the dose administered, and the age of the recipient (or ovarian reserve).

Ovarian reserve: The ovarian reserve represents the number of potentially fertilizable oocytes in the ovary and indirectly represents future fertility potential. Ovarian reserve is made up of about one million primordial follicle oocytes at birth, and this number is reduced to approximately 500,000 at the onset of puberty. These numbers are reduced to about 25,000 at age 37 and nearly exhausted at menopause. Moreover, after 38–40 years of age, there is an apparently accelerated phase of follicle depletion. While there is no noninvasive method for assessing primordial follicle reserve directly, the current most accurate indirect methods of fertility assessment include antral follicle count by ultrasound examinations and serum anti-Müllerian hormone (AMH) measurements.

Fertility preservation: Application of assisted reproduction, cryopreservation, and surgical techniques to minimize the impact of cancer and other gonadotoxic treatments on fertility. Examples of

fertility preservation procedures include sperm banking, egg or embryo freezing with in vitro fertilization, ovarian tissue cryopreservation and transplantation, and fertility sparing cancer surgery.

Controlled ovarian hyperstimulation-standard: Assisted reproduction technique in which gonadotropins (follicle-stimulating hormone [FSH] with or without luteinizing hormone [LH]) are used to induce the growth of multiple ovarian follicles. A GnRH agonist or antagonist is added to prevent spontaneous ovulation, so that the ovulation trigger can be timed for the assisted reproduction procedure.

Controlled ovarian hyperstimulation-random start: First described by our team, in random-start controlled ovarian hyperstimulation, gonadotropin's administration starts randomly regardless of the menstrual cycle. This approach is utilized when there is insufficient time to wait for next menstrual period.

Ovulation trigger: Administration of a drug that acts like the luteinizing hormone and causes ovulation after 34–36 h after administration. Typically, human chorionic gonadotropin (hCG) is used for ovulation trigger when at least two follicles measuring over 17 mm in average diameter are observed on ultrasound. When patients utilize letrozole in controlled ovarian stimulation protocols, hCG injection is usually given when leading follicles reach 19–21 mm in average diameter. Gonadotropin-releasing hormone (GnRH) antagonist cycles permit the use of GnRH agonists to trigger an endogenous LH ovulatory surge, with reduced risk of ovarian hyperstimulation.

Aromatase inhibitors: Class of drugs that reduce the estrogen action by inhibiting the production of estrogen from granulosa cells in ovarian tissue, breast tissue, fat tissue, and sources.

Clinical Case

A 27-year-old nulligravida female presented with the diagnosis of breast cancer which was a moderately differentiated invasive ductal carcinoma, size 1.6 cm, second stage, nuclear grade 2, ER+, PR-, and *HER2*- with positive sentinel lymph node. She had a pathogenic *BRCA2* mutation. After lumpectomy, she was referred to a reproductive specialist for fertility preservation due to possible gonadotoxic effects of adjuvant treatment. The patient and her husband wished to have at least two children. As the oncologist agreed to wait for at least 2 weeks before starting dose-dense adjuvant chemotherapy (doxorubicin hydrochloride and cyclophosphamide, followed by paclitaxel treatment), the patient signed the informed consent form to start COH with gonadotropins and letrozole. Ultrasound examination to evaluate her ovarian reserve was performed. Her antral follicle count was 24, and her serum AMH level was 3.05 ng/mL. The patient underwent a random-start COH because the patient had just ovulated at the time of the first consultation. The COH was performed using recombinant follicle-stimulating hormone (Follistim®, follitropin beta from recombinant DNA, Merck Connecticut) at the dose of 300 IU in conjunction with letrozole 5 mg (Femara®, letrozole, Novartis) daily. On the sixth day of COH, the patient began receiving a GnRH antagonist in accordance with the short protocol. Ovulation was triggered with GnRHa (40 IU, Lupron®, leuprolide acetate, Takeda) on the 11th day of COH with eight follicles ≥17 mm (largest one with a mean diameter of 20.7 mm). Her serum estradiol level was 701 pg/ml on the trigger day. Ultrasound-guided transvaginal follicular aspiration was performed 35 h after GnRH agonist administration, and 26 oocytes were retrieved. Seventeen metaphase II (MII) oocytes were subjected to ICSI, and 13 blastocysts were obtained. Preimplantation genetic testing revealed that seven blastocysts were euploid and two were aneuploid. While the genetic examinations of two embryos were unknown due to noisy signal, two blastocysts were not biopsied. Of the seven euploid embryos, three were found not to carry the pathogenic BRCA2 mutation.

Practical Clinical Tips

- Method of fertility preservation should be individualized based on the patient's specifics such as her age, desired size of family, expected delay in attempting pregnancy after breast cancer treatment, level of gonadotoxicity of the cancer treatment, and the available time.
- Ovarian suppression should not be used as a method of fertility preservation in women with cancer.
- When using aromatase inhibitor protocols, preferred trigger is GnRHa, and the trigger should be performed at least using a lead follicle size criterion that is 2-mm larger than that is used for standard IVF cycles.
- In most women, there is no need to wait for the beginning of menstrual cycle to start ovarian stimulation for oocyte or embryo cryopreservation; random-start protocols appear to be equally effective as early follicular start protocols.
- Women with BRCA mutations may have lower ovarian response and fewer embryos in response to controlled ovarian stimulation. They also have lower ovarian reserve and likely lose larger ovarian reserve after chemotherapy. Consider earlier and more proactive approach to fertility preservation.
- Ovarian tissue cryopreservation and autotransplantation are no longer considered experimental in the United States and several other countries. Given its increasing success rates and ability to restore natural fertility, it should be a solid alternative to other methods of fertility preservation. However, ovarian tissue cryopreservation may not be as effective in women older than 40 years of age. To compensate for age-induced loss of ovarian reserve and declining overall quality, larger amounts of ovarian tissue may need to be frozen with increasing age of the woman.

Take-Home Messages

- Women should be informed of the gonadotoxic effect of the cancer treatments and should be referred to reproductive endocrinologist to preserve fertility.
- Gonadotoxic effects of chemotherapy depend on age, ovarian reserve, chemotherapy type, and dose, and fertility preservation decisions should be made accordingly.
- Controlled ovarian hyperstimulation for oocyte or embryo cryopreservation is feasible and safe in hormone-sensitive patients with the use of aromatase inhibitors or possibly tamoxifen.
- Controlled ovarian stimulation can be initiated regardless of the menstrual cycle day (random start) with similar success rates when compared with conventional ovarian stimulation.
- If there is no time for oocyte retrieval with ovarian hyperstimulation, ovarian tissue cryopreservation should be considered.
- GnRH analogue suppression should not be used for fertility preservation.
- Women carrying *BRCA* mutations may need to be specially counselled for fertility preservation due to increased risk of diminished ovarian reserve with age and chemotherapy.
- Pregnancy after breast cancer survival does not seem to increase recurrence risk.

Key Readings

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Managing the Infertility Patient with Endometriosis

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Introduction

Endometriosis is an estrogen-dependent, chronic inflammatory condition defined by the presence of endometrial-like tissue outside of the uterus. Up to 10% of reproductive-aged women—176 million women worldwide—and 50% of those with infertility are affected by endometriosis [1–7]. There are three discrete categories of endometriosis, with distinct and overlapping pathophysiology, that may be seen alone or in combination: superficial peritoneal lesions, ovarian endometriomas, and deeply infiltrating disease. All three forms can contribute to infertility and pelvic pain. Treatment decisions are contingent on whether the patient's primary goal is the resolution of pain or the optimization of fertility.

Infertility contributes to impaired quality of life among endometriosis patients. Women with infertility report a negative impact on their relationships, as well as feelings of inadequacy and symptoms of depression [8]. Thirty to fifty percent of women with endometriosis are subfertile, and fecundity is reduced from 15 to 20% per month in healthy women to 2–5% in women with endometriosis [9–11].

The mechanisms of infertility in endometriosis are both pathologic and iatrogenic, as surgical treatment has the potential to compromise the ovarian reserve. Decisions to pursue surgery must be made judiciously, with a patient's fertility goals in mind. Additionally, the consent process for surgery should include a discussion of effects on the ovarian reserve. When surgery is undertaken, it should be performed meticulously, by expert surgeons, to avoid ovarian injury. Repeat surgery with the goal of improving fertility outcomes should be avoided. Limited data are available about the role of fertility preservation in endometriosis patients. We recommend that fertility preservation with oocyte or ovarian tissue cryopreservation be considered on an individual basis prior to surgery in patients at high risk for ovarian damage, including women with bilateral endometriomas and those with recurrent endometriomas. The chapter that follows will review the mechanisms of infertility in women with endometriosis and strategies for managing the fertility patient with endometriosis and discuss the role of fertility preservation.

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Mechanisms of Subfertility in Endometriosis

Endometriosis impacts fertility through multiple mechanisms. In moderate to severe disease, distorted pelvic anatomy and dense adhesions may impair tubal function and fertilization [11–13]. Additionally, the inflammatory milieu of the peritoneal fluid, characterized by increased numbers of macrophages, proteases, prostaglandins, and cytokines, creates an unfavorable environment for conception [11]. This inflammatory environment impairs both sperm motility and tubal function [14–17]. Fallopian tubes exposed to the peritoneal fluid of women with endometriosis exhibit decreased beat frequency compared to healthy controls [11, 17].

Baseline ovarian reserve may also be impaired in endometriosis. Endometriomas expose the ovary to free radicals and mechanical stretch, reducing the primordial follicle pool [18–20]. Anti-Müllerian hormone (AMH) levels are lower in women with endometriomas than in healthy women; furthermore, AMH is lower with bilateral compared to unilateral ovarian lesions [21]. The clinical significance of these decreased AMH levels is unclear, as AMH levels do not predict spontaneous fertility [22]. Additionally, although women with endometriomas undergoing ovarian stimulation have a lower oocyte yield and a higher rate of IVF cycle cancellation, clinical pregnancy and live birth rates are unchanged [23].

In addition to impaired sperm and tubal motility and decreased ovarian reserve, oocyte quality and embryonic development are also thought to be affected. Inflammatory factors in the peritoneum, as well as free radicals in the endometrium, exert negative effects on embryo development and survival [24–26]. These observations are supported by studies of embryo development in endometriosis patients. Embryos obtained from women with endometriosis demonstrate slower development and higher rates of arrested and aberrant growth [12, 24, 27]. Additionally, studies of donor oocytes suggest that oocyte quality is poor. Women with moderate to severe endometriosis who undergo transfer with embryos derived from healthy controls have improved implantation and pregnancy rates compared to healthy women who receive embryos from endometriosis patients [27–32]. Although these data suggest that reduced oocyte and embryo quality result in poor implantation and pregnancy rates, outcomes for in vitro fertilization (IVF) in women with endometriosis are comparable to those for other infertility diagnoses [16, 33–35].

Endometrial dysfunction further contributes to infertility in endometriosis. Aberrant function of the eutopic endometrium may result in implantation failure. Progesterone receptor dysfunction leads to progesterone resistance and subsequent impairment of endometrial receptivity and luteal function [14, 16, 36–38]. Additionally, autoantibodies to antigens in the endometrium may further affect endometrial receptivity and implantation [11].

Despite the hostile conditions for conception created by the inflammatory milieu in endometriosis, IVF appears to overcome these challenges and yields pregnancy rates that are similar to IVF outcomes for other causes of infertility [16, 33–35].

Fertility Outcomes After Endometriosis Surgery

Data from randomized trials indicate that surgery improves fertility outcomes in women with minimal to mild endometriosis [39, 40]. The largest of these RCTs, published in 1997 by the Canadian Collaborative Group on Endometriosis, randomized 341 women aged 20–39 with stage 1–2 endometriosis to diagnostic laparoscopy or laparoscopy with excision or ablation [39]. Participants were monitored for 36 weeks after surgery. In the excision/ablation cohort, 50/172 (30.7%) became pregnant, whereas only 29/169 (17.7%) conceived in the diagnostic laparoscopy group ($p = 0.006$). Spontaneous miscarriage rates were similar in both groups (20.6% and 21.6%, respectively). Similarly, a small RCT of 41 patients also demonstrated a benefit of excision/

ablation; 57% of patients in the operative laparoscopy group became pregnant compared to 45% in the diagnostic laparoscopy group [41]. Two additional randomized trials showed no difference in pregnancy rates between diagnostic and operative laparoscopy; however, both studies were limited by small sample sizes (96 and 38 patients, respectively) and insufficient power [42, 43]. A Cochrane review including three of these trials concluded that operative laparoscopy results in improved clinical pregnancy (CPR) and live birth rates (LBR) (CPR OR 1.89, 95% CI 1.25–2.86; LBR OR 1.94, 95% CI 1.20–3.16) [44], with a number needed to treat (NNT) of eight. Given that not all women will have endometriosis at the time of laparoscopy, the NNT increases to 40 assuming a prevalence of 30% [42].

There are no randomized trials to inform the treatment of women with advanced endometriosis who desire fertility. Although IVF is the mainstay of fertility treatment in these women, cohort studies suggest that surgery may have a role in optimizing spontaneous conception rates. Available data suggest that spontaneous conception rates in the 3 years following laparoscopy for stage 3–4 endometriosis range from 30 to 60% [43]. Therefore, women who are younger, highly symptomatic, or whose personal beliefs preclude IVF may consider surgery followed by a trial of spontaneous conception, whereas those with decreased ovarian reserve, advanced reproductive age, or male factor infertility are likely to derive greater benefit from proceeding straight to IVF [42].

The impact of surgery on IVF outcomes for women with deep infiltrating disease is controversial. Although some studies suggest that surgery compromises ovarian reserve and IVF outcomes [45], others suggest that a combined approach achieves greater pregnancy rates than either treatment independently [46, 47]. A prospective cohort study compared surgery followed by IVF to IVF alone and found improved implantation (32% vs. 19%) and pregnancy (41% vs. 24%) rates in the group that underwent surgery prior to IVF [47]. These improvements occurred in spite of the need for higher gonadotropin doses and a smaller oocyte yield in the surgery group [47]. Similarly, studies evaluating pregnancy rates after colorectal surgery for DIE affecting the bowel demonstrate favorable pregnancy rates postoperatively [48–51]. Pregnancy rates were evaluated as part of an RCT comparing outcomes after rectal shaving or disc excision to segmental bowel resection in a subgroup of 36 women who attempted conception postoperatively. The pregnancy rate in this cohort was 81% (29/36), and 59% of patients who became pregnant did so without assisted reproduction (17/29) [51].

There may be a particular role for laparoscopic surgery in patients with DIE who have previously failed IVF. A recent 2019 retrospective cohort study included 104 women with DIE who had previously failed at least two IVF cycles. The authors reported a postoperative pregnancy rate of 43.8%, with a mean time of 11 months from surgery to conception [52]. Another retrospective study of 78 women with advanced endometriosis, a mean of 53 months of infertility, and 6 failed IVF cycles reported a pregnancy rate of 42% after operative management of endometriosis [53]; only 9% of pregnancies in this cohort were achieved spontaneously and most required IVF [53].

Fertility outcomes after surgery are likely related to the success of the surgery at restoring normal anatomy. Adamson et al. established the Endometriosis Fertility Index (EFI) to predict pregnancy rates following surgery [54, 55]. The predictive tool, which has now been validated by several investigators, takes into account patient age, years of infertility, history of prior pregnancy, ASRM endometriosis score, and the condition of the fallopian tubes, fimbriae, and ovaries at the conclusion of laparoscopy [54, 55]. Mild tubal dysfunction is characterized by "slight injury to the serosa" or "slight injury to the fimbriae with minimal scarring," whereas a nonfunctional tube is characterized by "severe injury to the fimbria, with extensive scarring, complete loss of fimbrial architecture, complete occlusion or hydrosalpinx, or extensive salpingitis isthmica nodosa." Similarly, a nonfunctional ovary in this classification system is described as absent or "completely encased in adhesions" [54]. For women with good EFI scores of 9–10,

spontaneous conception rates were 46% at 1 year, 58% at 2 years, and 91% at 5 years; however, for those with low EFI scores of 0–1, the probability of pregnancy was 0% [56]. This score may be used to aid counseling about the pursuit of immediate IVF versus a trial of spontaneous conception postoperatively.

The risks and benefits of surgery in women with deep infiltrating endometriosis must be carefully evaluated. Overall, expert consensus and society guidelines suggest that IVF, rather than surgery, should be the first-line approach for women with advanced endometriosis who desire fertility as a primary treatment goal [12, 42, 57].

Management of Endometriomas in Women with Infertility

Randomized trial evidence demonstrates that excising large ovarian endometriomas enhances spontaneous conception rates; however, the management of endometriomas prior to in vitro fertilization is controversial. A Cochrane review including two randomized trials totaling 164 patients evaluated spontaneous pregnancy rates after cystectomy compared to ablation of endometriomas larger than 3 cm [58, 59]. Results demonstrated increased conception rates in the cystectomy group (OR 5.24, 95% CI 1.92, 14.97) [60]. Therefore, in women planning to attempt spontaneous conception, endometrioma excision should be considered.

Although endometriomas are commonly excised prior to IVF, evidence to support this practice is lacking. Several randomized trials address the management of endometriomas in the setting of in vitro fertilization. Despite the fact that women with endometriomas undergoing IVF require more gonadotropin, have increased cycle cancellation rates, and produce fewer oocytes, neither ovarian cystectomy nor aspiration improves pregnancy and live birth rates [23, 61, 62]. A 2015 meta-analysis of 33 studies, including three randomized trials of 246, 99, and 171 women, respectively, supports these results [23]. Two other meta-analyses, including a Cochrane review, also failed to demonstrate improved pregnancy rates after the surgical treatment of endometriomas [62, 63]. Notably, none of these studies considers the impact of endometrioma size, and cystectomy may allow for better ovarian access during oocyte retrieval and reduced risk of endometrioma rupture [12, 13]. Although excision of an endometrioma prior to IVF confers limited benefits in terms of IVF outcomes, poses risks to the ovarian reserve, and may result in decreased response to ovarian hyperstimulation [13], cystectomy may be considered on an individual basis in cases of significant pain or to optimize ovarian access for retrieval.

Impact of Surgery on the Ovarian Reserve

Decisions about pursuing surgery to optimize fertility must be made judiciously. The benefits of surgery must always be weighed against the possibility of injury to the ovarian reserve [12]. The risk of injury is greatest when endometrioma excision is planned. During ovarian cystectomy, ovarian damage may occur when excess ovarian cortex is inadvertently removed or due to thermal injury from the use of electrosurgery [12, 13]. Studies of ovarian reserve testing in women undergoing cystectomy for endometriomas consistently demonstrate decreases in AMH levels postoperatively [54–56]. AMH levels decrease by up to 30% after unilateral endometrioma excision and 53% after bilateral cystectomy [18, 42]. The clinical significance of this decline in AMH is unclear, as AMH is a poor predictor of spontaneous fertility [22], and some data suggest partial recovery of AMH levels 6 months after surgery [64]. Still, the surgical consent process should include a discussion of potential effects on the ovarian reserve, and surgery should be performed with care to avoid ovarian injury.

In addition, repeat endometrioma surgery poses a substantial risk of ovarian injury with little fertility benefit. Women who undergo repeat surgery experience a greater decline in AMH and antral follicle count (AFC) than after an initial procedure [65, 66]; therefore, women who do not become pregnant after the first surgery should be

advised to undergo IVF rather than a second procedure. A retrospective study evaluating IVF versus repeat surgery for women with advanced endometriosis revealed a pregnancy rate of 70% after two cycles of IVF compared to 24% 9 months postoperatively [67].

Minimizing the Iatrogenic Effects of Surgery on Ovarian Reserve

A number of strategies can be employed to mitigate ovarian injury during surgery, particularly when performing ovarian cystectomy. First, the plane between the ovarian cortex and the endometrioma must be developed carefully. Endometriomas are typically surrounded by a fibrotic capsule that makes the development of this plane challenging. Bleeding often occurs at the hilum, and special care should be taken during dissection in this region to avoid injuring viable ovarian tissue. Hydrodissection with vasopressin can help establish the correct plane and minimize bleeding; however, it is not approved by the FDA for this indication. The use of electrosurgery should be limited, and, where possible, alternative hemostatic agents should be used. Randomized controlled trial data support the use of suture or hemostatic sealants rather than electrosurgery for the preservation of ovarian reserve [68–71].

Additionally, ablation rather than excision of endometriomas may be considered when maintaining the ovarian reserve is of primary concern. Randomized trials, including a Cochrane review, demonstrate that ovarian cystectomy for endometriomas larger than 3 cm results in improved spontaneous conception rates and lower recurrence rates compared to ablation [60]; however, ablation may result in less damage to the ovarian reserve [18, 42]. Therefore, women who are unlikely to conceive without assisted reproduction may benefit from ablation over excision. Data suggest that ablation performed with CO_2 laser or plasma energy results in less thermal injury than ablation achieved via monopolar or bipolar electrosurgery [72, 73]. Given the benefits of ablation over excision for the preservation of the ovarian reserve, Donnez et al. propose combining ablation with medical therapy [74]. They describe a three-step approach to the surgical management of endometriomas greater than 3 cm, including [75] laparoscopic drainage of the cyst with biopsy to obtain pathologic confirmation of endometriosis, [1] administration of 12 weeks of a gonadotropin receptor hormone (GnRH) agonist to reduce endometrioma size and mitotic activity, and [2] laparoscopy with ablation of the cyst wall using CO2 laser [74]. Although randomized controlled trial data demonstrate that this three-stage approach results in improved postoperative AMH levels compared to ovarian cystectomy with acceptable recurrence rates (8%) at 2 years [76], the need for serial laparoscopies limits the practicality of this method [12]. In order to minimize the negative impact of excision and eliminate the need for multiple surgeries, combined excision/ablation approaches have also been described [77]. In the combined approach, 80–90% of the cyst is excised, and the remainder is ablated at the hilum, where bleeding and follicular damage most commonly occur. A small prospective cohort study of 52 women evaluating this combined excision/ablation method with CO_2 laser demonstrated a 41% spontaneous pregnancy rate 8 months postoperatively with a low recurrence rate of 2% [74]. Conversely, the results of a randomized controlled trial comparing cystectomy to combined excision/ablation with a bipolar device showed no difference in AFC or recurrence rate; however, the studies were underpowered (n = 51) [78]. Given the improved spontaneous conception and recurrence rates after excision compared to ablation of endometriomas [60], cystectomy remains the standard of surgical care; however, ablation has reasonable recurrence rates and may be considered either alone or in combination with excision to reduce damage to the ovarian reserve.

Surgeon experience also plays a role in fertility outcomes after endometrioma excision. Experienced surgeons tend to remove less ovarian cortex during cystectomy, and retrospective data suggest that surgeon experience may be correlated with live birth rate after cystectomy [79]. A retrospective study at an academic Taiwanese

hospital showed increased live birth rates after IVF in patients who underwent endometrioma excision by an attending physician compared to a surgical trainee [80].

In addition to ovarian injury, fertility may be impaired by the formation of postoperative adhesions that distort anatomy and inhibit tubal function. The pathogenesis of adhesion formation is incompletely understood; however, it appears to hinge on an inflammatory reaction that occurs in the setting of tissue injury, resulting in the release of histamine, cytokines, and growth factors. Vascular disruption due to tissue injury impairs the delivery of oxygen and nutrients, as well as the removal of metabolic waste [81]. Adhesion barriers do not directly address the pathogenesis of adhesions, but randomized controlled trial evidence support a role in their reduction via mechanical separation of healing surfaces. In a Cochrane review that included 18 randomized trials, oxidized regenerated cellulose (Interceed), expanded polytetrafluoroethylene (Gore-Tex), sodium hyaluronate and carboxymethylcellulose (Seprafilm), and fibrin sheets were all shown to successfully mitigate postoperative adhesion formation [82]. Hemostasis was must be excellent in order to for these barriers to function effectively [12]. Other anti-adhesion agents that more directly target the pathogenesis of adhesion formation are under study, including statins, non-steroidal anti-inflammatory agents, and the antimicrobial, anti-inflammatory agent xanthorrhizol [83–85].

Role of Medical Therapy in Fertility Preservation

The utility of medical treatments in fertility patients is limited as hormonal therapies inhibit ovulation, and none have been shown to improve fertility outcomes [86]. However, there is a role for medical therapy in preventing endometriosis recurrence in women who are not actively trying to conceive.

Although early data regarding postoperative hormonal suppression demonstrated no benefit in terms of disease recurrence, the duration of suppression in these trials was short at 3–6 months [87]. Longer durations of therapy (18–24 months) have shown significant improvements in endometriosis recurrence rates [88]. Because endometriomas reduce baseline AMH levels even in women who have never undergone surgery, preventing endometrioma recurrence is of paramount importance for fertility preservation. A randomized trial including 239 women demonstrated that postoperative hormonal suppression with oral contraceptive pills (OCPs) reduced endometrioma recurrence from 29% in untreated women to 14.7% in women on cyclic OCPs and 8.2% in continuous OCP users [89]. Furthermore, among those who did have an endometrioma recurrence on OCPs, endometriomas were smaller at diagnosis and demonstrated slower growth [90]. Notably, although levonorgestrel intrauterine devices are effective at reducing postoperative pain recurrence, they do not reduce endometrioma formation. The European Society of Human Reproduction and Embryology (ESHRE) endometriosis guidelines have been updated to recommend postoperative hormonal suppression to prevent endometriosis recurrence [91].

Another potential role for medical therapy in fertility patients is pretreatment prior to IVF cycles. Some studies suggest that treatment with GnRH agonists prior to ovarian stimulation yields improved clinical pregnancy rates; however, data are mixed. A 2006 Cochrane review included three randomized trials of women with endometriosis undergoing IVF and concluded that 3–6 months of treatment with a GnRH agonist prior to initiating IVF resulted in a fourfold increase in clinical pregnancy rate [92]. Live birth rate was reported in only one of the three RCTs but was also increased with GnRH treatment. Despite these initially favorable data, more recent RCT results demonstrated no increase in oocyte yield after GnRH pretreatment; however, this study was not powered to evaluate pregnancy rates [93]. Ovarian suppression prior to IVF remains controversial, and the potential benefits must be weighed against the increased cost of medication to stimulate a suppressed ovary and delays in the start of IVF. More data are required to determine the optimal duration of suppression and the subgroup of patients likely to derive the greatest benefit [12].

Fertility Preservation

Women with endometriosis are at risk for decreased ovarian reserve, both due to due the pathophysiology of the disease and iatrogenic causes. Although oocyte and ovarian tissue cryopreservation are frequently used for fertility preservation in women undergoing gonadotoxic therapies or those wishing to delay childbearing, endometriosis is increasingly being considered an indication for fertility preservation. Literature evaluating the clinical outcomes and cost-effectiveness of fertility preservation in endometriosis patients is limited.

In 2009, Elizur et al. of Montreal published the first report of oocyte cryopreservation in a young woman with advanced endometriosis and decreased ovarian reserve [94]. The patient was a 25-year-old nulliparous woman with a history of multiple prior laparoscopies, including a unilateral salpingo-oophorectomy due to an endometrioma. She presented with recurrent pain, and her antral follicle count was found to be decreased at three. Ultrasound demonstrated a normal appearing left ovary with no endometrioma present. She was counseled about the possibility of fertility preservation prior to repeat laparoscopy. She underwent three ovarian stimulation cycles, with suppression using a GnRH agonist prior to each cycle. She required high doses of follicle-stimulating hormone (FSH) for her stimulations, with maximum doses of 600 IU/day. A total of 21 oocytes were cryopreserved [94]. Since 2009, there have been few case reports of fertility preservation in women with endometriosis. Several studies have included women with endometriosis in their cohorts but did not evaluate outcomes for these patients separately [95]. In 2018, Raad et al. published a series of 49 patients with endometriosis who underwent ovarian stimulation for fertility preservation [96]. The authors divided patients into three groups depending on their endometriosis phenotype: superficial disease, deep infiltrating disease, or endometrioma. The mean number of oocytes retrieved across all subgroups was 9.5 ± 6.1, and no differences were observed between groups. There was a significant reduction in oocytes retrieved among women who reported prior endometrioma excision compared to those without prior ovarian surgery (11.2 ± 6.5 vs. 8.3 ± 5.2, $p < 0.01$) [96], further emphasizing the impact of adnexal surgery on ovarian reserve. Pregnancy outcomes after oocyte thaw and transfer were not evaluated in either of these studies.

Any woman freezing oocytes should be counseled that oocyte cryopreservation does not guarantee a live birth and that the efficiency of oocyte cryopreservation varies by age. The live birth rate per warmed vitrified oocyte ranges from 5% in women 38 and older to 7.4% in women under the age of 30 at the time of oocyte retrieval [97]. Therefore, in order to optimize the chance of a live birth, women under 38 should be advised to freeze 15–20 oocytes, whereas women 38 and older are recommended to cryopreserved 25–30 oocytes [98]. IVF outcomes are similar for women with and without endometriosis; however, oocyte yields are lower in women with endometriomas [23]. Although some data can be extrapolated from studies of elective fertility preservation, there is a need for more data about pregnancy rates after oocyte cryopreservation specific to women with endometriosis, particularly those with ovarian disease. While there is a theoretical risk of endometriosis progression or worsening of pain with ovarian hyperstimulation, several retrospective studies have demonstrated that undergoing IVF does not worsen pain or increase the risk of disease recurrence, even for women with deep infiltrating disease [99–102]. Prospective studies of larger cohorts are needed to confirm these results.

For women unable to undergo oocyte or embryo cryopreservation, ovarian tissue transplantation is an option. Donnez et al. have reported two cases of fresh ovarian cortex transplantation in women with advanced endometriosis who required oophorectomy for large endometriomas (8–9 cm in size) [103]. Two cortical strips were transplanted in a peritoneal window adjacent to the ovarian hilum at the time of oophorectomy. One of the two patients became pregnant after three cycles of IVF; however, the outcome of the pregnancy was not reported [103]. Data from high-volume fertility preservation centers indicate that pregnancy and

live birth rates after ovarian tissue cryopreservation compare favorably to those for oocyte cryopreservation in women undergoing gonadotoxic therapy, and as of December 2019, the Practice Committee of the American Society for Reproductive Medicine lifted the experimental label on this technology [104, 105]. Some data, however, suggest that the efficacy of ovarian tissue cryopreservation may be reduced among patients with decreased ovarian reserve, which is highly prevalent among endometriosis patients [106]. Additionally, the removal of healthy ovarian tissue may further compromise the ovarian reserve. If ovarian tissue cryopreservation is to be more widely implemented among women with endometriosis, this technology requires additional study in this patient cohort [12]. Table 1 describes the benefits and disadvantages to fertility preservation with ovarian tissue and oocyte cryopreservation.

Table 1 Options for fertility preservation in women with endometriosis

	Oocyte and embryo cryopreservation	Ovarian tissue cryopreservation
Benefits	High success rates, particularly with embryos	Option for women who are unable or unwilling to undergo ovarian stimulation
	Avoids a laparoscopic procedure	Option for women who require oophorectomy
	Avoids risk of damage to ovarian tissue	Could be performed at the time of excision surgery for at-risk patients
Risks	Reproductive potential of follicles from endometriosis patients requires further study	Potential for damage to viable ovarian tissue
	Need to cryopreserve large numbers of oocytes (15–20 in women <38 and 25–30 in women ≥38)	Risks of laparoscopy
	Possibility of impaired oocyte and embryo quality	

Adapted from Llarena NC, Falcone T, Flyckt RL. Fertility Preservation in Women With Endometriosis. *Clin Med Insights Reprod Heal*. 2019. https://doi.org/10.1177/1179558119873386

Choosing Surgery Versus IVF for Fertility Patients

Decision-making about whether to pursue surgery, fertility preservation, or IVF with embryo transfer in women with endometriosis who desire fertility can be complex (see Fig. 1). Centers where minimally invasive gynecologists are performing the majority of laparoscopies for endometriosis should provide consultation with a reproductive endocrinologist for women with advanced disease who desire fertility.

Preoperative discussion should include the impact of adnexal surgery on the ovarian reserve, as well as a discussion of tubal patency. Women who have not had an evaluation of tubal patency may benefit from undergoing a preoperative hysterosalpingogram to evaluate for hydrosalpinx or other tubal pathology. This allows for preoperative counseling regarding the condition of the fallopian tubes and its potential impact on fertility. For example, in a woman with hydrosalpinx who is likely to require IVF, consent should be obtained for salpingostomy and/or salpingectomy. The reproductive endocrinologist may also offer ovarian reserve testing prior to endometriosis surgery. Because AMH does not correlate with spontaneous conception rates, this testing is most useful in women who are likely to require IVF. When choosing to pursue fertility preservation, a low AMH result should be considered along with other patient factors such as age, surgical history, and the presence of endometriomas; decreased AMH alone is not a compelling indication for fertility preservation.

In young women with debilitating pain who also happen to desire fertility, we typically recommend surgery as the primary intervention. Conversely, women with advanced disease and minimal symptoms who primarily desire fertility should proceed with IVF. Women of older reproductive age and decreased ovarian reserve or those with prior laparoscopies who desire both pain management and fertility optimization require more careful consideration. These are the patients likely to benefit from undergoing ovarian stimulation for fertility preservation prior to surgery. Others who may benefit from fertility preservation are those at risk for injury to the bilateral

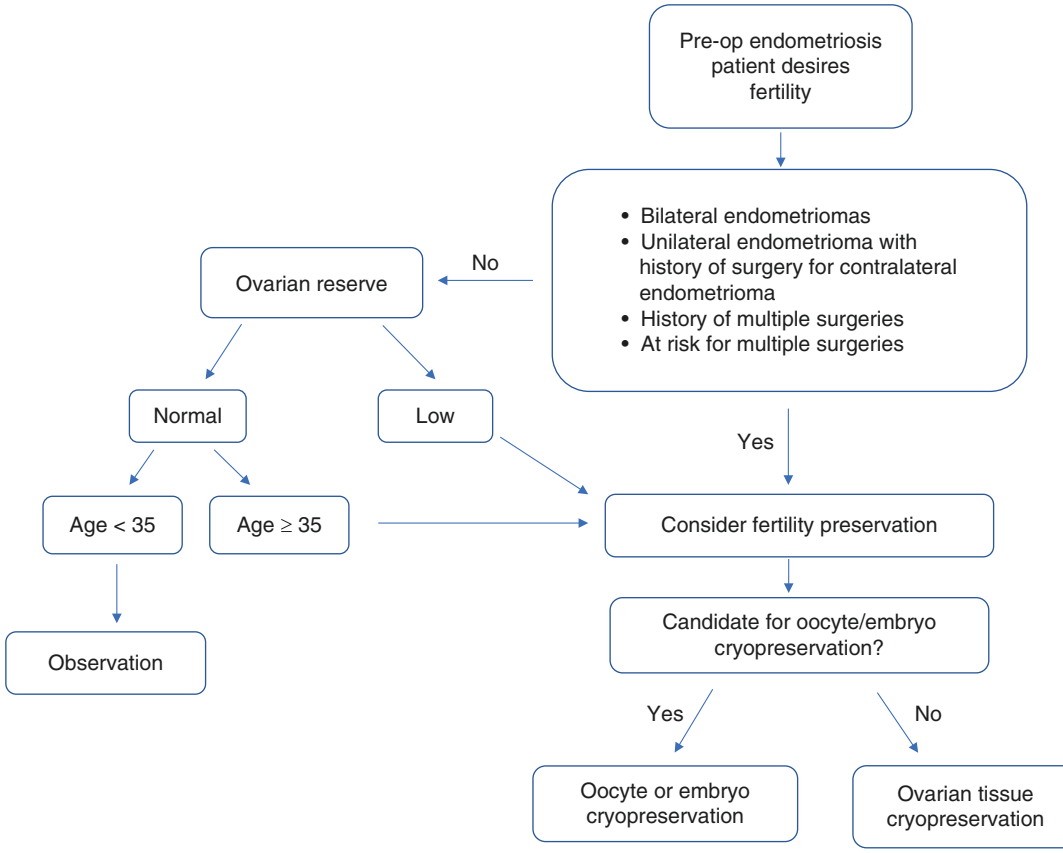

Fig. 1 Practical approach to fertility preservation in pre-surgical endometriosis patients. (Adapted from Llarena NC, Falcone T, Flyckt RL. Fertility Preservation in Women With Endometriosis. *Clin Med Insights Reprod Heal*. 2019. https://doi.org/10.1177/1179558119873386)

ovaries, including women with bilateral endometriomas, or a history of unilateral endometrioma with a contralateral recurrence. Decision tree models of elective oocyte cryopreservation have found that the intervention is most cost-effective at the age of 37; however, given the threats to the ovarian reserve in women with endometriosis, we recommend consideration of fertility preservation in women undergoing adnexal surgery at the age of 35. Figure 1 describes a practical approach to fertility preservation in the patient planning surgical treatment for endometriosis.

The following patient cases provide examples of decision-making about sequencing surgery, IVF, and fertility preservation, taking into account individualized patient goals and medical histories.

Case #1: Oocyte Cryopreservation Prior to Ovarian Cystectomy

A 32-year-old G0 female presents to the office with pelvic pain and endometriosis confirmed by prior laparoscopy. She previously had a right ovarian cystectomy for a 6-cm endometrioma. The patient underwent pelvic MRI demonstrating evidence of deep infiltrating endometriosis affecting the posterior cul-de-sac and left ovary. A 5-cm left ovarian endometrioma was identified. Her current treatment goal is pain management. She has tried oral contraceptive pills in the past without relief. The patient does not currently have a partner

but has a strong desire to become pregnant in the future.

Although pain control is the primary goal for this patient at the present time, she desires future fertility. She is at risk for ovarian injury due to her history of ovarian cystectomy and new contralateral endometrioma recurrence. The patient was counseled about the risks and benefits of laparoscopy with excision of endometriosis, including the potential for damage to the ovarian reserve. She was offered oocyte cryopreservation prior to surgery and accepted.

Her AMH level was found to be 1.2. She underwent ovarian stimulation with an antagonist protocol, and nine oocytes were retrieved, eight of which were mature and frozen. Because freezing 15–20 oocytes is recommend for an increased chance of achieving a live birth, the patient was offered a second cycle; however, she declined as she felt her pain was too great and did not wish to further delay surgery.

She then underwent laparoscopic excision of endometriosis. The cystectomy was approached with care to minimize damage to the cortex. A combination excision/ablation approach was used. The endometrioma was almost completely excised and gently peeled away from the cortex. Near the hilum, a small remnant of the cyst wall was ablated using a helium plasma device, which results in less depth of tissue injury than monopolar electrosurgery [107]. The posterior cul-de-sac disease was excised in its entirety. Postoperatively, she was put on continuous oral contraceptive pills with the goal of preventing pain and endometrioma recurrence.

Practical Clinical Tips

- Patients at risk for bilateral ovarian injury, including those with bilateral endometriomas, or a history of ovarian cystectomy and a contralateral recurrence, may benefit from fertility preservation prior to surgery.
- Women under the age of 38 should freeze 15–20 oocytes to optimize the chance of a live birth. Those over 38 should freeze 25–30.
- When the preservation of the ovarian reserve is a high priority, consider a combined excision/ablation approach to ovarian cystectomy.

Case #2: Fertility in a Woman with Endometrioma and Diminished Ovarian Reserve

A 35-year-old G3 P1021 woman presents to the office with infertility. She was diagnosed with unexplained infertility 6 years ago and underwent three IVF cycles at an outside facility. The first cycle done 6 years ago yielded 15 oocytes, and the patient became pregnant and delivered a term infant. Two years ago, she underwent another cycle that yielded nine oocytes; however, none fertilized. A third cycle was done several months prior to her presentation, and the cycle was cancelled due to poor ovarian response to stimulation. Ultrasound was suggestive of an endometrioma, and MRI demonstrated deep infiltrating endometriosis involving the left ovary and rectum. A 4-cm endometrioma was seen. AMH resulted at 0.76, consistent with diminished ovarian reserve. Although the patient reported moderate dysmenorrhea, her symptoms were improved with ibuprofen, and her primary treatment goal was fertility. She underwent hysterosalpingogram, which demonstrated patent fallopian tubes bilaterally without hydrosalpinx.

The patient was counseled about the options of laparoscopy versus immediate IVF. Given her diminished ovarian reserve and history of poor response to ovarian stimulation, immediate IVF was recom-

mended, and the patient accepted this recommendation. She underwent ovarian stimulation using a microdose flare protocol, which yielded nine oocytes, six of which were mature. Three oocytes fertilized with ICSI and one grew to a blastocyst. She became pregnant after single embryo transfer.

Practical Clinical Tips

- In patients with endometriosis who have minimal symptoms and desire conception as their primary treatment goal, we recommend pursuing IVF rather than surgery.
- Consider hysterosalpingogram prior to surgery to aid in preoperative counseling about the need for salpingostomy or salpingectomy prior to fertility treatment.

Case #3: Hydrosalpinx and Implantation Failure in a Patient with Endometriosis

A 36-year-old G1 P1001 F with a history of chronic pelvic pain and endometriosis presented to the office for infertility follow-up. She had stage 4 endometriosis diagnosed 10 years prior to her presentation and had undergone laparoscopy with excision of endometriosis and extensive lysis of tubo-ovarian adhesions. Approximately 1 year after her surgery, at the age of 28, she underwent IVF. At the time of her IVF cycle, AMH was 2.4. She became pregnant after single embryo transfer and had a live birth. Six embryos were frozen.

At the time of her presentation to the office, she denied pelvic pain and desired a frozen embryo transfer. She had an embryo thawed and transferred but did not become pregnant. She then began preparing for a second frozen embryo transfer; however, ultrasound was suspicious for bilateral hydrosalpinx. Hysterosalpingogram was performed and confirmed bilateral hydrosalpinges. The decision was made to proceed with bilateral salpingectomy. At the time of laparoscopy, extensive adhesive disease was found encasing the fallopian tubes and ovaries. She underwent laparoscopy with lysis of adhesions and bilateral salpingectomy. Following surgery, she underwent single embryo transfer and became pregnant. This case highlights the importance of evaluating for tubal pathology in women with endometriosis and implantation failures.

Practical Clinical Tips

- In women with a history of deep infiltrating endometriosis and recurrent implantation failure, consider hydrosalpinx as a potential cause of implantation failure.

Conclusions and Take-Home Messages

Women with endometriosis are at risk for decreased ovarian reserve due to both pathologic and iatrogenic causes. Decisions to pursue surgery should be made with patient's fertility goals and the risks of ovarian injury in mind. Repeat surgeries in patients with the primary goal of fertility optimization should be avoided. Long-term postoperative hormonal suppression reduces the risk of endometrioma recurrence and should be offered to women who are not actively trying to conceive. Although data about oocyte and ovarian tissue cryopreservation in women with endometriosis are limited, these interventions may be considered for women with endometriosis who are planning to undergo surgery, particularly those at risk for bilateral ovarian injury.

Definitions

Endometriosis: An estrogen-dependent, chronic inflammatory condition defined by the presence of endometrial-like tissue outside of the uterus.

Ovarian reserve: The number of oocytes remaining in the ovary, or oocyte quantity that is best measured by anti-Müllerian hormone or antral follicle count.

Diminished ovarian reserve: In order to meet the criteria set forth by ESHRE for diminished ovarian reserve, also called poor ovarian response to stimulation (POR), at least two of the following criteria must be met [75]:

- Advanced maternal age (≥ 40 years) or any other risk factor for diminished ovarian reserve.
- A previous poor response to ovarian stimulation (≤3 oocytes with a conventional stimulation protocol).
- An abnormal ovarian reserve test (AFC < 5–7 follicles or AMH < 0.5–1.1 ngmL).

Fertility preservation: An intervention designed to extend fertility in women who are at risk for ovarian injury, oophorectomy, or diminished ovarian reserve due to pathologic or iatrogenic causes. These interventions include oocyte, embryo, and ovarian tissue cryopreservation.

Oocyte cryopreservation: Freezing of mature oocytes after controlled ovarian hyperstimulation in postmenopausal women. Cryopreservation is typically done via vitrification.

Ovarian tissue cryopreservation: Cryopreservation of ovarian cortical tissue. This is the only method of fertility preservation that is effective in pre-pubertal girls. In December 2019, ASRM removed the experimental label from this technology.

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Severe Autoimmune Diseases

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Indications and prognosis

Autoimmune diseases often affect young women of reproductive age. Approximately 7% of all patients presenting at *Ferti*PROTEKT network clinics suffer from benign diseases, which include autoimmune diseases. Of these 7% of women, about 25% suffer from systemic lupus erythematosus (SLE) and 8% from vasculitis [1].

Above all, rheumatological systemic diseases such as connective tissue diseases and vasculitis and also haematological or neurological diseases such as multiple sclerosis - despite great therapeutic progress in recent years - continue to be an indication for the use of relatively undirected but highly immunosuppressive cytotoxic drugs. Cyclophosphamide (CYC) is used almost exclusively for this purpose, orally or as intravenous pulse therapy. CYC also forms the cytotoxic central pillar for autologous stem cell transplantation, as the maximum therapy for immunosuppression in autoimmune diseases.

Diseases in which CYC therapy may be necessary:

- Severe organ manifestations (glomerulonephritis, alveolitis or manifestations in the central nervous system) in *connective tissue diseases* (SLE, systemic sclerosis, Sjogren's syndrome, Sharp's syndrome, polymyositis or dermatomyositis).
- Severe organ manifestations (mostly pulmonary or renal) in anti-neutrophil cytoplasmic antibodies (*ANCA*) *associated vasculitis* (granulomatosis with polyangiitis [formerly: Wegener's granulomatosis], eosinophilic granulomatosis with polyangiitis [formerly: Churg–Strauss syndrome] or microscopic polyangiitis).
- Treatment-refractory forms of *large vascular vasculitis*, whereby only Takayasu arteritis occurs during reproductive age.
- Autoimmune *neurological diseases*: e.g. multiple sclerosis.
- *Non-malignant haematological diseases*: e.g. immune thrombocytopenia, acquired haemophilia, autoimmune haemolysis.

With the exception of ANCA-associated vasculitides, these diseases usually peak before fam-

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ily planning is complete. A cure is not possible. However, by early diagnosis and initiation of the appropriate treatment, most patients can now be treated adequately on a permanent basis. As a result, their life expectancy has also become increasingly closer to that of the normal population, which means that the desire to have children and fertility preservation also plays an important role for these patients.

Gonadotoxity of the treatments

The ovarian reserve, determined by the concentration of Anti Müllerian hormone (AMH), is limited in many autoimmune diseases due to the chronic disease per se and especially in cases of high disease activity [2–6]. For this reason, advice on fertility preservation should be given prior to CYC therapy, especially in autoimmune diseases.

CYC significantly increases the risk of premature ovarian insufficiency (POI) in autoimmune diseases. The percentages in the literature vary between 12 and 54% and are mainly influenced by the age of the patient at the time of therapy and the cumulative dose of CYC (Table 1).

The age and dose dependency of cyclophosphamide on ovarian toxicity are shown in a Chinese study of 216 women and in a study by Di Mario et al., in which ovarian toxicity was determined by AMH concentration [6, 16] (Fig. 1). According to these studies, other immunosuppressive drugs used in the treatment of SLE, such as mycophenolate, azathioprine, prednisolone, ciclosporin, tacrolimus and hydroxychloroquine, do not lead to a significant reduction in AMH concentration [6, 16].

Probability of exacerbation of the underlying disease

CYC treatment for autoimmune diseases is only indicated if there is high disease activity. A rapid initiation of therapy is usually necessary; however, the influence of fertility preservation therapy on the underlying disease must also be considered.

Due to the pathogenesis and gender distribution of many autoimmune diseases, it must be assumed that an increase in female hormones has a negative influence on the disease, and further exacerbation of the underlying disease can occur

Table 1 Studies on POI rate after cyclophosphamide (CYC) treatment

Study	Origin of study	Diseases	Number of women	POI rate (%)	Risk factors identified
Boumpas et al. 1993 [7]	USA	SLE	39	12–39	Age, CYC dose
Mc Dermott et al. 1996 [8]	UK	SLE	52	54	Age, CYC dose
Mok et al. 1998 [9]	China	SLE	70	26	Age, CYC dose
Ioannidis et al. 2002 [10]	Greece	SLE	67	31.3	Age, CYC dose disease duration
Huong et al. 2002 [11]	France	SLE, GPA	84	22.6	Age
Park et al. 2004 [12]	South Korea	SLE	67	14.9	Age
Singh et al. 2007 [13]	India	SLE	35	31.4	Cytochrome P450 polymorphism
Appenzeller et al. 2008 [14]	Canada	SLE	57 (CYC 750 mg/m^2) 50 (CYC 500 mg/m^2)	17.5 0	Age, CYC dose
Alarfaj et al. 2014 [15]	Saudi Arabia	SLE	188	13.1	Age, CYC dose
Di Mario et al. 2019 [6]	Italy	SLE	14	–	Age, CYC dose

SLE systemic lupus erythematosus, *GPA* granulomatosis with polyangiitis and identified risk factor for premature ovarian insufficiency (POI)

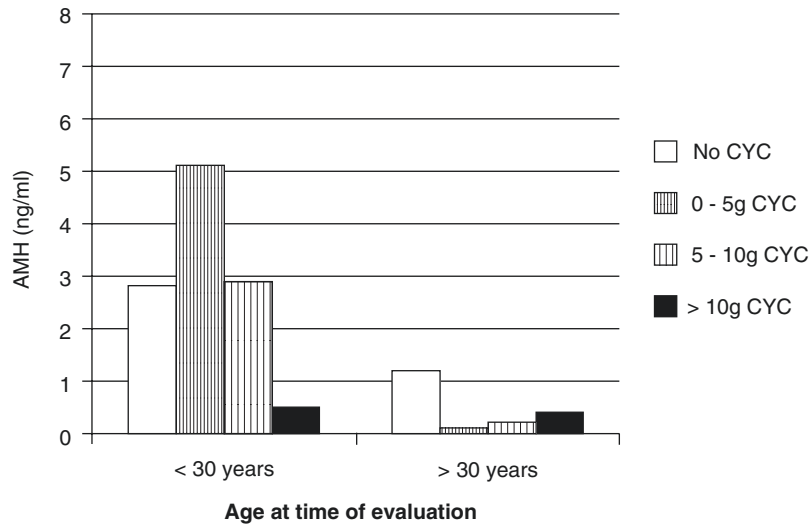

Fig. 1 AMH serum concentration after CYC treatment in women with SLE is dependent on age and dose. (Modified according to [16])

during ovarian stimulation for egg collection. Furthermore, other studies suggest that down-regulation with a GnRH agonist has a positive effect on SLE [17]. A transfer of these findings to other autoimmune diseases is reasonable, but due to the rarity of the diseases, they have not been sufficiently and conclusively investigated.

Overall, there are only a few studies/recommendations on fertility preservation specifically for autoimmune diseases [18–21]. The other recommendations are mostly based on findings from the treatment of SLE patients. The European League against Rheumatism (EULAR) also includes fertility preservation in its 2017 recommendations [22].

Effectiveness and risks of fertility preservation

The ovarian reserve is often reduced in autoimmune disease. Lawrenz et al. [3] and Di Mario et al. [6] found a 32 and 29% lower AMH concentration in women with systemic lupus erythematosus compared to a control collective. Lower AMH concentrations were also found in women with rheumatoid arthritis, Bechet's disease and spondyloarthritis [2], multiple sclerosis [5] and Takayasu's arteritis [4]. However, according to one study in lupus patients, AMH reduction appears to occur only in a severe form of autoimmune disease [6].

However, it is questionable whether the lowered AMH concentration also leads to fertility preservation measures being less effective. If oocytes are to be cryopreserved, the stimulation dose can often be adjusted. If ovarian tissue is cryopreserved, the AMH concentration plays a rather minor role. Important, however, is the density of primordial follicles, which in contrast to the AMH concentration, is not reduced in women with Hodgkin's lymphoma [23].

GnRH agonists

The effectiveness of GnRH agonists (GnRHa) (see chapter "GnRH Agonists") has now been proven in patients with breast cancer (see chapter "Breast Cancer"). For autoimmune diseases only very limited data is available. However, it can be assumed that the data on efficacy in breast cancer can also be transferred to autoimmune diseases, since the risk of POI is comparable in both disease groups and the same cytotoxic drug is used (CYC).

Somers et al. [24] and Koga et al. [25] treated women with lupus erythematosus with CYC and GnRHa and compared the POI rate with a control group without GnRHa. The cumulative CYC doses administered were 12.9 g and approx. 5.0 g, respectively. The POI rate was 5 and 6% with GnRHa therapy and 30 and 50% in the control group. Further studies investigated the effect

of GnRHa [26] and its tolerability in children with SLE based on AMH concentration [27].

GnRHa can therefore be considered in individual cases as a singular method if a higher cumulative cyclophosphamide dose is planned.

Ovarian Stimulation

The procedure (see chapters "Ovarian Stimulation to Collect Oocytes" and "Cryopreservation of Unfertilized and Fertilized Oocytes") should be discussed individually if stimulation therapy for cryopreservation of fertilised or unfertilised eggs is to be carried out.

In principle, two risks should be emphasised:

1. Risk of exacerbation of the disease under stimulation.

   In cases of connective tissue diseases in particular, especially SLE, stimulation can lead to a deterioration in disease activity. However, the available data are limited. Guballa et al. examined 17 women (10 with anti-phospholipid antibody syndrome (APS) and 7 with SLE) who underwent stimulation [28]. Stimulations with clomiphene citrate and with high-dose gonadotropins were included in the evaluation. No exacerbation was documented in women with APS. Women with SLE showed a slight exacerbation in 3/7 (43%) women in 3/16 (16%) stimulation cycles.
2. Risk of thrombosis.

   In general, the risk of thrombosis is increased in autoimmune disease, particularly in connective tissue diseases, and especially in SLE. Antiphospholipid antibodies are found in 40% of SLE patients, depending greatly on the patient's ethnicity [29–31]. The risk of thrombosis is highest in active APS and active SLE. According to a meta-analysis, if the serum marker "lupus anticoagulant" is increased, the risk of thrombosis increases by about six times, even in patients without SLE [32]. Other markers such as anticardiolipin antibodies, anti-β2 glycoprotein antibodies, anti-prothrombin antibodies, anti-phosphatidylserine antibodies and anti-phosphatidylethanolamine antibodies were only associated with a slight and insignificant increase in the risk of thrombosis in this study.

   There are little data available on the risk of thrombosis during stimulation. In the above-mentioned study by Guballa et al. [28], none of the 17 women stimulated with clomiphene citrate or gonadotropins had a thrombosis. However, all women received a thrombosis protection (heparin, aspirin or corticosteroids).

   In assisted reproduction, stimulation is also possible in SLE patients with special caution [28, 33]. In the event of an acute worsening of the underlying disease with the need for therapy escalation, the basic requirements for safe stimulation are not met. Therefore, this option should only be indicated with extreme caution in cases of active APS or SLE. Adequate thrombosis protection, depending on the risk profile, must be ensured [34].

Cryopreservation of Ovarian Tissue

Cryopreservation of ovarian tissue (see chapters "Removal of ovarian tissue" and "Transportation, cryopreservation and storage of ovarian tissue") is a good option for young women under the age of 35 and up to a maximum of approximately 40 years. Good pregnancy rates are particularly evident in women up to 35 years of age, and the method can also be successfully carried out in SLE patients [35, 36]. Since autoimmune diseases are chronic diseases, this method offers fertility preservation even if renewed CYC therapy is necessary. Due to the often reduced ovarian reserve, however, an adequate reserve should first be ensured by AMH measurement and determination of the AFC using ultrasound. A case report of a successful pregnancy in a patient with SLE after retransplantation of cryopreserved ovarian tissue is available [36].

Practical Approach

The choice of fertility preservation methods is always an individual decision, which should be made in close consultation with the patient, the gynaecologists and rheumatologists in charge.

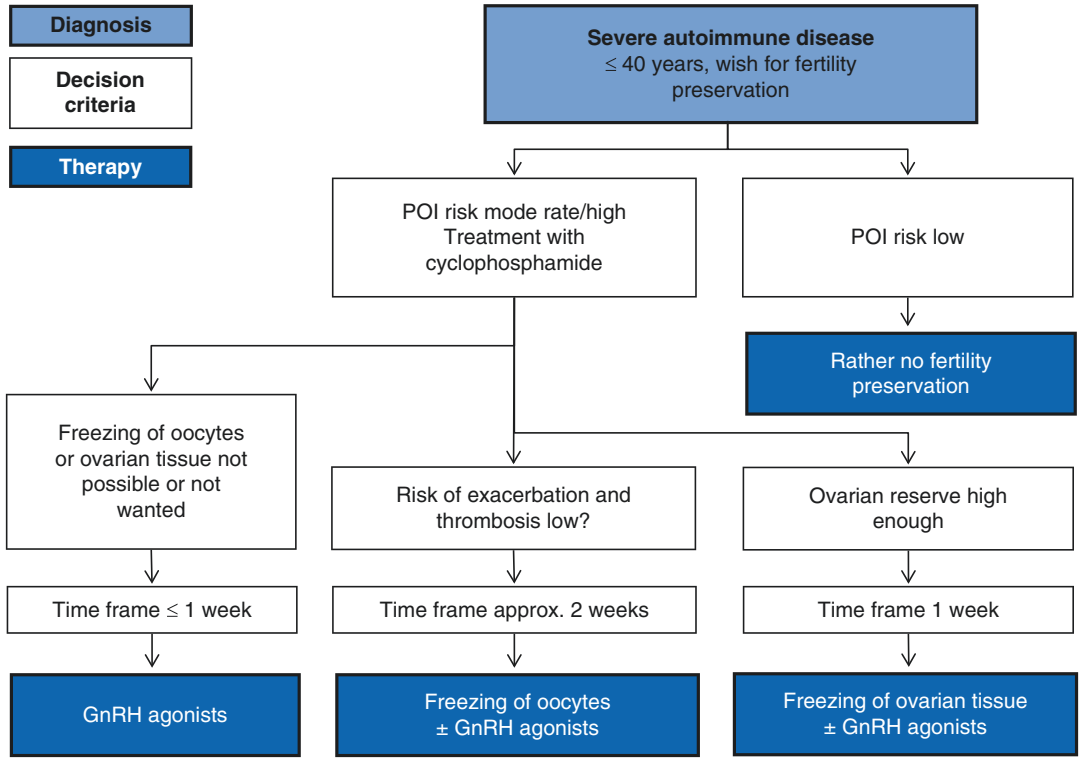

Fig. 2 Algorithm for fertility preservation in women with autoimmune diseases

In principle, patients should be introduced to a reproductive medicine centre as early as possible in order to ensure the greatest possible time frame for the implementation of fertility preservation methods. Figure 2 shows the procedure for carrying out fertility preservation measures for autoimmune diseases.

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Fertility Preservation Considerations in Female Patients with Benign and Malignant Hematologic Disease

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Introduction

Historically, hematological malignancies were considered incurable diseases with high mortality. Now most hematological malignancies have favorable long-term survival rates, and some patients with non-malignant hematologic diseases can also be "cured" by bone marrow transplantation. Treatments may however carry a risk of irreversible damage to future fertility. The impact varies substantially depending on the type of hematological diseases and therefore the treatment required. Thus, fertility preservation is an important consideration in the management of this group of patients.

Leukemia and non-Hodgkin lymphoma are the most common childhood malignancies of children aged less than 5 years old [1] and account for around one-third of childhood malignancy around the world [1, 2]. Lymphoma is also the commonest malignancy in young adults of 15–24 years old. Four decades ago, the 5-year relative survival rate for leukemia and lymphoma ranged from 14 to 40% [2], but due to the evolution of chemotherapy regimens, targeted therapy, and supportive care, the 5-year survival rate is now up to 65–88% [2].

Benign hematologic diseases are also very important in this context, as both the condition and its treatment may have substantial effects on fertility. Hemoglobinopathies are more prevalent in people of Southeast Asian, Arabic, and Mediterranean descent and are the commonest inheritable genetic diseases. The carrier rate ranges from 5% worldwide to 40% in Arab nations and Southeast Asia [3]. The complication of iron overloading with transfusion-based supportive therapy and preconditioning chemotherapy for hematopoietic stem cell transplantation (HSCT) can both lead to significant fertility impairment.

In this chapter, we review the impact of different malignant and benign hematologic diseases on fertility, followed by discussion on additional precautions in terms of fertility preservation in this specific group of patients. Finally, safety concerns relating to ovarian tissue autotransplantation in blood-borne malignancies are discussed, with a brief introduction regarding potential new techniques in transplantation.

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Impact of Malignant Hematologic Disease on Fertility

The impact of hematologic cancers on fertility greatly depends on the nature, type, and dose of treatment received and age of the patient. The risk of gonadotoxicity of commonly used chemotherapy in hematological diseases is shown in Table 1 and more details on gonadotoxicity of chemotherapy was discussed in Chap. 2. Of the chemotherapeutic agents, alkylating agents have long been recognized to be among the most gonadotoxic agents [4]. Alkylating agents achieve their anti-cancer effect by inducing DNA damage by breaking the DNA double helix and adding an alkyl group, thus are not dependent on the cell replication cycle to have their effects. They will therefore affect oocytes within primordial follicles, as well as proliferating granulosa cells in growing follicles, thus directly diminishing the ovarian reserve.

In general, in young standard risk patients with leukemia, potentially gonadotoxic chemotherapeutic agents can be avoided in first-line therapy regimens. However, in high-risk and relapsed patients, chemotherapy regimens with higher gonadotoxicity or hematopoietic stem cell transplantation may be indicated. Hence, an individualized approach of fertility preservation needs to be considered and patients counseled accordingly.

Acute Lymphocytic Leukemia (ALL)

ALL is the commonest childhood hematologic cancer with incidence of around 42 million children in the USA and accounts for around 20% of all childhood cancer [2, 5]. The highest incidence is at age 1–4 years with an increasing prevalence over time, from 1.9 per 100,000 in 1975 to 3.1 per 100,000 in 2015 [2]. The remission rate is up to 90%. Standard treatment involves use of antimetabolite and vinca alkaloid chemotherapy, thus is of low gonadal toxicity. High-dose alkylating agents are usually not indicated unless in high risk or relapse cases.

Table 1 Gonadotoxic risks of commonly used treatment in hematological diseases

Diseases	Commonly used treatment	Gonadotoxic risks
Acute lymphoid leukemia	Vincristine Corticosteroids Asparaginase Anthracycline	Low
Acute myeloid leukemia	Cytarabine Anthracycline Fludarabine	Low
Chronic lymphoid leukemia	Fludarabine Cyclophosphamide Rituximab (anti-CD20 antibody)	Low
Chronic myeloid leukemia	Tyrosine kinase inhibitors	Low but potentially teratogenic
Classical Hodgkin lymphoma	ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) ± BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisolone) OEPA (vincristine, etoposide, prednisolone, and doxorubicin) and COPDAC (cyclophosphamide, Vincristine, dacarbazine, prednisolone)	Low for ABVD but high for BEACOPP Limitation being short duration between changes of regimen Low for OEPA and COPDAC
Non-Hodgkin lymphoma	CHOP (cyclophosphamide, hydroxydanuorubicin, oncovin, prednisolone) ± rituximab	Low
Sickle cell disease	Hydroxyurea	Low but potentially teratogenic
Both benign and malignant hematologic diseases	Hematopoietic stem cell transplantation with either high-dose chemotherapy conditioning or total body irradiation (TBI)	High

Acute Myeloid Leukemia (AML)

AML accounts for 15% of childhood leukemia with highest incidence in those aged less than 1 year in children and young adults. However, the highest incidence is at aged 60 years or older (incidence 1.6/100,000 in age less than 1 year versus 28.6/100,000 in aged 80–84 years) [2]. The regimen for patients with standard risk does not involve alkylating agents, and around half of the patients present with standard risk.

Chronic Lymphocytic Leukemia (CLL)

CLL is mainly a disease of the elderly population, with the average age at diagnosis of 60–70 years old [2]. Being the commonest adult leukemia, the standard regimen for low-risk patients is fludarabine, cyclophosphamide, and rituximab (anti-CD20 antibody) [6]. In early stage without active disease, watchful waiting is the recommended approach rather than any chemotherapeutic agents [6]. However in high-risk patients or in circumstances of relapse, more gonadotoxic agents would be required.

Chronic Myeloid Leukemia (CML)

Similarly to CLL, CML is mostly a disease of elderly. CML has the highest incidence in older population with a median age at diagnosis of 57–60 years old [7]. The incidence was 0.7–1.75/100,000 around the world. The incidence in children is 0.6–1.2 per million children per year which contributes to less than 5% of childhood leukemia [8]. Before the development of tyrosine kinase inhibitors (TKI), the 5-year survival rate was as low as 5% as compared to around 85% nowadays [7]. Most patients in the chronic phase, however, require potentially lifelong treatment with TKI.

Imatinib, which is the first licensed TKI, is not clearly associated with risk of damage to the ovarian reserve [9], although adverse effects on ovarian function have been reported [10]. Somewhat controversially, it may also have protective effects against the gonadotoxicity of cisplatin [11, 12]. Pregnancy should be avoided due to the potential teratogenicity and risk of miscarriage with high doses [13, 14]. Thus, for women with pregnancy intent, switching to TNF-α at least during the first trimester of pregnancy is recommended [8]. The evidence regarding second-generation TKIs is even more limited [15]. Hence, unless for patients considering HSCT, further discussion on fertility preservation in CML is usually not necessary.

Classical Hodgkin Lymphoma (cHL)

cHL is more common in adolescents and young adults aged less than 34 years, though the peak is at age 75–79 [2]. Five-year overall survival is up to 98.3% for those aged less than 20 years and 93.9% for those aged 45 years or less in 2018 [2]. Worldwide ABVD (doxorubicin/bleomycin/vinblastine/dacarbazine) with or without involved field irradiation is the standard treatment for those with early limited stage disease [16]. As this regimen does not contain alkylating agents, the chance of POI is low [17]. Current protocols for young adult patients often involve assessment by an interim PET scan after two cycles of ABVD to decide on further chemotherapy regimen [18]. In PET-positive patients, escalation of the chemotherapy regimen with several cycles of BEACOPP (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisolone) has been shown to be effective; BEACOPP carries a high risk of POI of up to 50% as compared with 10% with the ABVD regimen alone [19].

Assessment of ovarian function after treatment with ABVD or BEACOPP confirms the substantial differences in gonadal toxicity between these regimens, with recovery to pretreatment AMH levels within 12 months of the end of treatment after ABVD but little recovery after BEACOPP [20]. One year after ABVD treatment, the median of AMH was 10.5 pmol/l (IQR 4.3–17.3), which was similar to the pretreatment level, while the median of AMH of those received BEACOPP was 0.11 pmol/l (IQR 0.07–0.20). However, detailed analysis of post-

treatment AMH levels after ABVD indicated that full recovery was dependent on age, rather than pre-treatment ovarian reserve. Thus recovery in women aged ≥35 years was markedly reduced to 54% (95% CI 43–66%) compared with 83% (95% CI 77–88%) for those aged <35, indicating a specific impact of ageing distinct from the age-associated progressive reduction in the number of follicles in the ovary.

As there is only a short time interval between interval PET scan and the start of the subsequent BEACOPP regimen, fertility preservation may need to be counseled and provided before start of ABVD. From the large phase III studies, 19% of women had positive interim PET which warranted more toxic chemotherapy [18, 21, 22]. Thus if fertility preservation is to be offered to all patients before treatment starts, then it needs to be recognized that this will involve over-treating the majority of women, whose fertility will not be lost after ABVD-only therapy. Fertility preservation (with ovarian tissue cryopreservation) may be possible in the short interval between the PET scan after ABVD and starting BEACOPP: this requires a high level of planning and coordination between oncology and reproductive medicine centers.

Treatments of HL in children and adolescents are also evolving, with current trials assessing the impact of reducing radiotherapy administration and the substitution of the highly gonadotoxic alkylating agent procarbazine with dacarbazine, which may be significantly less toxic.

Non-Hodgkin Lymphoma (NHL)

NHL accounts for approximately 90% of all lymphoma. It is the commonest malignancy in adolescents and young adults [1, 2] with 5-year relative survival of 84.3% for those aged less than 20 years [2]. It involves a heterogeneous group of malignancies of lymphocytes, and the commonest is B-cell lymphoma accounting for 85–90% of the diagnoses. The standard chemotherapy regimen for mature B- and T-cell lymphomas – CHOP (cyclophosphamide, hydroxydaunorubicin, oncovin, and prednisolone) +/− rituximab – does not carry a high risk of POI [19]. Despite that, more gonadotoxic and intense treatment may be required in high-risk group or other subtypes of NHL.

Benign Hematologic Disease

Fertility preservation in benign hematologic disease is less widely reported in comparison with malignant diseases. Hemoglobinopathies are the most prevalent monogenic inheritable diseases worldwide. Supportive treatment with repeated transfusion can have a significant impact on fertility due to the complications of iron overloading. Excessive iron deposition leads to cellular damage by oxidative stress, and common sites of deposition include the heart, endocrine glands, and liver [23]. Hypogonadotropic hypogonadism is an important cause of infertility in these women, with additional impact influencing the fitness for pregnancy such as heart failure and pulmonary/portal hypertension. Additionally, ovarian toxicity with a direct impairment of the ovarian reserve was observed in these groups of women indicated by reduced serum AMH level and antral follicle counts [24, 25]. Iron overload can be reduced but not always completely prevented by iron chelating treatment.

In addition to transfusion-related hemochromatosis, maintaining fertility for women with sickle cell anemia is challenging. Hydroxyurea is the commonest disease-modifying drug used and is potentially teratogenic, with effects reported from both animal studies and clinical observation [26, 27]. Women are advised to avoid pregnancy for at least 6 months after discontinuation of hydroxyurea. Additionally, treatment with hydroxyurea for more than 12 months was associated with diminished ovarian reserve as indicated by AMH. 24.2% of women treated with hydroxyurea had AMH less than the fifth percentile as compared with none in those not treated with hydroxyurea [28].

Hematopoietic Stem Cell Transplantation (HSCT)

HSCT is increasingly utilized in both malignant and benign hematological diseases with more than 40,000 transplants performed in 2014 in Europe [29]. Despite achieving a high cure rate, it also comes with a high rate of fertility impairment of up to 90% [30, 31] with a pregnancy rate of <1% [32]. There are three different conditioning approaches for HSCT – myeloablative, reduced intensity conditioning, and non-myeloablative. The chance of POI is related to the myeloablative regimen and the age of the patient. Women who received busulfan and cyclophosphamide as conditioning had a higher chance of infertility, as did those who received high-dose total body irradiation [30]. Start of treatment in girls aged ≥13 years was associated with risk of fertility impairment as defined by FSH ≥15 IU/l [30]. The effective sterilizing dose of radiation decreases with age from 18.4 Gy at 10 years old to 14.3 Gy at 30 years old [33], but a dose of 2 Gy can reduce the number of oocytes by 50% [33]. Even in those whose menses returned after HSCT, earlier menopause is common [31] with OR 6.35, 95% CI 1.19–33.93, $p = 0.031$ from the US childhood cancer survivor cohort. A history of any dose of ovarian irradiation and use of procarbazine were both risk factors for POI [34].

Reduced intensity conditioning and non-myeloablative approaches were initially only used in the elderly with comorbidities, but more recently its use has been investigated in malignant disease [35]. The impact of adopting a reduced conditioning approach on the long-term survival in leukemia and lymphoma remains unclear at present [36]. In addition to the direct impact on the ovarian reserve, the possibility of graft versus host disease against granulosa cells leading to further damage has been reported [37]. Also, the actual benefit on endocrine function and fertility is yet to be determined [31, 38].

Other Impacts

Depending on the site of irradiation, fertility prospects may be compromised. Cranial irradiation may cause gonadotropin insufficiency, and radiation to the pelvis may compromise uterine development and function particularly in the young child [39]. Childhood cancer survivors are also at a higher risk of development of second primary malignancies, particularly where radiotherapy has been used. The chance of second neoplasm in Hodgkin's lymphoma and leukemia survivors is as high as 33.8% and 20.8%, respectively [40].

Methods of Fertility Preservation

Oocyte/Embryo Cryopreservation

The gold standard for fertility preservation is oocyte or embryo cryopreservation, with details discussed in previous chapters. Although cryopreservation of oocytes and embryos are promising and well-established methods for fertility preservation, they are often not feasible in acute hematological diseases. Firstly, acute leukemia is often a medical emergency, and patients are often not medically fit enough to have a delay in treatment of 2 weeks or more. Commonly they have abnormal blood counts including severe anemia, thrombocytopenia, and coagulopathy, which may render oocyte retrieval or laparoscopy for ovarian tissue preservation hazardous and inappropriate. Secondly, oocyte or embryo cryopreservation is not feasible in pre-pubertal girls.

In women with sickle cell anemia, in spite of having adequate time interval for ovarian stimulation, ovarian stimulation and oocyte retrieval can be potentially complicated. In a small cohort of five women undergoing ovarian stimulation and oocyte retrieval, one woman experienced painful crisis during the stimulation and another woman experienced chest pain and respiratory failure on the evening of oocyte retrieval [41]. Use of anticoagulant in patients with sickle cell anemia was common due to the thromboembolic risks which will complicate both oocyte retrieval and ovarian tissue cryopreservation [41]. In addition, sickle cell anemia and hemoglobinopathies are both hereditary diseases that are inherited in an autosomal recessive manner. Hence, screening of the partner and provision of

pre-implantation genetic testing of embryos will need to be considered and discussed before cryopreservation.

Ovarian Tissue Cryopreservation

Ovarian tissue cryopreservation may be the only option for some females with hematological disease, but it can pose challenges. One of the technical difficulties in acute malignancies is the risk of infection and bleeding due to leukocytopenia, thrombocytopenia, and anemia, and the possible contamination of the tissue with malignant cells is a major concern. There have been more than 100 reported cases of ovarian tissue autotransplantation in Hodgkin's lymphomas survivors with no reported case of recurrence [42–44], while the evidence was more limited in non-Hodgkin lymphoma [45]. While the first successful pregnancy after ovarian tissue re-implantation was in 2004 in a women previously treated for HL [46], the first baby after transplantation of cryopreserved ovarian tissue in a woman with leukemia was only reported in 2018 [47]. The ovarian tissue was harvested during complete remission, and the ovarian tissue autotransplantation was performed after extensive testing including xenografting on immunosuppressed mice. For pre-pubertal girls, data on fertility outcomes are rare, with pregnancies reported with the use of pre-/peri-pubertal tissues including in beta thalassemia and sickle cell anemia [48, 49].

Gonadotropin-Releasing Hormonal Agonist (GnRHa) Suppression

The rationale for giving GnRHa to protect the ovary against chemotherapy is to achieve ovarian suppression, although the mechanism for how this can achieve protection is unclear. The protective effect of GnRHa remains controversial. In a recent meta-analysis, the rate of persistent amenorrhea was lower with concurrent use of GnRHa with chemotherapy (RR 0.60, 95% CI 0.45–0.79) [50]. In another Cochrane review in 2019, a similar result was identified with a higher incidence of recovery of menstruation during a follow-up period no longer than 12 months (RR 1.60, 95% CI 1.14–2.24), but it was not observed in those with follow-up longer than 12 months (RR 1.08, 95% CI 0.95–1.22) [51]. Conversely, a beneficial effect of GnRHa in women with breast cancer was demonstrated in an individual patient data meta-analysis only after 12 months from chemotherapy [52]. Most of the evidence is from breast cancer survivors. In a subgroup analysis of women with lymphoma, the concurrent use of GnRHa did not reduce the chance of POI/amenorrhea (RR 0.70; 95%: 0.45–0.79) or spontaneous pregnancy (RR 1.13, 95% CI: 0.66–1.93) [50]. There were only 3 randomized controlled trials with just over 100 lymphoma survivors (both Hodgkin's and non-Hodgkin's lymphoma), while all the remaining 10 RCTs involve breast cancer survivors. One of the major concerns of these meta-analyses is the heterogeneity of the studies. The chemotherapeutic agents received varied greatly even in the same spectrum of malignancies, and the protective effect of GnRHa may be present with low/medium toxicity therapy than high toxicity chemotherapy [50]. Apart from different chemotherapeutic agents used in breast cancer and lymphoma, the discrepancy of age at treatment may also be a contributory reason. Also, there was no common endpoint in most studies which makes comparisons difficult. Furthermore, the type, dosage, and regimen of GnRHa also varied, particularly the issue of whether treatment needs to be started weeks prior to chemotherapy, to ensure ovarian suppression has been achieved: this is difficult to achieve in practice. In addition, GnRHa is not appropriate in pre-pubertal girls. In conclusion, GnRHa suppression is not an established method of fertility protection in hematological malignancy, and it should not be the sole method of fertility preservation offered to women where more effective methods are possible [53].

Additional Considerations

In children and adolescents, extra attention to psychological, cultural, and ethical aspects needs to be considered. Discussions on fertility and

pregnancy are sensitive and may not have been raised at school or in the family previously while patients and their families are facing a major life event at the same time. Additional cultural and religious issues need to be taken into consideration. In some cultures, sexuality and fertility is a forbidden topic for unmarried girls, and transvaginal ultrasound or oocyte retrieval may not be acceptable. Occasionally the wishes and best interests of the parents and the child may be different, requiring broad multidisciplinary discussion to determine the best way forward.

Safety of Ovarian Tissue Re-implantation

A major issue is the risk of ovarian tissue contamination in blood-borne malignancies. In an analysis of 5571 autopsy results of Japanese women aged 40 or less, the chance of ovarian metastasis was 22.4% with highest rate in gastrointestinal tract malignancies, 13.3% in lymphoma, and 8.4% in leukemia [54]. However, there was no detail on the disease statuses which is expected to be late stage for most of these cases. The risk of contamination is however regarded as high in leukemia patients, followed by non-Hodgkin lymphomas, while it is smaller in solid tumors and Hodgkin lymphoma [43, 45, 55]. Breast cancer is one of the more well-studied solid tumors in this context. In 146 ovarian samples, none were positive for ovarian metastasis either histologically or clinically [56]. For other solid tumors including gastrointestinal tract tumor, sarcoma, and tumors of the central nervous system, there are only scattered and limited reports, with negative results. Even in epithelial and non-epithelial ovarian cancer, xenografting of ten ovarian tissues in SCID mice did not show any evidence of recurrence [57], whereas in ovarian tissues from patients with leukemia, 60.6% of ovarian samples ($n = 33$) were positive by PCR and 10% ($n = 50$) induced recurrence in SCID mice [45]. The risk of inducing recurrence significantly hinders the use of cryopreserved ovarian tissues in leukemia survivors.

Before considering ovarian tissue collection for re-implantation, the "remission" status of the malignancy should be considered. Historically, morphological remission with less than 5% blast in the bone marrow was used as the criterion for stopping of treatment for ALL. However it was associated with high risk of relapse requiring more toxic chemotherapeutic agents with compromised survival, and it has been replaced by the concept of minimal residual disease (MRD). A positive MRD is defined as more than 1 malignant cell in 1000, which is associated with a high risk of relapse. Most studies in assessing MRD were conducted in either childhood ALL and adult CML [58, 59], and it has been extended to AML recently [60]. It gives better prognostic value than other parameters like age, cytological subtype, and white cell count at diagnosis [60]. Different methods have been investigated for detection of MRD including flow cytometry, real-time quantitative polymerase chain reaction (RT-qPCR), or even next-generation sequencing. They have different sensitivities, and some may even detect MRD up to 1 in 1,000,000. However, none define a safe level for recurrence with certainty.

Histology and Immunohistochemical Staining

The first-line screening test is histological examination with traditional hematoxylin and eosin staining and immunohistochemical staining. It is essential in screening for metastasis; however, the major drawback of this methods is the low sensitivity. Commonly used markers for AML are CD43 and c-KIT (CD117); Hodgkin lymphoma, CD15 and CD30; and non-Hodgkin lymphoma, CD20. The markers used should be directed by the positive markers in the primary malignant cells.

In conditions with lower risk of recurrence, examination by histology and immunohistochemical staining has been regarded as sufficient. No recurrence has been reported with the use of microscopic negative ovarian tissues in both Hodgkin lymphoma and non-Hodgkin lymphoma

[43, 55]. However, in leukemia more than half of the cases can be falsely regarded as negative by histological examination only as compared with PCR [47].

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Additional molecular testing like RT-PCR can improve the sensitivity of detection. In a recent review, 61.0% of cryopreserved ovarian tissues for leukemia were positive by PCR [47]. Even in ovarian tissue harvested after chemotherapy, 52.2% of cryopreserved ovarian tissues were positive by PCR. The sensitivity of PCR on ovarian tissue has been reported to be up to log 10^{-5} [61, 62]. However, molecular markers for the leukemia were only present in 28–89% of all cases and were especially infrequent in AML.

Other Tests

Multicolor flow cytometry for malignant cells has been investigated and can detect MRD up to log 10^{-5} [62]. It can be a method of choice especially in PCR-negative leukemia. Next-generation sequencing has been investigated in screening for ovarian metastasis by identifying specific gene mutations or re-arrangements. It can be performed either by comparing with the pre-treatment malignant cell genome or by screening for a myeloproliferative panel, but the former was the preferable method as it is patient specific [47].

Xenografting

Xenografting into severe combined immunodeficient (SCID) mice provides a more biological test of viable malignant cells within the ovarian tissue, first used in this context in 1996 [63]. Tissues are commonly transferred to the peritoneum, kidney capsule, or subcutaneous tissue of SCID mice and the mice sacrificed 3–6 months later [47, 64]. In an analysis of 5 samples with CML, 12 samples with ALL, and 1 sample with AML, xenografting of PCR-positive ovarian tissue on SCID did not always lead to recurrence, but all recurrences occurred in PCR-positive tissues [55, 61, 65, 66]. All recurrences occurred in xenografted of ovarian tissue that had been harvested before the start of chemotherapy. It is unclear why there was no recurrence in some cases despite PCR-positive testing, but this may reflect non-viability of the malignant cells within the tissue.

While these different methods have been used for screening for the presence of malignant cells, a limitation is that all of them are destructive and are thus not testing the pieces of tissue that will be transplanted into the patient. The decision regarding ovarian tissue autotransplantation needs to be discussed very thoroughly with the patient where this is relevant.

Future Potential: In Vitro Follicle Growth and the Artificial Ovary

Isolation of follicles with in vitro growth (IVG) and in vitro maturation (IVM) may provide a contamination-free method of fertility restoration in future [67], although it will not provide the hormonal restoration which is a positive feature of ovarian transplantation. Approaches include complete in vitro growth of follicles from the earliest, non-growing stage right through to isolation of mature metaphase II oocytes [68], or isolation of growing follicles at varying stages with support for further development. However, up till now, production of successful offspring through IVG has only been proven in mice [69]. Isolation of follicles from ovarian tissue with no contamination with leukemia cells can be achieved even in PCR-positive samples [61]. IVM of immature oocytes can be achieved from pre-pubertal ovary, but the success rate is related to the pubertal status with only 10.3% maturation rate collected from pre-pubertal tissue as compared to 28.1% in post-pubertal women [70].

Instead of autotransplantation of ovarian tissue or IVM, re-implantation of immature follicles and ovarian stromal cells into artificial ovaries offers a potentially attractive option. The artificial ovary ideally should able to support the growth and mat-

uration of follicles and probably also be eventually biodegradable. Different scaffolding materials have been evaluated, including collagen gels and three-dimensional alginate matrigel matrix and fibrin matrix [71–74]. 3D printing has also been used to generate a scaffold for the support of murine follicles, with very positive preliminary results [75]. These artificial ovaries eliminate the risk of malignant cell contamination, provided of course that none are introduced with the follicles as other ovarian cell types may be necessary for the complete function of the artificial ovary.

Conclusion

The impact of hematologic disease on fertility is very variable, depending on the nature of the disease, the treatment regimen and dosage of chemotherapeutic agents, and the age of the patient. Ovarian tissue cryopreservation is an important modality for fertility preservation in some groups of patients mainly due to the urgency of treatment initiation and their pre-pubertal status, although oocyte cryopreservation is generally achievable in adult women with lymphoma. Providing contamination-free re-implantation of ovarian tissue remains a critical issue. Methods including PCR, flow cytometry, and next-generation sequencing can aid exclusion of malignant seeding; however, all tests are destructive in nature. In future, in vitro maturation or the artificial ovary may provide a recurrence-free approach, but it will be some years before these reach clinical practice.

Practical Clinical Tips

- The risk of fertility impairment is related to age of the patient, the treatment regimen, and the dose of treatment received.
- Despite oocyte/embryo cryopreservation being well-established methods for fertility preservation in adult females, ovarian tissue cryopreservation is often the only feasible option in patients with hematological diseases who are pre-pubertal or those patients requiring to start treatment urgently.
- Ovarian tissue autotransplantation in leukemia carries a potential risk of malignant cell contamination.
- Different techniques for the detection of minimal residual diseases in ovarian tissues include histology, immunohistochemical staining, reverse transcription polymerase chain reaction, next-generation sequencing, and xenografting in mice.

Take-Home Messages

- The first-line treatment for standard risk leukemia and early stage lymphoma does not involve highly gonadotoxic treatment; treatments for relapse and with hematopoietic stem cell transplantation are associated with a high risk of POI.
- Risk of malignant cell contamination especially in leukemia remains a major concern, and all current available tests are destructive in nature.
- Isolation of follicles with in vitro maturation and the artificial ovary may be an option for future fertility restoration without risk of malignant cell transplantation.

Clinical Cases

Amelia is a 3-year-old girl who has just been diagnosed with acute lymphocytic leukemia. She presented with bruises and nose bleeding. Full blood count showed severe anemia, thrombocytopenia, and leukocytosis with predominant lymphoblast. She and her parents are now at your clinic asking for fertility preservation. What addition information do you needed?

– *We need to first evaluate the risk of POI of the treatment regimen intended, which require a good communication with the oncology hematologist. Generally if the chance of POI is higher than 50%, then fertility preservation should be considered.*

Amelia did not require agents with high gonadotoxic risk; thus fertility preservation is not provided. However, she has a relapse 2 years later and requires myeloablative conditioning therapy and a hematopoietic stem cell transplantation. So, what would you now recommend to her and her parents?

– *As HSCT carries a 90% of POI, ovarian tissue cryopreservation should be considered, which is the only option in prepubertal girls. However, we need to provide adequate counseling of the risks of the procedure especially in the presence of grossly abnormal blood counts and the potential risks of leukemia cells contamination during autotransplantation in future.*

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Fertility Preservation in Transgender Males

Kenny A. Rodriguez-Wallberg

Introduction

The reproductive needs of the transgender population are becoming more recognized, and an increasing number of transgender males, i.e. individuals assigned female at birth with a male gender identity, are showing interest in undergoing procedures that can provide options for future reproductive possibilities. The current guidelines for gender-affirming medical treatment from the World Professional Association for Transgender Health (WPATH) [1] and the Endocrine Society [2] recommend that all transgender individuals seeking gender-affirming medical interventions should be informed about the risk of future infertility associated to their treatments. The guidelines for Female Fertility Preservation, recently provided by the European Society of Human Reproduction and Embryology [3], encompass an updated overview of the literature, including also specific recommendations for the patient group of transgender males [3]. The ESHRE [4] and the American Society for Reproductive Medicine [5] have both discussed and proposed access to reproductive care and fertility preservation for this patient population previously.

Although it has been recommended that a discussion on options for fertility preservation should be included in reproductive counselling of young transgender individuals seeking gender-affirming treatment, many of them do not currently recall having this counselling and do not have access to reproductive care to date [6].

In this chapter the current status of fertility preservation for transgender males is presented, and practical information on aspects that should be considered when aiming at providing care to this specific patient group are discussed.

Fertility Preservation for Transgender Males: What Are the Current Barriers

Although the use of assisted reproductive technologies (ART) has expanded worldwide and ART application is established for fertility preservation of various patient groups, inclusion of transgender males in programmes for fertility preservation is relatively recent, and little data are available [3]. It is important to notice that most clinical centres dedicated to reproductive health and performing ART treatments may lack medical experience with transgender individuals. A main reason for this is the fact that the subject of transgenderism has been largely absent in

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most healthcare educational programmes at all levels [7]. This deficiency translates into the abundant evidence of transgender patients' negative experiences of healthcare in general [8–12].

The practice of fertility preservation requires multidisciplinary collaboration, and lack of knowledge or awareness at any of the disciplines involved may become the first barrier for transgender patients with regard to fertility preservation provision. Several studies indicate that transgender patients in general receive less frequently reproductive counselling than other patient groups. In Australia, a recent study including 409 transgender and non-binary individuals reported that a smaller proportion (about one third) of the group hoped to have children in future, but nearly all (94.6%) considered that fertility preservation should be offered to all [6]. In Europe, 189 transgender individuals surveyed in Germany found that only less than 10% of transgender females and 3% of transgender males had accessed fertility preservation methods [13]. Explanatory barriers include additional aspects relevant to healthcare providers [14], as well as the patients' fear of hormonal effects or the invasive procedures that are involved in the methods to obtain oocytes to cryopreserve [15]. In certain countries, the high costs of the procedures involved in FP are also an important barrier [16]. Awareness of explanatory barriers may help to improve the development of healthcare programmes for FP of transgender individuals.

Establishment of Fertility Preservation for Transgender Patients

In Sweden, fertility preservation programmes have been developed at large university hospitals, and those are publicly tax-financed including both the treatment of infertility using assisted reproductive technologies ART and also the performance of fertility preservation whenever it is indicated by medical reasons. A large experience of FP for cisgender females has thus been achieved and reported from Swedish FP programmes [17–19], but only relatively recently in 2013, it was allowed to offer FP to transgender individuals, after the prerequisite to undergo sterilization at time of legal gender change was removed by law. Following this change in the law, the Swedish reproductive medicine centres have actively worked to include transgender individuals in programmes for FP; however, the specialized centres lacked previous knowledge on this patient group.

At our reproductive medicine centre at Karolinska University Hospital in Stockholm, a healthcare programme for FP for transgender individuals was initiated in 2013. The clinical programme was specifically developed within a prospective clinical research project not only to investigate both short- and long-term clinical outcomes but also to obtain the feedback from the patients, which was planned using a qualitative research design to explore patients' experiences [15]. A similar study design was also proposed to identify the challenges that healthcare personnel would experience during the development of the programme [14]. The issues highlighted from these experiences are hereby summarized.

Importance of Specific Training to the Healthcare Team

The Fig. 1 illustrates the timeline associated with the development of the programme for fertility preservation for transgender patients at our centre. As no sufficient knowledge on this specific patient group was available among the healthcare personnel, the first steps included preparations to meet the new patient group's specific needs. The preparations included lectures, seminars and group discussions with specialized psychiatrists, psychologists, endocrinologists, andrologists and counsellors, all personnel categories from the multidisciplinary gender team of the hospital. Patient association representatives were also invited to our centre, including organizations working for LGBTQ people's rights. At the beginning, a small group of professionals including clinicians were assigned to be primarily responsible for the new patient group, received further education and participated in

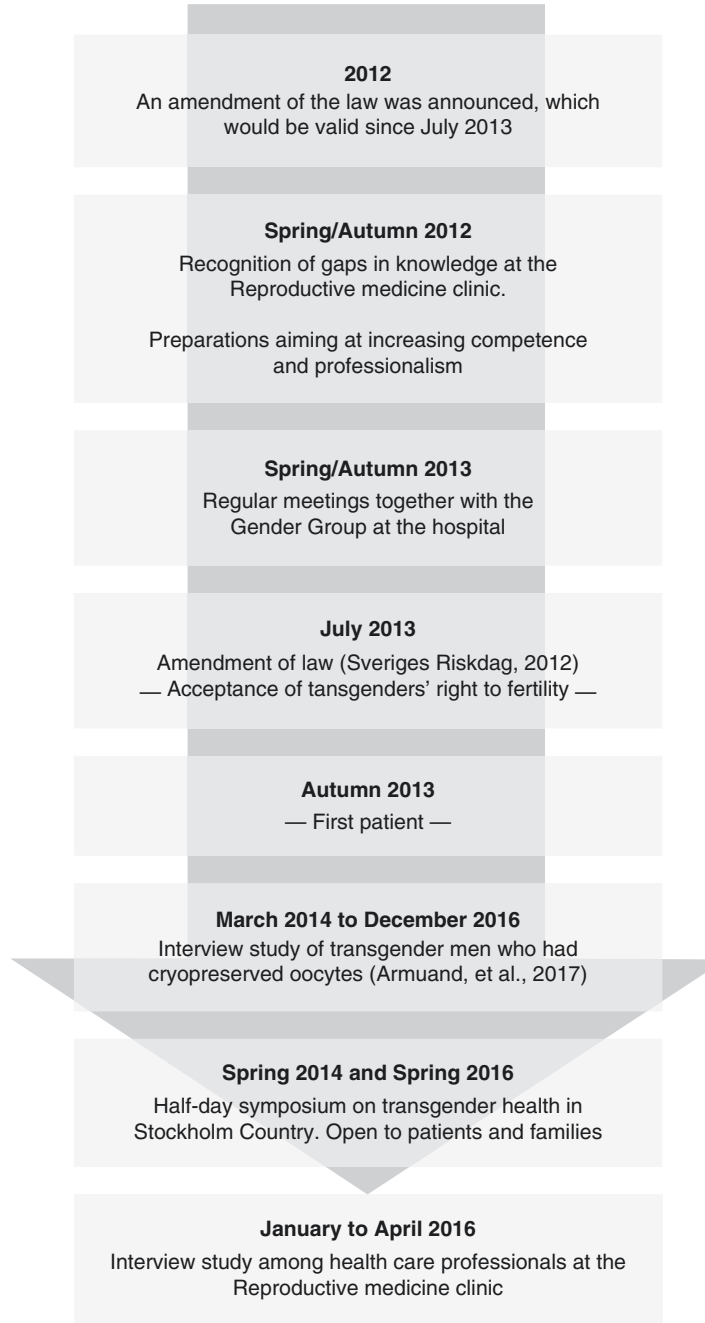

Fig. 1 Timeline of events leading up to the development of a fertility preservation programme for transgender individuals at Karolinska University Hospital, Stockholm, Sweden. Aiming at capturing individuals' experiences and achieving optimal care, a pilot research project involving patients and healthcare personnel was also created in parallel with the healthcare programme. (Reproduced with permission from Ref [14])

scheduled meetings together with the hospital gender team, developed the logistics and ensured continuity for the patients. Thereafter, when the clinical routines and experience were achieved, the knowledge and guidelines were shared with the larger group of healthcare providers at the centre.

To capture the patients' experiences, the framework suggested by INVOLVE [20] was used. A qualitative study was designed to investigate the patients' experiences through semi-structured interviews. The results of this work provided insights on the transgender male's vulnerable position in connection to fertility preser-

vation [15], and several improvements could thus be implemented in the programme.

How to Improve Fertility Preservation Information to Transgender Males

The importance of addressing the right pronoun was evidenced since the beginning of the work with this new patient group. It is important to ask the preferred pronoun early in the process and to document it in the patient chart to ensure that all personnel at the centre would use the right pronoun and the individual would feel recognized and respected by the fertility preservation team. The information about reproductive cells, and the organs that need to be evaluated, should be provided to transgender males in a sensitive way, considering their gender dysphoria. The words currently accepted by cisgender females may be offensive and should be avoided. For most transgender patients, the gender-neutral terms, such as patient, person and individual, are well accepted and should be preferred. Although transgender males may be interested in receiving information about the cells that could be potentially used in the future to achieve a pregnancy, i.e. the eggs, they usually do not wish to be reminded of the feminine association of eggs.

Fertility preservation for transgender males should include a complete description of the methods involved and a discussion of the expected efficacy and limitations of the methods available. Individuals that are referred for fertility preservation may have already initiated hormonal gender-affirming treatment. If these patients aim to cryopreserve eggs, it has been our policy to suspend the treatment and await for normalization of testosterone levels before starting gonadotropin stimulation [15]. Alternative methods such as the cryopreservation of ovarian tissue have been discussed also with the patients, but the need of re-transplantation of the tissue in the future has been regarded as a large limitation. Methods for in vitro follicle growth could be available in the future, but the plausibility of fully developed methods within a short delay is currently unlikely.

Illustrations for Counselling to Transgender Males and Information on Pelvic Exams

For transgender individuals that are interested in receiving information about egg freezing following hormonal stimulation, detailed information including the anatomical situation of the ovaries in the pelvis should be provided. This may appear obvious for healthcare personnel working with cisgender patients, but it is not for transgender males. Our reported experience with transgender males indicates that cisgender illustrations are not appropriate in the context of transgender people [15]. As follows of our study findings, specific illustrations have been produced at our centre for counselling of transgender males. The Fig. 2 illustrates the methods involved in fertility preservation aiming at egg freezing in pictures that do not remind of a feminine body. Figure 2a presents the situation of the ovaries in a body that could be interpreted as masculine. For egg retrieval, the use of transvaginal ultrasound has been standardized to evaluate and control follicle development during hormone stimulation aiming at obtaining eggs and to allow a safe and effective ovarian puncture (Fig. 2b). The procedures are usually performed under sedation and analgesia. People with gender dysphoria may have not undergone previously a vaginal examination or a transvaginal ultrasound exam. The need of vaginal exams using transvaginal ultrasound should be clearly explained. Some patients may feel discouraged or hesitant regarding undergoing vaginal exams during the first medical visit or may need repeated attempts to accomplish the exam.

Letrozole Supplementation During Controlled Ovarian Stimulation Aiming to Reducing Estrogenic Symptoms

For fertility preservation of transgender males aiming at egg freezing, we have used at our centre a stimulation protocol incorporating an aromatase inhibitor (letrozole) alongside gonadotropin stimulation. This protocol, initially developed for women with estrogen-sensitive

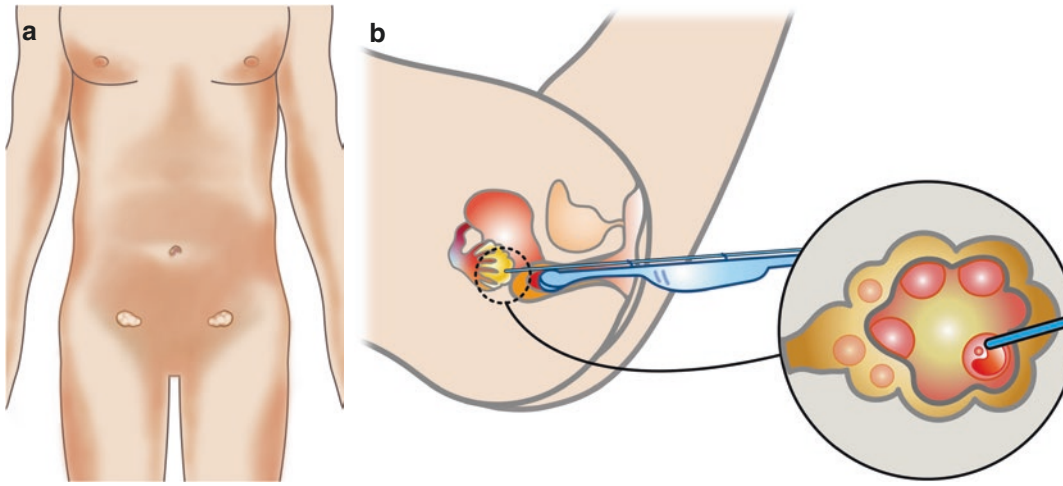

Fig. 2 (**a**) Illustration of the situation of the ovaries in the pelvis for transgender males. The figure has been included in a patient brochure on fertility preservation for transgender males. The illustration allows the conceptualization of ovaries in a male body. (Reproduced with permission from the Karolinska University Hospital, Stockholm, Sweden. Image provided courtesy of Dr. Rodriguez-Wallberg). (**b**) Retrieval through ultrasound-guided transvaginal follicle puncture. The figure has been included in a patient brochure on fertility preservation for transgender males. (Reproduced with permission from the Karolinska University Hospital, Stockholm, Sweden. Image provided courtesy of Dr. Rodriguez-Wallberg)

breast cancer aiming at fertility preservation, reduces significantly the systemic estradiol rise during stimulation and minimizes estrogenic side effects [21]. We have previously reported the use of this protocol in transgender males aiming at egg freezing with good acceptance [15, 22].

Healthcare Personnel Acting as Gatekeepers of Fertility Preservation and Lessons Learned

A large Australian study of 409 transgender and non-binary adults older than 18 years of age found that positive patients' experiences of FP were often associated to healthcare personnel that showed as professional and knowledgeable, providing affirming and caring services. Patients described negative experiences associated to healthcare professionals that acted mainly as gatekeepers of fertility preservation [23]. This Australian study clearly indicates that healthcare providers may act encouraging or discouraging towards fertility preservation [23]. The study discusses the WPATH Standards of Care guidelines published in 2011 [24] that do address the topic of fertility preservation generally but provides only brief information without covering specific issues.

The experience of our centre in Sweden indicates that continued education to ensure healthcare professionals' preparations to encounter a new patient group are needed. A major challenge towards optimal care is the attaining of good communication and confrontation with preconceived opinions and cis-normative assumptions [14]. For transgender men, it may be expected that the ovarian stimulation required and the transvaginal exams are likely to increase distress and gender dysphoria and the patients should receive information on this increased risk [15]. It is important that healthcare providers use non-gendered words as far as possible, such as "bleeding" or "pelvic examination" instead of "menstruation" or "gynaecological examination", and to use the right pronoun [15]. Information brochures should be specifically adapted to transgender patients to clearly explain the procedures needed and what to expect. Table 1 shows strategies of coping the distress of

Table 1 Overview of the main categories and subcategories identified by content analysis of individual in-depth qualitative interviews conducted with transgender men shortly after FP through ovarian stimulation and egg retrieval (from Ref [15])

The journey to FP	Reactions to the FP proceedings	Strategies for coping
Referral, assessment and diagnosis	Discontinuing the testosterone treatment to regain menstruation	Goal-oriented
A frustrating wait	Resumption of menstruation	Searching for support
Doubts and encouragement	The hormonal treatment	Changing the focus
	Becoming exposed by pelvic examinations and being seen by others	A cognitive approach
	Not as bad as anticipated	

fertility preservation procedures reported by transgender men [15]. These ways to handle the situation such as focusing on the goal, searching support from friends or relatives or using distractors during the exams should be discussed with the patients. Contextual sensitivity during FP procedures is important, and healthcare providers should have knowledge of transgender patients' vulnerable situation in connection to FP. With that knowledge, providers can help to reduce distress through their actions, or at least not increase it.

Conflict of Interest The author declares no conflicts of interest.

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Fertility Preservation in Turner Syndrome and Other Gonadal Dysgenesis

Julie Labrosse and Michael Grynberg

Introduction

Contrarily to men, no de novo gametogenesis occurs in women. Since their follicular stockpile cannot be replenished, the ovarian reserve irremediably declines in time. For instance, the initial supply of germ cells present during midgestation (up to seven million) has already decreased at birth. At the onset of puberty, only 300,000 germ cells remain. Follicular depletion occurs irrespectively of whether cycles are ovulatory and is known to accelerate at the age of 37–38 years old [1]. At the time of menopause, less than 1000 primordial follicles are left [2].

Besides this age-related physiological decline of the ovarian reserve, some women suffer from premature follicular loss or dysfunction, known as primary ovarian insufficiency (POI). POI is a particularly heterogeneous gonadic disorder that comprises a wide spectrum of etiologies such as infectious, inflammatory, cytogenetic, genetic, or iatrogenic causes. POI can be related to an insufficient initial pool of primordial follicles established in utero, an accelerated decline of the ovarian reserve, and/or to toxic or autoimmune follicular destruction [3]. Although the majority of POI remain unexplained, an increasing number of genetic diseases have recently been identified and could explain ovarian dysfunction. Notably, either structural or numerical sex chromosome anomalies can engender incomplete or defective gonadal development [4].

Turner syndrome (TS), also known as monosomy X, is the most common form of gonadal dysgenesis in females. TS affects 1/2500 girls and has been recognized as the most frequent genetic cause of POI [2]. Monosomy X karyotypes can be due to the partial or complete loss of one X chromosome in a 46,XX fetus or to the loss of a Y chromosome in a 46,XY fetus. TS is associated with short stature, skeletal deformities, a propensity for hearing deficits, as well as cardiac, renal, hepatic, and metabolic pathologies [5]. Patients with TS are also at higher risk of mental health conditions and social concerns compared to general population [6]. Most TS patients (50%) have a "classical" form of the disease, in which all cells are monosomic for X chromosome (45,X). Other forms of TS are referred to as "mosaic" TS, in which both normal and abnormal cell lines are found [7]. Mosaic TS include patients with 45,X/46,XX karyotypes (25%), 46,XX karyotypes with partial losses in one X chromosome, and other forms of structural

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anomalies of the X chromosome such as the presence of a ring X chromosome or of an isochromosome in the long arm of X chromosome. Y chromosome fragments are detected in 10–11% of cases [8].

The exact mechanisms underlying ovarian failure in TS patients remain to be elucidated. Physiologically, one copy of the X chromosome is inactivated in 46,XX females [9]. However, up to 15% of the genes on the silenced X chromosome escape inactivation and are therefore expressed by both chromosomes. Abnormalities observed in TS are thought to be caused by the haploinsufficiency of genes that are normally expressed by both chromosomes [9]. Haploinsufficiency of genes is known to contribute to advanced follicular atresia, primary amenorrhea, and infertility [10]. In TS, the main mechanism of follicular depletion presumably relies on an accelerated germ cell apoptosis that may start as early as 18 weeks of fetal life [11]. The primordial follicular pool might also be significantly lower from the start. TS comprises a variety of phenotypes and variable degrees of ovarian immaturity or dysfunction, ranging from streak ovaries devoid of follicles to patients with continuous follicular development leading to spontaneous puberty. Altogether, about two-thirds of patients with TS do not achieve spontaneous puberty and menarche. A majority of them require hormone therapy to initiate puberty [12]. Studies reported that TS patients with 45,X/46,XX mosaicism were more likely to experience spontaneous puberty compared to those with a complete monosomy 45,X, which suggests that the presence of functional ovarian tissue is correlated with the presence of 46,XX germ cells in the ovaries [13, 14]. Interestingly, a completely non-mosaic 45,X karyotype in peripheral blood leucocytes does not preclude the coexistence of 45,X/46,XX mosaicism in the ovary [2].

Despite the various comorbidities that may negatively impact their overall quality of life, TS patients consider POI and fertility issues as one of the greatest challenges that they have to face [15]. Similarly to other adolescent and young adult populations at risk of infertility such as cancer survivors, fertility concerns represent a central source of pain and hardship across their life span [15, 16]. Although up to 98% of them suffer from infertility [7], 97% of TS patients expressed the wish to conceive in a recent study [17]. The rare cases of spontaneous pregnancies are more likely found in patients with mosaic TS [18–20]. Faced with this reality, the significant improvements in the field of cryopreservation of female reproductive cells and tissue have raised the possibility of fertility preservation (FP) for these patients suffering from POI. Hereby, we discuss the different options for preserving female fertility in patients with TS and other forms of gonadal dysgenesis and the ethical questions raised by these approaches.

Gonadal Dysgenesis and Fertility Preservation: When and for Whom?

The possibility of preserving fertility for patients with gonadal dysgenesis has initially been raised by Abir et al. in 2001 [21]. However, the exact criteria on which the decision of FP should be based and the right timing remain to be established. The depth of ovarian dysfunction is highly variable from a patient to another and may start at a very young age. Hence, timely diagnosis of these pathologies is essential. This prerequisite can be challenging. Despite short stature which seems to be the general clinical characteristic of TS, all other clinical symptoms are inconsistent, even in individuals with non-mosaic 45,X karyotypes. Although some TS cases are diagnosed at birth (mostly those with a 45,X karyotype due to the presence of dysmorphic features or cardiac abnormalities), diagnosis of TS is sometimes delayed until adulthood, when it is already too late to preserve fertility.

The cornerstone of the decision to preserve fertility relies in a precise evaluation of the ovarian reserve and of the quickness of its decline. Unfortunately, reliable markers of the ovarian reserve for young patients are lacking. Before puberty, serum FSH and estradiol levels are low due to a physiologic state of hypogonadotropic hypogonadism. Although gonadotrophin levels tend to be higher in patients with TS, a significant overlap exists between them and other girls, notably during

mid-childhood [22]. In addition, ultrasonographic antral follicular count, which is probably the best marker of the ovarian status when performed transvaginally, is most often infeasible in young virgin patients. It is therefore performed by transabdominal imaging in most cases, which is less precise.

Described over the past decades as the most reliable hormonal marker of the follicular stockpile in adults, anti-Müllerian hormone (AMH) may also be a promising marker of ovarian function for patients with gonadal dysgenesis. AMH is specifically produced by granulosa cells of non-selected growing follicles. Serum AMH levels are known to strongly correlate with antral follicular count for women of reproductive age. Its age-related decline precedes the changes of traditional markers of ovarian reserve such as FSH, inhibin B, and oestradiol [22]. An AMH level < 3 pmol/L has been suggested as a cutoff value to identify TS patients suffering from premature ovarian insufficiency [23]. Measurable AMH levels in TS patients seem to be correlated to signs of spontaneous puberty such as breast development and menarche [23]. AMH levels have also been described as statistically significant predictive factors of the presence of follicles in the ovaries of TS patients [24]. Moreover, an association between the presence of measurable AMH levels and karyotypes has been described. A recent study including 270 TS girls observed that AMH levels were measurable in 77% of patients with mosaic TS versus in only 10% of patients with 45,X karyotypes [22]. Consistently, ovarian biopsies of TS patients with mosaic karyotypes were more likely to have follicles [24, 25]. However, AMH may be measurable in only a minority of TS patients and importantly fluctuates during childhood and adolescence [22, 26, 27]. Indeed, AMH levels are detectable at birth and transiently increase in infancy, before steadily increasing during childhood. Levels then peak during the final pre-pubertal year and slightly decrease at puberty, with a final peak in early adulthood [28]. Constructing age-specific reference intervals is difficult due to these physiological fluctuations and because of the variety of assays and differing interval width for age used in studies to measure AMH [29].

Since most adult women with gonadal dysgenesis have already established ovarian failure with high serum FSH levels at the time they wish to start a family, preserving fertility at a young age seems to be the best option [2]. However, major progress has to be made concerning timely management of these patients. Studies revealed that proper counseling is still lacking and that patients with a limited window of reproductive potential are not adequately guided [5]. A review of 469 medical records of TS patients receiving care from March 2013 to March 2018 showed that only 10% of patients had been referred to a fertility specialist on time [15]. Discussions should be comprehensive and consider the psychological impact of infertility, counseling on the pros and cons of fertility preservation, realistic risks of pregnancy, and the scope of family building beyond genetic children. Approaches vary greatly, depending on the patient's pubertal status, ovarian function, and degree of psychological maturity. Fertility-related discussions should also be part of routine care because parents often feel that they have insufficient knowledge to discuss fertility issues with their daughters. Altogether, given the complexity of the management of patients with gonadal dysgenesis, recent recommendations highlight the importance of a prompt referral to fertility specialists [5].

Fertility Preservation Options

Oocyte Cryopreservation After Ovarian Stimulation

Oocyte freezing after ovarian stimulation is the privileged option to preserve fertility of post-pubertal women [2, 30]. Ovarian stimulation consists in the exogenous administration of FSH during 10–15 days. High doses of recombinant FSH are usually required, ranging from 225 to 450 IU/day. For adolescents, luteinizing hormone supplementation can be administered on the day of gonadotropin-releasing hormone antagonist administration to ensure adequate steroidogenesis despite the relative immaturity of the hypothalamic-pituitary-ovarian axis. Oocytes are then

retrieved by transvaginal ultrasound-guided pickup and frozen. Cryopreservation by vitrification is now the standardized freezing technique. Recent progress have enabled significantly higher post thaw oocyte survival, fertilization, and pregnancy rates with vitrification techniques compared to slow freezing [31]. Furthermore, vitrified/warmed oocytes yield to similar results in terms of fertilization and pregnancy rates compared to fresh oocytes [31–33]. No increased risks of obstetric and neonatal complications, nor increase in chromosomal abnormalities or congenital anomalies, have been reported with cryopreserved oocytes [31].

Oocyte cryopreservation has been confirmed as a possible FP measure for patients with gonadal dysgenesis [34–38]. Published cases of oocyte freezing in TS patients performed so far are detailed in Table 1. The most recent one describes successful oocyte retrievals in seven TS patients undergoing controlled ovarian stimulation for fertility preservation, despite relatively low serum AMH concentrations [39]. Six out of the seven patients had a mosaic form of TS. A mean of 9 (±3.16 SD) oocytes were cryopreserved, which is comparable to the number obtained in patients without TS. Although not all young women undergoing these procedures were sexually active, all agreed to transvaginal oocyte collection under anesthesia. Treatments were well tolerated, and all patients recovered promptly after oocyte retrieval.

Despite these encouraging results, questions have risen on the fact that patients with gonadal dysgenesis may be poor responders to controlled ovarian stimulation. Failure of ovarian stimulation or low oocyte yield due to a lower number of FSH-sensitive follicles in these patients can indeed adversely affect the success of oocyte cryopreservation. In clinical practice, a trial to test follicular response to stimulation by gonadotropins can be performed, notably for patients with baseline FSH below 20 UI/L [2]. Furthermore, the possible detrimental effect of a sporadic increase in serum estradiol levels has been subject to debate for many years. In adolescent girls, high doses of estrogen may have a deleterious effect on growth. Although the expected impact of a five- to tenfold increased estradiol levels during a mean of 5 days is poor, robust data are still lacking to rule on this issue. The question of a possible deleterious effect of high estrogen levels concomitant to ovarian stimulation is notably relevant for TS patients for whom final height is a major concern. Recent protocols applied to breast cancer patients undergoing fertility preservation before potentially gonadotoxic treatments have used letrozole, an aromatase inhibitor, to help prevent an excessive rise of estradiol levels [16, 40]. Adjunction of letrozole to ovarian stimulation enabled the retrieval of mature oocytes while maintaining serum estradiol levels at normal ranges. It is conceivable that indications of such protocols might be extended to TS patients in a near future.

The quality of cryopreserved oocytes retrieved from patients with gonadal dysgenesis is also a subject of debate. Not all cryopreserved oocytes obtained might be suitable for fertilization or might develop with a normal karyotype. The possibility of genetic anomalies is increased compared to general population. This point might be added to a possible suboptimal competence of oocytes recovered in young patients. Indeed, although the rate of aneuploidy increases with age, relatively high rates of aneuploidy have been observed in patients under 25 years old [41]. The number of frozen oocytes required to ensure a realistic prospect of future pregnancy in patients with gonadal dysgenesis remains to be established. Data on post-warming oocyte survival is still lacking. In order to maximize the number of vitrified oocytes preserved, FP for patients with gonadal dysgenesis might also be considered not as a single attempt, but rather in a comprehensive strategy of repeated stimulations and accumulation of oocytes.

Recently, an algorithm has been established to help decide on the different FP strategies available for patients with TS. Indications were based on assessments of the ovarian reserve including the measurement of serum AMH [42]. The algorithm suggests that if the initial ovarian reserve is age-appropriate and serum AMH levels are higher than 2 ng/mL (lower quartile for girls aged 5–13 years of age), AMH can be controlled peri-

Table 1 Results of oocyte cryopreservation by vitrification in women with TS

	Peripheral blood karyotype	Age at ovarian stimulation	Baseline FSH (IU/L)	AMH (pmol/L)	Antral follicular count	Protocol	Total gonadotropin dose	Number of cryopreserved oocytes
Lau et al., 2009	45,X(98)/47,XXX (2)	16	6.3	NA	6	Mid-luteal GnRH-a downregulation by buserelin acetate followed by rFSH 450UI + hMG 150UI starting day 6	rFSH 4500UI + hMG 750UI	2
Balen et al., 2010	45,X(28/30)/46,XX(2/30)	28	3.3	43.8	NA	Antagonist protocol with stimulation by rFSH 150UI	rFSH 1350UI	3 cycles: 9;7;20
El-Shawarby et al., 2010	45,X(86)/47,XXX (11)/46,XX (3)	22	4.6	8.52	7	Short protocol with hMG 375UI starting day 3, increased to 450UI on day 5 and onwards	hMG 3450UI	8
Oktay et al., 2014	45,X(27/30)/47,XXX(3/30) 46,XX(11/20)/45,X(9/20) 46,XX(16/20)/45,X(4/20)	13 14 13	5.7 5.3 5.6	11.36 6.4–12.1 5.43	6 12 6	rFSH+rLH rFSH+hMG hFSH+rLH	2475UI + 150UI 1800UI + 450UI/3750UI + 2100UI 20125UI + 75UI	9 + 1IVM 2 cycles: 8;4 7 + 5(IVM)
Talaulikar et al., 2019	45,X(100) 45,X(83)/46,XX (17) 45,X (61)/46,XX (39) 45,X/46,XX/47,XXX 45,X (63)/46,XX (37) 45,X (50)/46,XX (50) 45,X(88)/46,XX (12)	22 18 18 25 21 22 26	6.9 3.2 7.4 2.9 6.2 8.4 7.5	3.5 3.05 7 9.5 3.5 21.3 12.1	7 9 11 12 5 14 11	Antagonist protocol with stimulation by hMG 225–450UI	3375UI 3375UI 3375UI 2025UI 675UI 2700UI 4725UI	9 13 9 10 4 6 12

GnRH-a gonadotropin-releasing hormone agonist, *rFSH* recombinant follicle-stimulating hormone, *hMG* human menopausal gonadotropin, *rLH* recombinant luteinizing hormone, *IVM in vitro* maturation

odically. If AMH levels do not significantly decline, FP by oocyte freezing should be performed at an appropriate age. If AMH levels importantly decline before there are any signs of puberty and that oocyte freezing is not feasible, ovarian tissue cryopreservation combined with in vitro maturation should be considered. In post-pubertal girls, oocyte cryopreservation should be privileged regardless of AMH levels. If the ovarian reserve is sufficiently high, ovarian tissue freezing as a supplement to oocyte freezing can be considered to preserve gonadal function and enhance chances of natural fertility [42].

The application of technologies such as pre-implantation genetic testing for aneuploidy (PGT/A) may enable a widespread screening of embryos for numerical chromosomal abnormalities. Although outcomes might be adversely affected by additional procedures such as PGT, advances in embryo culture have made embryo biopsy applicable at the blastocyst stage, and it is possible to biopsy a higher number of trophectoderm cells without impairing embryonic implantation potential [43]. Maternal chromosomal aberrations can also be detected by analyzing polar body biopsies of oocytes, which is an alternative to blastomere and trophectoderm biopsy [44]. Given the technological advances in oocyte and embryo screening, it is anticipated that at least a fraction of oocytes frozen from patients with gonadal dysgenesis should lead to successful pregnancies.

Cryopreservation of Oocytes Recovered During Natural or Modified Natural Cycle

Natural cycle protocols may be a promising alternative for poor responders to ovarian stimulation [45, 46]. Requiring no exogenous treatment by gonadotropin, natural cycle protocols can be performed in patients having ovulatory menstrual cycles to retrieve and freeze oocytes. Natural cycle procedures might yield oocytes of better quality, thus allowing the transfer of higher quality embryos into a more receptive endometrial environment [47]. However, since very few oocytes per cycle are retrieved using natural cycle, the main limit of this strategy is the number of cycles required to obtain a sufficient number of frozen oocytes [48]. Knowing that pregnancy rates after oocyte cryopreservation increase in parallel with the number of gametes preserved, using natural cycle prior to oocyte retrieval may slow down FP processes. Although low pregnancy rates have been reported using modified natural cycle IVF in patients over 35 years (presumably due to poor oocyte quality), it is conceivable that collecting many oocytes through recurrent natural cycles in young gonadal dysgenesis patients might be satisfying in terms of oocyte competence [2]. Hence, although natural cycle might not be ideal when fertility preservation has to be performed promptly (such as prior to cancer treatments), it may be considered as an option for patients with gonadal dysgenesis that are expected to respond poorly to ovarian stimulation.

In Vitro Maturation of Oocytes

In vitro maturation (IVM) has recently emerged in the field of female FP. IVM consists in retrieving immature oocytes from unstimulated small antral follicles. IVM applies to both pre- and post-pubertal patients, since cumulus-oocyte complexes either can be recovered transvaginally or from cryopreserved ovarian tissue [49]. After retrieval, oocytes are cultured in vitro from germinal vesicle to metaphase II stage and cryopreserved.

Since the number of oocytes retrieved at the germinal vesicle stage is strongly correlated to the number of antral follicles visible in the ovaries, IVM performed alone may not be suitable for young patients with genetic diseases due to their impaired ovarian reserve. For TS patients, IVM appears as a particularly interesting option when combined to ovarian tissue cryopreservation. A case report describes IVM performed at the same time as ovarian tissue cryopreservation in a 16-year-old TS girl with 20% 45,X0 and 80% 46,XX karyotype [50]. The technique consisted in aspirating every follicle visible on the

ovarian surface before performing ovarian tissue cryopreservation. Eleven immature germinal vesicle stage oocytes were found and taken for IVM. Eight oocytes matured satisfyingly and were cryopreserved by vitrification. The corresponding maturation rate was of 73%, suggesting that a high percentage of germinal vesicle stage oocytes can mature in patients with mosaic TS [50]. However, the relatively high number of preantral follicles and germinal vesicle stage oocytes identified may be related to the low percentage of 45XO karyotype (20%). It is possible that ovaries of patients with a higher percentage of 45XO karyotype or with a non-mosaic form of TS would contain fewer follicles. Finally, the pregnancy potential of in vitro matured oocytes obtained from ovarian tissue remains to be established.

Embryo Cryopreservation

Embryo cryopreservation is a well-established technique and has been used in fertility centers worldwide for the past 30 years in the management of infertile patients. Live birth rates obtained with cryopreserved embryos are similar to those obtained with fresh embryos [22]. Protocols and methods of oocyte retrieval are identical whether performed in an embryo cryopreservation or oocyte cryopreservation approach. However, embryo cryopreservation requires sperm from a partner or a sperm donor, which might limit its applicability as a relevant FP option for young patients and single young women. Moreover, frozen embryos may have to be discarded if there is a change in relationship status and the male partner does not consent to the use of frozen embryos.

Ovarian Tissue Cryopreservation

Cryopreservation of ovarian tissue is no longer considered an experimental procedure and is a promising FP option for young women with gonadal dysgenesis. At this time, it is the only FP strategy that can be offered to pre-pubertal girls and is the only procedure that can preserve both endocrine and reproductive ovarian functions. Ovarian tissue cryopreservation consists in the surgical removal of ovarian cortex fragments. It is most frequently performed by laparoscopy. The harvested ovarian tissue is then cryopreserved by promoting follicular viability, integrity of tissue compartments, and cell-to-cell contacts. The choice of cryoprotectant is specific to each cell and tissue type. It should ideally ensure maximum permeation capacity while minimizing the risk of toxicity and ice crystal formation. On the basis of current knowledge, the standard method for human ovarian cryopreservation is slow programmed freezing using a combination of permeating cryoprotectants such as propanediol, dimethyl sulfoxide, or ethylene glycol, with non-permeating substances such as human serum albumin-containing medium or sucrose [51].

Cryopreserved ovarian tissue remains viable during many years. The tissue can then be grafted orthotopically in its original site (in or onto the remaining ovary or into a peritoneal pocket in the pelvic peritoneum of the fossa ovarica) or heterotopically in other sites (subcutaneous tissue of the abdominal wall, forearm, or chest wall) [51, 52]. To date, thousands of young women have had their ovarian tissue cryopreserved for fertility preservation, and at least 360 transplantations of frozen-thawed ovarian tissue have been reported [53]. In addition, the first live birth obtained after transplantation of ovarian tissue recovered from a premenarchal girl has recently been reported [54]. Nevertheless, data is still lacking concerning the potential of pre-pubertal ovarian tissue after thawing and transplantation [55, 56]. Ovarian follicles of children and adolescents have been described as remarkably different from those of adult and as having specific histologic characteristics. Notably, pre-pubertal ovaries may contain a higher proportion of abnormal non-growing follicles and may have limited follicular growth compared to adult follicles [55].

Ovarian tissue transplantation provides a chance of natural conception, which may be a particularly interesting option for patients with gonadal dysgenesis and clinicians before considering assisted reproductive technology (ART). Although true efficacy remains to be established,

first reports observed that 30% of women having undergone ovarian tissue transplantation with a wish to conceive achieved a pregnancy [57]. No factor has been identified to be predictive of successful pregnancy after ovarian transplantation, possibly due to the heterogeneity of populations on which the technique was performed. Neither age at cryopreservation, age at first transplantation, the amount of tissue grafted, nor the follicular density of the grafted tissue was found to be predictive of successful conception. Cases of women becoming pregnant >5 years after transplantation have been reported [57]. On the other hand, the pool of follicles is largest immediately after transplantation, and the chance of reproductive success could thus be increased if ART treatment is initiated as soon as hormone levels have been restored.

Since the function of frozen-thawed ovarian tissue after grafting is suboptimal, an important question is the quantity of ovarian cortex to be withdrawn for cryopreservation. It seems that the quantity of ovarian tissue removed should be influenced by the expected probability of premature ovarian insufficiency [58]. After grafting, hypoxia and hyperactivation of follicular growth induce the substantial loss of primordial follicles and reduce oocyte quality after transplantation [2, 59]. Coagulation, which is sometimes necessary for hemostasis, may damage the ovaries. In the specific case of TS patients in which ovaries are generally small with a poor density of follicles, damage to the remaining cortex after coagulation may have a dramatic impact. For patients with gonadal dysgenesis, it is usually recommended to remove as much tissue as possible, typically an entire ovary [16].

Current limitations of ovarian tissue freezing and transplantation may be accentuated for TS patients. First, ovarian tissue cryopreservation might be less appropriate due to their reduced ovarian reserve from the beginning. Ideally, ovarian ischemic damages after transplantation should be counterbalanced by a large initial ovarian reserve in the cryopreserved tissue. Since primordial follicles are the most resistant to ischemic damage after grafting, the chance of restoring fertility seems to be related to the number and quality of follicles endowed within the transplanted cortical tissue. The number and density of follicles in ovarian cortical tissue were assessed in adolescent girls with TS, who had had a quarter to one whole ovary removed for fertility-sparing procedures [25]. Eight of the nine tissues contained follicles. Primordial follicles were observed in both mosaic and non-mosaic TS patients. Follicle density was correlated with serum levels of FSH, as patients with the lowest FSH levels had the highest follicular density. One to 190 follicles were found in approximately 0.1–2.0 mm^3 of tissue analyzed, which resulted in a density of 1.5–499 follicles/mm^3 of ovarian cortical tissue. Younger girls and those with mosaic karyotypes had the highest number of follicles. Consistently, most live births with ovarian tissue transplantation are from women whose ovarian tissue was frozen before the age of 25, when oocyte quantity and quality are relatively satisfying. It is likely that TS patients with higher degrees of mosaicism will have a better chance of success after ovarian cryopreservation and transplantation.

Despite these limitations, ovarian tissue freezing and transplantation may be a very promising approach in the future. Since the performance of the first successful orthotopic ovarian transplantation in 1999, surgical methods for post-transplantation follicle survival and success have remarkably evolved. The use of robotic surgery combined with a neovascularizing human extracellular matrix scaffold could dramatically improve post-transplantation results. Another approach that has been tested in human ovarian xenograft models is sphingosine-1-phosphate (S1P), a ceramide-induced death pathway inhibitor known to have vasculogenic properties. Continuous infusion of S1P in human ovarian xenografts accelerated neovascularization, reduced tissue ischemia, and maintained primordial follicle density at levels similar to pretransplant measurements. Although S1P has never been tested in a clinical setting, a synthetic analog currently in use for the treatment of multiple sclerosis may be considered in future clinical trials. Recently, focus has been placed on the phosphatase and tensin homolog (PTEN)/

phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/forkhead box O3 (FOXO3) and Hippo signaling pathways. Akt stimulators (PTEN inhibitor and PI3K activator) are known to activate dormant primordial follicles in vitro, and ovarian fragmentation disrupts the Hippo signaling pathway, leading to the promotion of follicle growth [60]. In vitro activation by culturing ovarian tissue with Akt stimulators has been suggested as an option to potentialize results in POI patients [61, 62]. In general, improving the survival rates of primordial follicles after ovarian tissue transplantation may enlarge the indications of ovarian tissue cryopreservation not only for TS girls but also in other clinical scenarios where the initial ovarian reserve is low. This may also enable to use smaller amounts of tissue from younger patients and to increase the prospects for elective ovarian tissue cryopreservation without significantly compromising the remaining ovarian reserve.

Pregnancy in TS

Due to the numerous medical comorbidities associated to TS, pregnancies of TS women are at higher risk compared to general population [63]. These obstetric risks are present irrespective of whether the pregnancy was obtained naturally or medically and irrespective of the origin of the gametes (whether autologous or heterologous). Potential pregnancy complications are notably related to cardiac, renal, and other medical conditions. Specifically, pregnancies in women with TS are more likely to be complicated by diabetes, hypertension, thyroid dysfunction, low birthweight or intrauterine growth restriction, and preterm birth [16, 64]. Congenital heart disease occurs in up to 50% of girls with TS, including a high incidence of bicuspid aortic valve, coarctation of the aorta, and underlying vasculopathy that can lead to rare but often life-threatening dissection of the aorta. Several cases of aortic dissections and deaths in pregnant women with TS have been reported [65]. A Swedish national registry of 124 childbearing women with TS karyotype also identified a high rate of circulatory and endocrine diseases, as well as cases of aortic aneurysm [66]. Nonetheless, no maternal deaths during a 10-year follow-up period were reported. Overall, maternal mortality in women with TS has been reported to be as high as 1–2%, which is 100–200 times greater than in the general population [2].

Currently, no sufficient data are available to adequately counsel women with TS having a wish to conceive. Modalities of screening, supervision, and long-term follow-up have to be determined. The 2012 American Society for Reproductive Medicine Practice Committee Opinion states that TS in itself is a *relative* contraindication to pregnancy and an absolute contraindication in the presence of "any risk factor or significant abnormality" [67]. The definition of "significant abnormality" remains unclear. It seems that all women with TS need to undergo an intensive pre-pregnancy health screening and that pregnancy and postpartum should carefully be handled by a multidisciplinary team.

Recent recommendations prioritize preconception cardiac evaluation including measurement of the aortic size index for all TS women with a short-term parental project. Imaging of the thoracic aorta and heart by transthoracic echocardiography and cardiac magnetic resonance scan should be performed within 2 years before planned pregnancy or ART [5]. Women with aortic dilatation, bicuspid aortic valve, elongation of the transverse aorta, coarctation of the aorta, and/or hypertension should be advised that pregnancy carries a high risk of aortic dissection. An ascending aortic size index of >2.5 cm/m^2 or an ascending aortic size index 2.0–2.5 cm/m^2 with associated risk factors for aortic dissection (which include bicuspid aortic valve, elongation of the transverse aorta, coarctation, and hypertension) should be considered as important arguments against the occurrence of a pregnancy, whether obtained spontaneously or by ART. If aortic dilatation or other risk factors are absent, follow-up during pregnancy should optimally include at least one cardiac evaluation, at approximately 20 weeks of gestation. It is also important to maintain strict control of blood pressure (135/85 mmHg) in all

pregnant women with TS. Exercise testing before pregnancy can also be useful to reveal exercise-induced hypertension, notably for patients with coarctation of the aorta. Delivery by cesarean section is recommended, particularly for patients with a history of aortic dissection. The higher risks of maternal and neonatal complications in case of multiple pregnancies are increased for patients with gonadal dysgenesis. The risk of aortic dissection is fivefold higher in multiple pregnancies compared to singletons. Hence, recommendations present single embryo transfers as the unique possible embryo transfer strategy in these patients.

Furthermore, spontaneous pregnancies in women with TS are associated to an elevated risk of miscarriage and chromosomal abnormalities in the offspring [19, 20, 68]. Consistently, early reports relate an excess of miscarriage, fetal malformations, and chromosomal defects such as trisomy 21 in spontaneous pregnancies obtained by patients with TS [69, 70]. Consistently, a large national cohort analyzing 480 TS patients reported a significantly higher spontaneous miscarriage rate compared to general population (30.8% versus 15%, respectively) [19]. Among the 17 daughters derived from this cohort, 2 were diagnosed with TS. In addition to an increased production of gametes with chromosomal anomalies, these higher pregnancy loss rates could be explained by a relatively smaller uterine size and/or reduced endometrial thickness and receptivity [21, 71, 72]. The higher prevalence of autoimmunity disorders in patients with gonadal dysgenesis could also contribute to an unfavorable uterine environment [21].

Altogether, the importance of balancing FP with the underlying risks related to pregnancy appears essential. Pregnancies of patients with gonadal dysgenesis should be handled with extreme caution. Optimal cohesion between the patient and the different medical actors is crucial and even more so since reports show that patient care in this context is highly insufficient. Indeed, a recent multicentric retrospective study evaluating the application of French guidelines on the management of pregnant TS patients leads to very unsatisfactory results [73]. The analysis included 103 pregnant TS patients (spontaneously or by ART) between January 2006 and July 2017. A total of 170 pregnancies were reported: 35 spontaneous, 5 by means of ART using their own oocytes, and 130 with oocyte donation. The study revealed that guidelines were very poorly respected, as one in four patients had no preconceptional assessment and no cardiologic follow-up during pregnancy. Postpartum cardiac ultrasonography was performed for 45% of pregnancies, but only 11% were performed within 8 days postpartum.

Ethical Concerns

Preserving fertility of patients with gonadal dysgenesis raises numerous ethical considerations. The primary objective of fertility preservation procedures is to improve the psychosocial well-being of individuals and enhance the possibility to have biological children. However, the strong yearnings for a biologic child must be counterbalanced with the associated risks, difficulties, and uncertainties. The desire to bear one's own genetic child does not in itself establish its legitimacy. Three prominent points are subjects of debate: insufficient evidence of the efficacy of fertility-sparing procedures for this specific category of patients, risks associated to pregnancy, and risks of chromosomal anomalies in children [58].

So far, FP has essentially been discussed for cancer patients. The benefit of preserving fertility before cancer treatments seems legitimate since chemotherapy and/or pelvic radiation can be highly gonadotoxic and dramatically damage ovaries that were initially sane and normally functioning. The benefit is not as clear-cut for patients with gonadal dysgenesis. Cryopreserved oocytes retrieved from patients with gonadal dysgenesis may be of poor quality and poor candidates for fertilization from the start. Data are lacking concerning ovarian tissue cryopreservation. The invasive character of each technique and the burden associated to these procedures (injections and/or surgery) have to be counterbalanced with potential risks and yet unproven ben-

efits. Patients might put excessive hope in the possibility of FP, while relatively low success rates have been reported.

One of the major ethical questions is the age at which fertility-sparing procedures should be performed. Evidence suggests that FP has to be discussed promptly, as early as during childhood/adolescence. Although ovarian follicles are lost from gestation onwards, there is uncertainty about the optimal age for removal and cryopreservation. There may be a trade-off between the odds of success and the prospect of meaningful assent. In particular, young patients may not have the psychological resources to make their own decision. Discussions should consider each patient's degree of psychological maturity. As in any medical intervention, parents also have to be counseled with respect and sensitivity. Parents often play an important role and are in most cases the major source of support. However, their position is delicate since staying neutral may be challenging. Indeed, parents can be influenced by the social stigma of infertility, their personal desire for their daughter to have biological children, and their own potential fear of not having a biological grandchild [74]. In virgin adolescents, parents and/or the patient may have difficulty to accept the fact that oocyte retrieval is performed transvaginally. Accepting fertility preservation with transvaginal procedures collides with individual feelings and the burden of cultural weight.

Because evidence indicates that fertility preservation should be performed early in life, the possibility of a patient undergoing preservation and later choosing not to utilize the cryopreserved material (whether oocytes or tissue) is significant. Although it is a personal choice, the decision to spare fertility is reinforced by powerful social and cultural expectations about motherhood. At an adult age, patients having preserved their fertility might realize that their life goals do not meet that of parentality and family building. Although some patients may be grateful of the decision to preserve a possibility of genetic motherhood, others may feel pressured to procreate when they otherwise might prefer a life without pregnancy or child-raising.

Other Options

Egg Donation

Until recently, oocyte donation was the only reproductive option for patients with gonadal dysgenesis experiencing ovarian failure. Clinical pregnancy rates per embryo transfer in women with TS vary between 16 and 40%, which is similar to that of other oocyte donation recipients [5]. However, despite some successful pregnancies obtained, ongoing pregnancy rates are statistically significantly lower in TS patients when compared with matched, non-TS patients having received an oocyte [75]. A review of 23 women with TS following egg donation reported a miscarriage rate of 44% and take-home baby rate of 18% per transfer [76]. Consistently, a 33–60% pregnancy loss rate has been a constant finding in most series analyzing TS patients who have undergone oocyte donation. Possible mechanisms could be related to uterine hypoplasia/hypovascularization or some inherent endometrial receptivity defect. Other factors such as the higher rate of autoimmune disorders might also play a role. Adequate hormonal replacement therapy is thought to be of utmost importance to reduce pregnancy losses in patients with gonadal dysgenesis after oocyte donation. A retrospective cohort study lead in three Nordic countries (Finland, Denmark, Sweden) between 1992 and 2011 including 106 women with TS having delivered after oocyte donation reported 122 deliveries and 131 newborns [77]. Neonatal outcomes were reassuring, with similar rates of preterm birth and low birthweight as after conventional ART procedures.

Gestational Surrogacy and Adoption

Gestational surrogacy has become an accepted option for couples experiencing fertility issues. Gestational surrogacy consists in the planned pregnancy of a woman carried on behalf of another woman. Women with certain medical conditions for whom having a pregnancy would be at high risk but for whom long-term prospects

for health are good can be considered as potential candidates for surrogacy. Patients with gonadal dysgenesis present various comorbidities. Complications are exacerbated during pregnancy, and even patients with a normal cardiac evaluation before pregnancy may be at risk for cardiovascular complications that arise during the third trimester of pregnancy or postpartum. Hence, in this specific context, gestational surrogacy seems to be both a reasonable and advisable alternative in countries where it is legal.

The American Society for Reproductive Medicine recommends that all patients with TS should be counseled about resorting to gestational surrogacy and adoption as alternatives to pregnancy [67]. Two distinct types of surrogates can be considered. In most cases, the surrogate gestational mother (gestational carrier) carries the pregnancy without providing the genetic component for reproduction. In sporadic cases, the surrogate mother provides both the genetic and gestational component for reproduction (true surrogacy). Gonadal dysgenesis patients can also use autologous oocytes for in vitro fertilization if the ovarian reserve can enable the retrieval of fresh oocytes of quality, or if oocytes had previously been cryopreserved. The use of autologous oocytes provides an opportunity to these women to be biological parents. Alternatively, gestational surrogacy can serve to carry pregnancies resulting from the use of donor oocytes or embryos, if needed.

Although few data exist on these issues, the stigma associated to patients with gonadal dysgenesis may make it more difficult for these women to adopt. Nonetheless, adoption is another viable option for women with gonadal dysgenesis who desire to be parents, without taking the risks of increased maternal and fetal complications.

Conclusion

Fertility preservation in patients with gonadal dysgenesis is highly challenging. Determining the best strategy to manage these patients and the adequate indications of fertility-sparing techniques is complex. The present challenge is to identify potential candidates to fertility preservation procedures as early in life as possible to enable timely management. Future advancements in the field of genetics are expected to enhance the identification of these patients at an earlier age. Given the remarkable lack of data on the competence of frozen gametes, patients and their family should be aware that results of fertility preservation in these situations remain to be documented. Discussions on the risks of genetic transmission and risks of pregnancy are mandatory. Beyond the desire of becoming genetic parents, other alternatives such as egg donation, gestational surrogacy, and adoption also have to be discussed.

It is possible that the biggest discoveries and solutions to premature ovarian insufficiency in patients with gonadal dysgenesis will come from improved understanding of the mechanisms of accelerated primordial follicle loss. If these mechanisms are understood, future targeted treatments to prevent accelerated ovarian aging could be developed. In the meantime, as the optimal age at which fertility should be preserved remains to be determined and is presumably patient-dependent, ensuring timely referral of potential candidates to fertility preservation techniques towards specialists is crucial. Promoting multidisciplinary approaches and the creation of strong networks of fertility experts will enable the safest and most successful management of these patients.

Definitions

- **Premature ovarian insufficiency:** the presence of primary or secondary amenorrhea >4 months, occurring before the age of 40 years old, with low estradiol levels (<50 pg/mL) and high gonadotropin levels (FSH > 25 IU/L, determined by two measurements obtained at least 4 weeks apart).
- **Gonadal dysgenesis:** the defective embryonic development of gonads.
- **Turner syndrome (TS)**, *aka* **monosomy X**: the partial or complete loss of one X chromo-

some in a 46,XX fetus or the loss of a Y chromosome in a 46,XY fetus. There is a "classical" form of the disease, in which all cells are monosomic for the X chromosome (45,X), and a "mosaic" form, in which both normal and abnormal cell lines are present.

Practical Clinical Tips

- The depth of ovarian dysfunction is highly variable in patients with gonadal dysgenesis and may start at a very young age. Timely diagnosis is essential.
- Approaches should consider the patient's pubertal status, ovarian function, and degree of psychological maturity.
- In clinical practice, a trial to test follicular response to stimulation by gonadotropins can be performed, notably for patients with baseline FSH below 20 UI/L.
- Fertility preservation for patients with gonadal dysgenesis might be considered in a comprehensive strategy of repeated stimulations and accumulation of oocytes in order to maximize the number of vitrified oocytes preserved.
- Due to the numerous associated medical comorbidities, pregnancies of TS women are at higher risk compared to general population and should be handled with extreme caution.

Take-Home Messages

- TS patients consider fertility issues as one of the greatest challenges that they have to face. Up to 98% of them suffer from infertility.
- Oocyte cryopreservation has been confirmed as a possible FP measure for patients with gonadal dysgenesis.
- IVM performed alone may not be suitable for patients with genetic diseases but is a particularly interesting option when combined to ovarian tissue cryopreservation.
- Although still experimental, cryopreservation of ovarian tissue provides a chance of natural conception and is the only strategy that can be performed in pre-pubertal girls and preserve endocrine ovarian function.
- The psychological impact of infertility and wish to conceive have to be balanced with the insufficient evidence of the efficacy of FP procedures in case of gonadal dysgenesis, the risks associated to pregnancy, and the risks of chromosomal anomalies in children.

Clinical Case

A young TS patient of 14 years old underwent FP procedures. She had a mosaic form of TS, an AMH level of 2.1 ng/mL, and an antral follicular count of 21. Ovarian stimulation was performed by an antagonist protocol using 300 IU of recombinant FSH from Day 1 to Day 6. At Day 6, E2 level was 1566 pg/mL. The gonadotropin dose was lowered to 187.5 IU from Day 6 to Day 9. The antagonist was introduced at Day 6 until Day 9. Ovulation was triggered by recombinant HCG at Day 10, with E2 levels at 1313 pg/mL. Oocyte retrieval was performed 36 h after trigger. Twenty oocytes were retrieved and cryopreserved.

Key Readings

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Follicle Activation by Physical Methods and Clinical Applications

Melody Devos, Isabelle Demeestere, and Johanne Grosbois

Introduction

The ovarian reserve is defined as the pool of follicles available to provide egg cells capable of fertilization throughout the reproductive life span of women and is generally represented as the population of quiescent primordial follicles. Some of these dormant follicles are continuously recruited into the growing pool via a process called primordial follicle activation, supplying the ovary with its population of growing follicles. This process starts early in life during the prepubertal period, but ovulation of the selected growing follicles occurs only from menarche through the end of a woman's reproductive life. The number of primordial follicles is established in utero, and the population of dormant follicles naturally and gradually declines with age. Menopause occurs once this supply has been almost exhausted (<1000 follicles) [1].

The incidence of female infertility has risen worldwide in recent decades [2]. One of the main reasons is the social trend towards delayed childbearing, which is associated with reproductive aging and diminished ovarian reserve (DOR). DOR is associated with a loss of normal reproductive potential that occurs as women get older due to reductions in oocyte quantity and quality. In contrast, women suffering from premature ovarian insufficiency (POI) have a complete loss of ovarian function before the age of 40 due to a premature exhaustion of the ovarian reserve. POI is defined by ESHRE guidelines as amenorrhea for at least 4 months and abnormal FSH levels measured at least twice (FSH > 25 IU/L) [3]. POI patients display a shortened reproductive life span and eventually experience infertility. The clinical need for effective approaches in women with DOR or POI is high due to the fact that alternative infertility treatment options beyond oocyte donation do not currently exist. Importantly, ovaries from DOR and POI patients often contain residual dormant follicles, and when these women achieve pregnancy, the offspring are healthy [4, 5]. Therefore, one of the main challenges of reproductive medicine is to find a way to rescue these residual follicles in order to offer these women the possibility of having their own 'genetic' progeny.

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In recent years, a new approach, called in vitro activation (IVA), has emerged as a potential infertility treatment [6, 7]. IVA relies on the use of physically disruptive procedures to activate the residual follicles from POI and DOR patients and boost their growth to maturity. Implementation of IVA in a clinical setting has shown promising results and has successfully led to the live birth of several babies [6, 8–15]. However, the safety and efficacy of the procedure remain unclear, and more basic and pre-clinical studies are required before implementation into clinical practice based on the results of experimental studies. In this chapter, we discuss the emergence of physically disruptive procedures to promote ovarian follicle activation and growth, debate their clinical relevance and limitations as an infertility treatment, and identify future work that may help to refine current IVA protocols.

Regulation of Primordial Follicle Activation

The primordial follicle is the first follicle stage formed by an oocyte meiotically arrested at the diplotene stage of prophase I surrounded by a single layer of flattened granulosa cells (GCs) [16]. This elementary unit composes the non-renewable reproductive stockpile of each woman, established at birth, and is localized to the cortical area of the ovary. The total number of primordial follicles in the ovary reaches a maximum of seven million during prenatal life, but only around one million remain at birth. Although most of these follicles are dormant, with low transcriptional and translational activity, a few are continuously recruited into the growing pool before and during the reproductive life span, irrespective of menstrual cycles or the hormonal environment [17]. Activated primordial follicles progress into primary follicles that are characterized by one layer of cuboidal GCs surrounding the oocyte. Primary follicles may further develop through the proliferation and the expansion of GCs to reach the secondary then antral stage and finally be selected as a dominant follicle and achieve nuclear maturation. However, most germ cells are lost by atresia and not selected to grow or are subjected to atresia during the growing process, and it has been estimated that less than 0.1% of them will reach ovulation [18]. Therefore, only around 400,000 follicles remain at puberty, and menopause occurs when the stockpile reaches approximately 1000 follicles. During reproductive life, the different morphological and functional follicular stages to reach competence for fertilization occur in an environment of balanced molecular signalling until the secondary stage which is governed by cyclic gonadotropin stimulation through the preovulatory stage. Due to their lack of receptors [19] and limited vascularization, primordial follicle recruitment occurs independently of pituitary gonadotropin control and is highly regulated by local factors including intra-follicular signalling pathways and environmental signals (reviewed in [20]).

The PI3K/Akt/mTOR Signalling Pathway

The first major signalling pathway that was found to govern follicle activation is the phosphoinositide 3-kinase (PI3K)/AKT and mammalian target of rapamycin (mTOR) signalling pathway. Initiated by different cell growth factors, such as insulin and insulin-like growth factor (IGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and Kit Ligand, PI3K/AKT/mTOR is regulated by phosphorylation and is well known to coordinate essential cellular processes including proliferation and survival. Ligand-receptor binding activates PI3K, leading to its phosphorylation of membrane phosphoinositides, a reaction that is reversed by phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Fig. 1a). This process activates basal signalling, inducing phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) activities on AKT. After phosphorylation, AKT is fully active and monitors several effectors promoting cell survival and cell cycle entry such as forkhead box O3 (FOXO3) protein, Bad, tuberous sclerosis com-

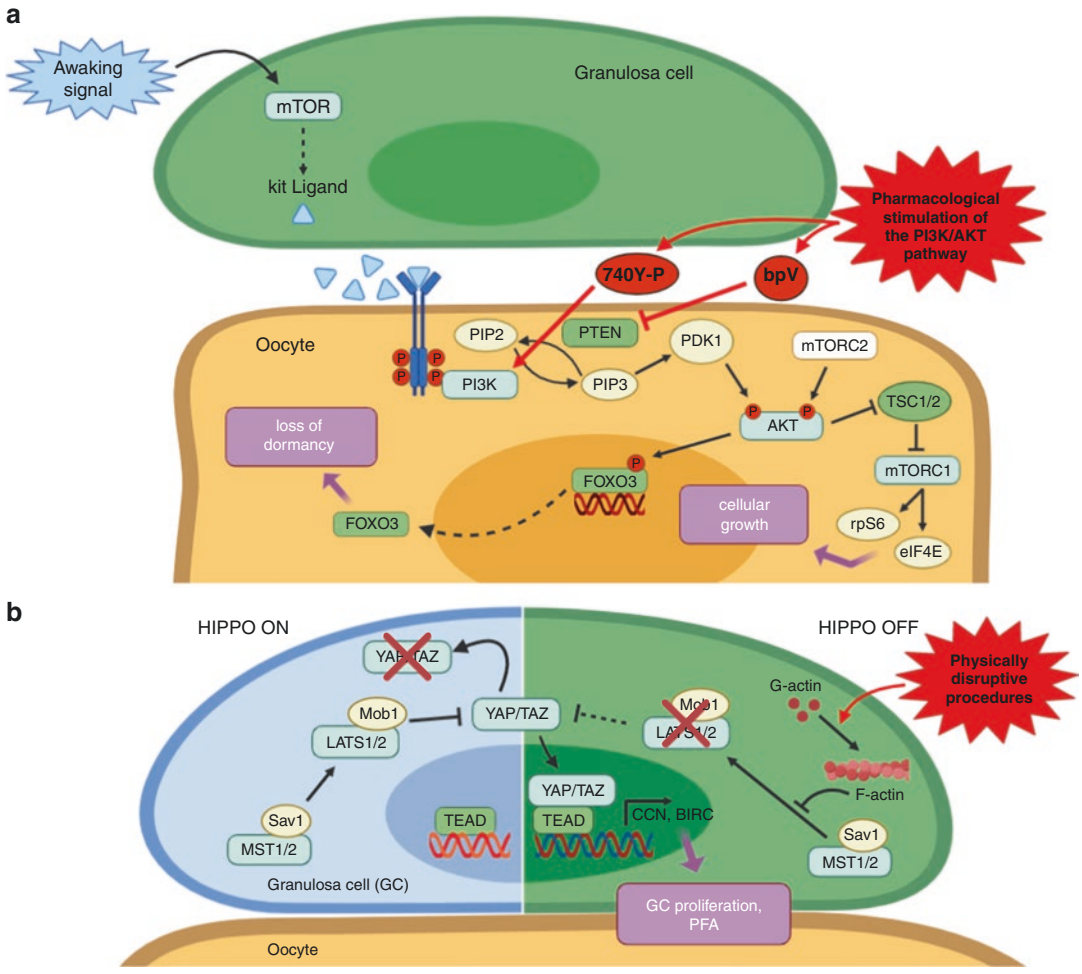

Fig. 1 The PI3K/AKT and Hippo signalling pathways regulate primordial follicle quiescence and entry into growth. (**a**) GC induction of mammalian target of rapamycin (mTOR) leads to the secretion of Kit ligand which binds its c-KIT receptor on oocytes, triggering the phosphoinositide 3-kinase (PI3K) pathway. Activation of the AKT pathway induces the exclusion of forkhead box O3 (FOXO3) from the nucleus and activation of the mTOR pathway to promote primordial follicle activation (PFA). Drug IVA relies on the use of pharmacological drugs that activate the PI3K/AKT cascade to promote follicle activation and growth. (**b**) Under basal conditions (Hippo ON), activated large tumour suppressor homolog 1/2 (LATS1/2) induces Yes-associated protein (YAP) and transcriptional co-activators PDZ-binding motif (TAZ) exclusion from the nucleus by phosphorylation and sequestration in the cytoplasm. Physically disruptive procedures trigger a switch in the G-actin/F-actin ratio, resulting in the inhibition of LATS1/2 activity. YAP/TAZ becomes hypophosphorylated and can translocate into the nucleus to induce TEAD-mediated transcriptional activity of target genes such as connective tissue growth factors (CCN) and baculoviral IAP repeat containing (BIRC), leading to granulosa cell proliferation and primordial follicle activation. (Adapted from [20] with permission). Figure created with BioRender

plex 1/2 (TSC1/2), and Cdk inhibitor p27 [21]. Of interest, FOXO3 phosphorylation by AKT induces its nuclear exclusion and loss of its inhibitory signal initially responsible for follicle dormancy. This function has been reported in knockout mouse model experiments on the PI3K pathway and was further confirmed in patients affected by premature ovarian insufficiency who have been reported to have FOXO3 mutations, identifying FOXO3 as a potential candidate gene responsible for follicle depletion [22, 23]. Besides its nuclear activity, AKT also inhibits the TSC1/2 complex leading to

mTORC1 activation. This other important protein complex regulates two main effectors, 40S ribosomal protein S6 (rpS6) and eukaryotic translation initialization factor 4E (eIF4E), acting directly on protein synthesis and cellular growth [24]. Genetic deletions in mouse models have highlighted the impact of the TSC/mTOR axis. Lack of TSC1/2 results in an increase in mTORC1 activity leading to massive follicle activation [25]. These results were confirmed in human transcriptomic analyses in which primordial follicle activation has been correlated with an enrichment of PI3K/AKT and mTOR signalling [26]. Follicle recruitment is thought to arise from a dialog between GCs and the oocyte through Kit ligand-KIT receptor (c-KIT) interactions [16]. The awakening signal is first perceived by the GCs and processed through the activation of the mTOR cascade in these cells, leading to the secretion of Kit ligand. Kit ligand then binds to its receptor, c-KIT, at the oocyte surface, which activates the PI3K/AKT/mTOR pathway in the oocyte and eventually ensures coordinated oocyte growth with cuboidalization and proliferation of its surrounding GCs.

The Hippo Signalling Pathway

Although the Hippo pathway is known to be integrated into the cellular structural environment, its role during physiologic follicle awakening remains to be established. Highly conserved, the Hippo pathway controls organ size and includes, among others, the negative growth regulators mammalian Ste-20-like kinase 1/2 (MST1/2) and large tumour suppressor homolog 1/2 (LATS1/2). Under basal conditions, activated LATS1/2 induces Yes-associated protein (YAP) and transcriptional co-activators PDZ-binding motif (TAZ) exclusion from the nucleus by phosphorylation and sequestration in the cytoplasm (Fig. 1b). Disruptions in cell contacts or actin polymerization disturb the Hippo pathway, leading to YAP/TAZ translocation into the nucleus where they interact with the TEAD transcription factors and promote the transcription of proliferation genes, such as connective tissue growth factor (CCN) and baculoviral IAP repeat containing (BIRC). Due to permanent waves of follicular development, ovulation, corpus luteum formation, and regression, the ovarian stroma is perpetually remodelled and undergoes major structural changes during each reproductive cycle. The Hippo pathway is thought to regulate the size of the ovary as in other organs. Moreover, several proteins in this pathway have been found to be expressed in follicles from mouse and human ovaries [6, 27, 28]. Mouse model experiments including gene deletions have further described a direct link between the Hippo pathway and follicle activation [20], while in humans, polycystic ovarian syndrome, which is characterized by enlarged ovaries containing an increased density of small preantral and early-growing follicles, has been associated with aberrant Hippo signalling [29].

In parallel to the PI3K/AKT/mTOR and Hippo signalling pathways, follicle activation has also been reported to be influenced by other signalling networks, such as the transforming growth factor-β (TGFβ)/mothers against decapentaplegic homolog (SMAD) [30–32], Janus kinase (JAK)/signal transducer and activator of transcription (STAT) [33], and the mitogen-activated protein kinase (MAPK) pathways [34–37]. Follicle activation is also influenced by several secreted factors derived from stromal cells, neighbouring follicles, and the blood supply, such as growth factors, cytokines, and hormones (reviewed in [20]). While each of these pathways has been described as directly or indirectly involved in the follicular activation process, the comprehensive view of cell signalling should be considered without neglecting the potential interactions between them, forming an intricate global network.

Development of the IVA Approach

A better understanding of the factors governing follicle recruitment is essential to improving current infertility treatments. Despite the progress that has been made in the past few decades, the field still struggles with many challenges, such as poor response to stimulation, in vitro fertilization

(IVF) failure, and resistant ovary syndrome (ROS) observed in POI patients [7]. Harnessing the reproductive potential of the residual follicles present in the ovaries of POI and subfertile patients can offer new possibilities for fulfilment of their desire to achieve motherhood.

The benefit of physically disruptive procedures for promoting follicle growth and maturation was first reported in 1935 by Stein and Leventhal as a treatment for anovulatory PCOS patients. By hypothesizing that mechanical forces generated by cysts were the cause of amenorrhea and sterility, they developed a protocol involving the resection of from 1/2 to 3/4 of the ovary to decrease the internal pressure on the healthy remaining follicles, the first ovarian wedge resection procedure. The seven patients included in their study resumed menses after surgery, and one live birth was reported [38]. The ovarian wedge resection protocol was thereafter applied in the clinic to induce ovulation in PCOS patients and resulted in a pregnancy rate of 59% among 1766 bilateral wedge resections [39] (Table 1). By damaging or removing a part of the ovarian stroma, this procedure induces a reduction in the production and conversion of androgen and inhibin levels, inhibiting factors, allowing a resumption of ovulation. It may also relieve inhibition of follicle growth by changing the physical environment of the ovary and creating a more permissive milieu for follicle expansion [44]. Laparoscopic ovarian drilling (LOD), using insulated needle cautery or laser vaporization, has also been proposed to decrease the number of growing follicles and induce spontaneous ovulation in PCOS women who are resistant to clomiphene citrate therapy. While ovarian wedge resection consists of the ablation of a part of the ovary, laparoscopic ovarian drilling is a surgical technique using heat (diathermy) or laser to create several perforations in the surface and in the inner area of the ovary. Drilling is considered less traumatic and induces fewer adhesions than wedge resection. Spontaneous ovulation was obtained in more than 70% of patients with a pregnancy rate of almost 70% after 24 months [42] (Table 1). Both techniques have shown promising results in terms of ovulation and pregnancy rates as well as live births [45], offering comparable benefits to medical ovulation induction with clomiphene citrate or gonadotropin induction, the first-line therapy, but without the risk of resistance or ovarian hyperstimulation syndrome (OHSS) [46]. Although ovarian wedge resection and drilling techniques offer an in vivo therapeutic option, by acting on cytoskeletal remodelling and with the advantage of a single surgery, the long-term impacts of these cauterization procedures include pelvic tissue adhesion and a decreased ovarian stockpile that might increase the risk of infertility all the more [47].

It is only recently that the impact of fragmentation has been associated with a disruption of the Hippo pathway. Cutting mouse and human ovaries has been correlated with an increase of actin polymerization from its globular (G-actin) into its filamentous (F-actin) form, which induces inhibition of LATS1/2 activity leading to increased non-phosphorylated YAP inside of the nucleus and an increase in expression of downstream growth genes, CCN and BIRC, leading to follicle activation [6, 48–50]. Experiments using chemical inductors of actin polymerization, jasplakinolide or sphingosine-1-phosphate, confirmed the impact of the cytoskeleton on the regulation of the Hippo signalling pathway [51]. It has been established that the extracellular matrix (ECM) and local ovarian thickness are essential regulators of follicle growth and survival [52]. Moreover, ECM proteins are secreted by both GCs and surrounding stromal cells, indicating local modulation of the environment by the follicle itself to accommodate its expansion. Similarly to fragmentation, treatment of mouse ovaries with enzymes that degrade the ECM decreases internal tissue pressure and compression of the follicles, leading to oocyte activation [53]. The impact of pressure modulation on follicle growth has also been reported in mouse grafting experiments. Indeed, several studies have reported that fragmented ovarian tissues provide a better outcome after grafting than whole ovaries [7]. Altogether, these results provide evidence that mechanical approaches can modulate molecular components and promote follicle awakening and growth.

Table 1 Example of studies reporting the outcomes of ovarian wedge resection or drilling in polycystic ovary syndrome (PCOS) patients

Procedure	Number of patients	Patients age (years)	Spontaneous cycle recovery (%)	Pregnancies (%)	Live births (%)	Refs
Ovarian wedge resection	1766 patients (from 1935 to 1983)	Unknown	Unknown	58.8	Unknown	[39]
	149 patients	Unknown	Unknown	83.7	74.4	[40]
	134 anovulatory patients	26.3 ± 6.2	Unknown	90.3	Unknown	[41]
Ovarian drilling	729 patients (from 1983 to 1993)	Unknown	84	55.7	Unknown	[39]
	112 patients resistant to CC	30.2 ± 4.2	73.2	58	56	[42]
	289 patients (from 2004 to 2013)	30.8 [30.3–31.3]	Unknown	47.4	40.5	[43]

CC clomiphene citrate

In addition, acting on signalling pathways that govern follicle activation could also maximize the yield of mature oocytes retrieved. As a major regulator of physiological follicle activation, it has been hypothesized that treatment of mouse ovaries with activators of PI3K/AKT could force the activation of the pool of quiescent follicles [54] (Fig. 1a). To induce the PI3K pathway, several molecules can be used that act at different levels of the signalling pathway; these include phosphatase inhibitors and kinase activators. Notably, in vitro exposure of an inhibitor of PTEN, bpV, a vanadate derivative, together with an activator of PI3K, 740Y-p, on mouse ovaries induces a massive follicle activation and can successfully lead to the generation of healthy offspring after grafting and IVF. Similarly, human cortical tissue treated in vitro with bpV and then transplanted into immunodeficient mouse allows follicle growth [54]. Finally, Kawamura et al. combined AKT stimulators with ovarian sectioning and hippo disruption and observed additive effects on follicle growth in mouse ovaries [6].

Application of IVA in a Clinical Setting

Considering the encouraging experimental results following manipulation of both the local environment and the PI3K and Hippo signalling pathways on follicle growth, IVA procedures have been introduced into the clinic to treat infertile patients with diverse types of ovarian dysfunction, with varying degrees of success (Table 2).

IVA combining PI3K/AKT activating drugs and mechanical stress through ovarian fragmentation has been used to treat POI patients characterized by prolonged amenorrhea and displaying early secondary or smaller follicles in their ovaries (Fig. 2). Following their success in mice, Kawamura and colleagues extended their protocol in the clinic and cultured fragmented cortical tissues from POI patients with AKT stimulators before autograft. Patients underwent ovarian stimulation, followed by oocyte retrieval and IVF, leading to one healthy birth [6]. Since then, the drug-IVA procedure has been used in different countries to treat POI patients and has resulted in two additional live births [8, 9].

A simplified drug-free IVA protocol was later introduced to treat POI patients experiencing recent cessation of menses with ovaries containing late secondary and smaller follicles (Fig. 2). This multistep surgery includes the retrieval of an ovarian cortical tissue piece, the fragmentation of the biopsy into small cubes, the creation of an artificial pocket, and the grafting of the ovarian cubes into this pocket [15]. This refined procedure demonstrated that fragmentation only was sufficient to promote follicle growth and maturation. A case report related the successful pregnancy of a POI patient after ovarian hyperstimulation [10], and another study reported 4 successful pregnancies in 14 POI patients with

Table 2 Reproductive outcomes of the in vitro activation technologies using various physically disruptive techniques in premature ovarian insufficiency (POI), diminished ovarian reserve (DOR), or poor ovarian responder (POR) patients

Procedure	Ovarian tissue	Number of patients	Patients age (years ± SD)	Residual follicles visualized	Follicle development	Mature oocytes	Pregnancies (method)	Live births	Refs
Drug IVA	Vitrified tissue	27 POI patients	37.3 ± 5.8	13/27	8/27	5/27	2/27 (IVF)	1/27	[6]
	Vitrified tissue	10 POI patients	Unknown	7/10	1/10	1/10	1/10 (IVF)	1/10	[8]
	Fresh tissue	14 POI patients	29.2 ± 4.2	7/14	6/14	4/14	1/14 (IVF)	1/14	[9]
Drug-free IVA	Fresh tissue	1 POI patient	32	1/1	1/1	1/1	1/1 (IVF)	1 ongoing (25 weeks)	[10]
	Fresh tissue	1 POI patient	33	0/1	1/1	1/1	0/1	0/1	[55]
	Fresh tissue	20 DOR patients	37.4 ± 2.5	18/20	3/20	1/10	12/20 (3 spontaneous, 9 IVF)	10/20	[12]
	Fresh tissue	11 POR patients with DOR	36.2 ± 6.0	9/11	11/11	11/11	4/11 (1 spontaneous, 3 IVF)	1/11 + 2 ongoing (6 and 9 months)	[14]
	Fresh tissue	14 POI patients	32.79 ± 2.12	3/14	7/14	5/14	4/14 (IVF)	3/14 + 1 ongoing (33 weeks)	[13]
	Fresh tissue	15 POI and DOR patients	Unknown	Unknown	13/15	13/15	6/15 (1 spontaneous, 5 IVF	4/15 + 1 ongoing	[15]
Ovarian scratching	Fresh tissue	80 POI patients	29.36 ± 3.41	12/80	11/80	3/80	1/80 (IVF)	1/80	[11]
Ovarian incision	Fresh tissue	11 ROS patients	Unknown	Unknown	7/11	7/11	4/11 (IVF)	3/11 + 1 ongoing	[15]

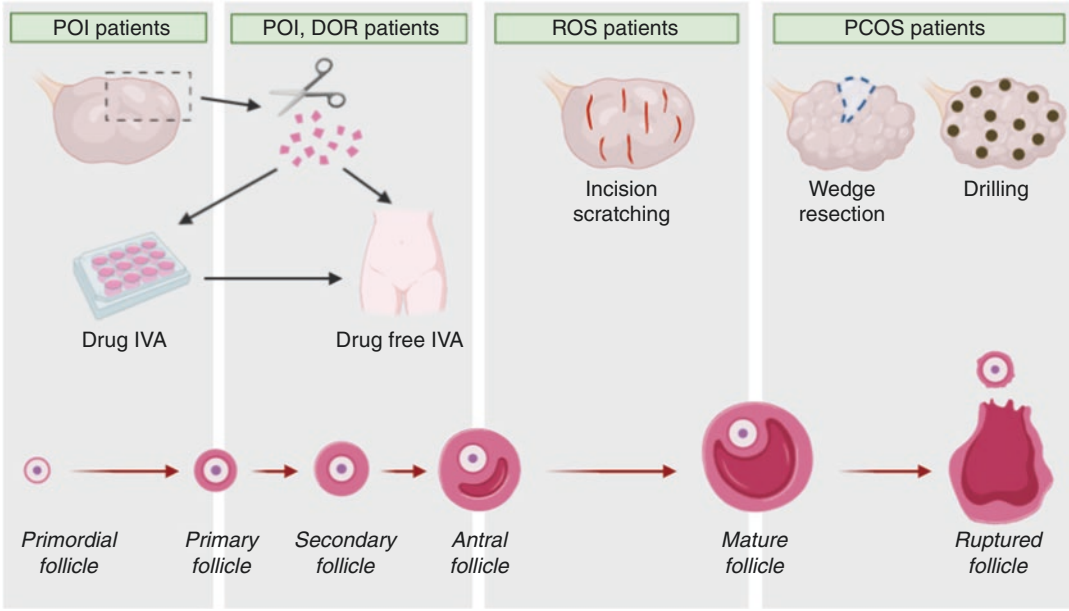

Fig. 2 Summary of the physically disruptive ovarian techniques currently used in the clinic as infertility treatments. Drug IVA has been applied to premature ovarian insufficiency (POI) patients with prolonged amenorrhea and only early-stage follicles in their ovaries. Drug-free IVA has been successful in treating POI patients experiencing recent amenorrhea with ovaries that contain late secondary and smaller follicles as well as diminished ovarian reserve (DOR) patients with a few antral and earlier stage follicles. Ovarian incision or scratching is effective for a subgroup of resistant ovary syndrome (ROS) patients who are unresponsive to follicle-stimulating treatments, while wedge resection and drilling are effective techniques to induce ovulation in polycystic ovary syndrome (PCOS) patients. Figure created with BioRender

a pregnancy rate of 57% per oocyte retrieval [13]. Considering the positive effect on follicle activation in POI patients, this procedure was extended to subfertile patients who had a few antral, early antral, secondary, and earlier stage follicles but a poor ovarian response (POR). One study using the drug-free protocol on 11 POR patients reported an increase in growing follicle numbers on multiple waves after FSH treatment. This enhancement of activated and growing follicles allowed the retrieval of mature oocytes to perform IVF. Sixteen embryo transfers were performed in 5 of the 11 patients and led to one live birth and two ongoing pregnancies [14]. However, a recent study from Lunding and colleagues using the same approach to treat patients with diminished ovarian reserve (DOR) challenged the procedure. Despite achieving pregnancy in 12 out of 20 patients, the authors were less optimistic about the efficacy of the procedure, stating that, after 10 weeks, the whole process of fragmentation and grafting did not enhance the number of growing follicles suitable for fertilization [12]. The conclusion of this study raised questions regarding drug-free IVA. However, the methodology and timing of analyses of the study (10 weeks) have been criticized, and it has been suggested that the timing may have been inappropriate for full analysis of follicle growth [7].

Patients displaying resistant ovary syndrome (ROS) are also in line to benefit from physically induced follicle activation procedures. These patients display detectable antral follicles but secrete low hormone levels and are unresponsive to standard gonadotropin stimulation protocols [56] (Fig. 2). Due to the fact that these patients exhibit symptoms similar to POI patients and because sectioning appears to be an effective approach for rescuing fertility in POI and POR patients, Tanaka et al. evaluated the benefit of using laparoscopic ovarian incision (LOI) to promote the final growth of the follicles in ROS

patients. This procedure is performed in one surgery and consists of in situ cortical tissue sectioning followed by ovarian stimulation [15]. The study included 11 ROS patients, and the technique demonstrated enhanced follicle growth in 7 patients. The simple incision into the ovary offers an attractive perspective for retrieval of mature oocytes from these patients in order to perform IVF/ICSI. Nevertheless, it seems less effective in POI patients, since a large trial including 80 POI patients reported only one live birth following ovarian scratching [11].

Limitations of the IVA Procedure

In light of the results achieved, not only DOR and POI patients could benefit from IVA technology but also cancer patients and pre-pubertal girls for fertility preservation purposes. In the future, it is likely that IVA might also open new perspectives for the increasing number of women who have a desire to achieve pregnancy in their late reproductive life, when their ovarian reserve is low. However, IVA remains an experimental procedure, with limited available data and the requirement of invasive procedures. The evidence regarding its applicability and success for accelerating follicle development and increasing the chance of pregnancy is based on small case series with limited number of patients, and as such, these data should be interpreted with caution. Moreover, the safety and convenience of IVA are currently uncertain and have been criticized with regard to different aspects of the procedure [12, 57, 58] which impede the wide application of this novel approach to treat infertility.

Some of the main concerns for IVA surround its safety with regard to both maternal and neonatal health. First, the initial IVA protocol relied upon the fragmentation of ovarian tissue combined with the use of pharmacological activators of the PI3K/Akt pathway in order to promote follicle growth. The exposure of the ovary to chemical stimulants, even temporarily, represents a significant safety issue. In vitro, treatment of human ovarian cortex with bpV, a PTEN inhibitor originally used in the IVA protocol, results in increased activation of primordial follicles but compromises development of growing follicles, which feature histomorphological abnormalities, steroidogenesis defects, and low survival [49, 59]. Similarly, bovine follicles exposed to bpV display increased DNA damage and reduced DNA repair capacity [60]. The long-term implications of such mechanical ovarian alteration and chemical exposure on the oocyte and subsequent foetus remain unknown. Therefore, thorough investigation is urgently needed and will be essential before these techniques can be adopted for widespread clinical use. In addition, the use of potentially oncogenic chemicals requires special caution. Indeed, alterations of the Hippo and the PI3K/Akt signalling pathway have been found in several cancers, including gynaecological malignancies [61, 62]. Second, some authors have argued that a 2-day in vitro culture of ovarian cortex may trigger tissue damage and necrosis and, thus, programmed cell death [63]. Third, the original protocol required two laparoscopic surgeries, one for ovarian cortex retrieval and a second for the autotransplantation of ‘activated’ ovarian cortex fragments. Besides the invasiveness of the procedure, both the biopsy and transplantation processes have been demonstrated to compromise the ovarian reserve. A recent prospective clinical cohort study, which used ovarian fragmentation and transplantation as a means to increase the number of recruitable follicles for IVF on 20 DOR patients, reported a lower antral follicle count in the biopsied ovaries compared to the intact ones, suggesting a potentially persistent damage of the biopsied ovary due to tissue and follicle removal [12]. A massive and premature follicular activation is also commonly observed following transplantation [64], leading to large-scale atresia of the follicles (‘burnout effect’) and limiting the graft life span. The loss of large numbers of primordial follicles is decisive in cases of low reserve. The short-term focus of IVA might be beneficial when the patient desires immediate outcomes. However, it is unlikely to be able to offer long-term reproduction post-transplantation [57]. Although the emergence of a new drug-free IVA procedure, which does not require use of either the chemi-

cals or the culture of ovarian tissue, could offer a safer and less invasive option with a single surgical procedure, questions regarding activation versus preservation of the dormant pool of primordial follicles persist. Importantly, the IVA approach does not improve age-related decline in egg quality, including genetic and cellular damage that are accumulated in oocytes with advancing age and which reduce pregnancy success rates [65].

The efficacy of the procedure also remains uncertain. The chance for spontaneous conception in POI patients has been estimated at 4–10% [66]. The effect of ovarian biopsy or scratch has shown very little progress towards boosting reproductive outcomes. A recent clinical trial that performed this strategy in 80 POI patients demonstrated a low rate of follicle development (13.75%) and reported only one live birth (1.25%) [11] (Table 2). The application of drug IVA in clinical practice has yielded some advancements but not enough. Case series reported that the combination of mechanical and chemical ovarian stimulation promoted the growth of residual follicles in 15 out of 51 POI patients (29.41%), leading to four pregnancies (7.84%) and the delivery of three healthy babies (5.88%) [6, 8, 9] (Table 2). Nevertheless, the presence of residual follicles within cut and treated ovarian cortical pieces before being transplanted is random and uncertain, which certainly contribute to this low efficacy. Refining the IVA protocol into a drug-free procedure has significantly improved the reproductive outcomes of POI and DOR patients, achieving pregnancy and birth rates of 43.55% (27 of 62 patients) and 29.03% (18 of 62 patients), respectively, according to the latest published reports [10, 12–15] (Table 2). However, the evidence regarding a real benefit of these techniques in humans remains scarce and limited to a few uncontrolled trials. Moreover, Lunding and colleagues demonstrated that neither biopsy nor autotransplantation of fragmented ovarian tissue increased follicular recruitment at 10 weeks after the procedure compared to the contralateral control ovary [12]. They reported a similar follicular response to ovarian stimulation and a similar number of ovulations in the biopsied and control ovaries, while the antral follicle count was higher in the non-biopsied ovary compared to the biopsied ovary. Given the invasiveness of the procedure, failure of the auto-transplant and potential harm to the biopsied ovary, a recent editorial called for an abandonment of the procedure [67]. Overall, these findings highlight that not all POI and DOR patients are likely to benefit from IVA as an infertility treatment but also suggest that a subgroup of patients may respond to the procedure. An important challenge for the future will be to identify the most suitable candidates and establish practice criteria.

IVA is still considered to be experimental and needs to be thoroughly investigated before it can be considered for widespread application in a clinical setting. Its translation from bench to clinic has been incredibly fast, despite our lack of knowledge of IVA's mode of action, underlying mechanisms, and potential side effects. Elucidating the putative mechanisms regulating follicle growth after IVA is a priority and includes, among others, clarifying the role of Hippo pathway disruption and mechanical stress during early-stage follicle development. In addition, only randomized controlled trials can estimate the real magnitude and success of the treatment. Therefore, a collaborative effort is urgently needed to make clinically applicable conclusions. Once the biological principles of IVA are understood, the observed clinical effects are sufficiently promising, and the intervention is mature, safe, and replicable enough, further application and expansion of the technique can eventually be considered.

Future Prospects

Local Delivery of Hippo Signalling-Disrupting Drugs

The concept of IVA relies on the manipulation of the physical environment of follicles through ovarian fragmentation and disruption of the Hippo pathway in order to obtain a higher number of mature oocytes. An alternative to the current invasive IVA procedure would be to inject molecules that disturb the Hippo pathway or that

directly target Hippo components into the ovaries of patients. In vitro, manipulation of the Hippo pathway via the promotion of actin polymerization has been evaluated as a strategy to boost follicle development. Treatment of mouse ovaries and human cortical fragments with jasplakinolide or sphingosine-1-phosphate has been shown to successfully disrupt Hippo signalling, leading to increased nuclear YAP and CCN2 expression and ultimately stimulated follicle growth [51]. However, these results have been recently challenged by Pors and colleagues, who demonstrated that sphingosine-1-phosphate exposure, although associated with Hippo disturbance, did not significantly affect follicle activation dynamics [68]. Other attempts have been made using CCN growth factors, transcription of which is stimulated following Hippo disruption through YAP/TAZ translocation into the nucleus and interaction with TEAD factors. Treatment of mouse ovarian explants with CCN2, 3, 5, and 6 has been demonstrated to promote a dose-dependent increase in ovarian weight and supports the development of primary follicles to the late secondary stage [6]. The local delivery of Hippo signalling-disrupting drugs could allow new in vivo approaches for infertility treatments while minimizing ovarian damage-associated follicle loss. Nevertheless, the safety of using such drugs as well as the assessment of potential detrimental side effects should be carefully addressed before any clinical application.

Gaining Insights into Ovarian Mechanical Forces

The ovarian damage induced by physically disruptive procedures used in infertility therapies is similar to monthly ovarian remodelling. The ovary is a mechanically responsive structure that is subjected to the continuous and repetitive process of follicular development, ovulation, corpus luteum formation, and regression. During each reproductive cycle, the ovary undergoes major structural changes. The permanent turnover of follicular development and subsequent ovulation continuously modifies the mechanical forces within the ovarian stroma. The ovarian extracellular matrix (ECM) is the natural three-dimensional scaffold into which follicles and stromal cells are organized in vivo. Mechanical aspects of the ECM, including its local stiffness and elasticity, direct cell behaviour, while cells produce this ECM and can modify its organization, leading to the principle of dynamic reciprocity between ovarian cells with their surrounding microenvironment [69]. Histologically, in large mammals, including humans, follicles are distributed along a collagen gradient within the ovary. The majority of primordial follicles rest in the outermost collagen-rich cortical region, which offers a rigid physical environment that supports follicle architecture and limits follicle expansion, while growing follicles are localized in the inner medulla, a softer and more pliant environment that enables follicle expansion and growth [70]. Therefore, changes in ECM rigidity are likely to have a direct effect on follicle demise. A recent study confirmed that early-stage follicles are localized to the collagen-rich ovarian cortex and revealed that ECM deposition and remodelling evolve in an age- and follicle stage-related manner [71]. Another group uncovered the underlying causes of this regionalization of the follicles within the ovary. Using mouse ovaries, they demonstrated that oocytes in primordial follicles were compressed by surrounding granulosa cells secreting extracellular matrix proteins, leading to a state of high mechanical pressure [53]. Similar to the fragmentation approach, primordial follicles become activated upon loosening the structure with enzymes that degrade extracellular matrix, and follicle dormancy is restored by compression with exogenous pressure [53], suggesting that changes in surface tension could trigger follicle activation. These findings imply that fragmentation of the ovarian cortex could not only disrupt Hippo signalling to promote follicle growth but also confer low mechanical pressure and create a more permissive environment to enhance primordial follicle activation and growth.

As highlighted above, the role of the physical and mechanical properties of the ovary in regulating initiation and progression of folliculogen-

esis is being increasingly recognized. Therefore, any alterations to its normal physical or mechanical aspects may be implicated in ovarian disorders. Interestingly, the ovaries of PCOS patients classically show a densely collagenized thickened ovarian cortex, potentially related to defects in actin polymerization and/or abnormal biosynthesis of extracellular matrix protein [48]. These defects are likely to create a biomechanically non-permissive environment that could be linked with the arrest of follicle growth at the antral stage or explain subsequent anovulation in patients with PCOS. Future studies on the physical environment surrounding ovarian follicles during homeostasis and pathological conditions are essential for designing more refined treatments. Elucidating the underlying signalling pathways and regulators responsive to these mechanical cues will enable the search for drugs that exploit factors or proteins linked to a more permissive environment as potential targets, or the development of cell-based therapies that aim to reduce surface tension. A comprehensive characterization of the in vivo forces and mechanical landscapes present in the ovary has not yet been achieved and will also be of paramount importance to overcoming female infertility. As a more complete picture of the relationship between cells and their surrounding microenvironment comes into focus, new horizons with a broad range of future therapeutic and diagnostic applications are emerging.

Conclusion

IVA using physically disruptive procedures is an innovative and rapidly evolving technology that has brought new hope for the treatment of female infertility. The combination of mechanical and chemical ovarian stimulation has been correlated with the modulation of the Hippo and the PI3K/AKT pathways, respectively, promoting the activation and growth of early-stage follicles. Clinical application of this technique has rapidly led to pregnancies, and about 20 healthy babies have been born so far from both drug and drug-free IVA-treated POI and DOR patients. Nevertheless, IVA remains highly experimental, with limited data on outcomes and the requirement for invasive procedures. Additionally, low pregnancy rates and possible carcinogenic effects associated with IVA require special caution. If the benefit of IVA is substantiated with larger studies and the intervention is safe and replicable enough, the ability to exploit the pool of dormant primordial follicles and boost the development of bigger follicles could offer new possibilities for treatment to millions of women with diminished ovarian reserve, whether age-related or not. In the future, a better understanding of the mechanisms underlying the activation and growth of early-stage follicles, the influence of their surrounding ovarian microenvironment, and the impact of mechanical cues on follicle demise will be crucial to refinement of current IVA strategies and identification of the patients that are the most likely to respond to the procedure. Moreover, the introduction of trials testing new drugs targeting the Hippo pathway or modulating the ovarian ECM, or evaluating the effect of various types of physical damage such as drilling, scratching, wedge resection, or fragmentation, will be a further step towards improving the technique and its outcomes. Finally, advances in tissue culture and transplantation technologies might help to prevent the burnout of the dormant follicle pool, which could be decisive in cases of low ovarian reserve.

Definitions

Follicle activation: initial recruitment and awakening process of quiescent ovarian primordial follicles, characterized by the growth of the oocyte coordinated with the cuboidalization and proliferation of its surrounding granulosa cells. This irreversible phenomenon is the first step of folliculogenesis and corresponds to the entry into the pool of growing follicles.

In vitro activation (IVA): concept that relies on the use of mechanical stress, associated or not with chemical cues, to boost follicle activation and growth and maximize the yield of mature oocytes available for further in vitro fertilization.

Physically disruptive methods: set of techniques, such as ovarian fragmentation, scratching, biopsy, wedge resection, or drilling, which, by inducing a varying degree of damage of the ovary, trigger a disruption of the local actin cytoskeleton and Hippo pathway. This change in the physical and molecular follicle microenvironment impacts follicle behaviour and relieves inhibition of follicle activation and growth.

Take-Home Messages

1. Progress in understanding the underlying mechanisms governing follicular activation has allowed the emergence of new clinical procedures that induce awakening and development of quiescent ovarian follicles in infertile patients.
2. Mechanical disruption of ovarian tissue offers the option to induce activation of residual follicles by acting on cytoskeletal proteins and internal tissue pressure.
3. Physically disruptive procedures, including ovarian fragmentation, scratching, biopsy, wedge resection, and drilling, have been introduced in the clinic to treat infertile patients with diverse types of ovarian dysfunction, with varying degrees of success.
4. In vitro follicular activation (IVA) remains highly experimental, with limited data on outcomes and the requirement for invasive procedures, and needs to be thoroughly investigated before widespread application.
5. Future refinement of IVA might involve the use of new drugs that target the Hippo pathway or modulate the ovarian extracellular matrix.

Key Readings

1. Kawamura K, Cheng Y, Suzuki N, Deguchi M, Sato Y, Takae S, et al. Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment. Proc Natl Acad Sci USA. 2013;110:17474–9.
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Clinical Case and Practical Clinical Tips

A 32-year-old patient had been trying to get pregnant for 2 years without success. She was treated with chemotherapy during childhood for Hodgkin lymphoma. For the past year, she had been experiencing irregular cycles and had not menstruated for 3 months. Her AMH value was under the detection limit, and her FSH levels had risen above 25 IU/ml twice. Ovarian stimulation had been attempted, without success, over the past 6 months.

- Inform her that a diagnosis of premature ovarian failure (POI) was likely due to previous chemotherapy treatments.
- Investigate other possible causes of POI.
- Propose cyclic hormonal substitutive therapy to restore menstrual cycles.
- Offer her IVA (ovarian cortex biopsy, fragmentation, and re-transplantation) by laparoscopy to achieve pregnancy

with their own gametes as an experimental approach.
- Inform her about alternatives such as egg donation.
- **Practical Clinical Tips**

  IVA using mechanical techniques alone can be offered in cases of recent diagnosis of POI in young women but should be proposed within an experimental protocol. There is no evidence that addition of activation drugs increases the success of the procedure if few growing follicles are still present.

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Fertility Preservation in Children and Adolescents

Isabelle Demeestere

Introduction

Although a deceleration of the increase in the incidence of cancer in children has been observed, it is still increasing by 0.54% (0.44–0.65) per year in children (age 0–14 years), and the increase is quite clear for leukaemia, lymphoma, and malignant central nervous system (CNS) tumours that represent 70% of the cancer diagnosed in this population [1]. Overall, 1 in 300 newborns will develop cancer before the age of 20. Due to the progress in oncological therapy, 80% of these patients are disease-free 5 years after diagnosis, but two-thirds will face late side effects that can dramatically affect their long-term quality of life. In 2015, an International Society of Paediatric Oncology (SIOPE) Strategic Plan was presented at the European Society for Medical Oncology (ESMO) annual conference with the aim of improving both survival and quality of life in children diagnosed with cancer (SIOPE strategic plan: https://www.siope.eu/SIOPE_StrategicPlan2015/). The plan estimated that nearly half a million European citizens will be survivors of childhood cancer by 2020.

One of the major concerns of childhood cancer survivors is whether they will retain the future possibility of conception with their own gametes. The Childhood Cancer Survivor Study (CCSS), which included more than 3500 female cancer survivors diagnosed before the age of 21, confirmed the increased risk of infertility in this population (RR 1.48, 95% CI 1.23–1.78 compared to siblings) [2]. Factors associated with a high risk of infertility are pelvic irradiation and alkylating agent-based chemotherapy in both sexes [3].

The future fertility of children affected by benign disease may also represent a major concern as some of these require similar high-risk therapies such as conditioning regimens for haemopoietic stem cell transplantation (HSCT) in children with haematological benign diseases (e.g. sickle cell anaemia, thalassemia, and granulomatosis). Finally, progress in genetics has provided early diagnosis for genetic disorders at risk of premature ovarian insufficiency, such as Turner syndrome, blepharophimosis-ptosis-epicanthus syndrome (BPES), and galactosemia.

These young patients at risk of premature ovarian insufficiency due to a disease condition or gonadotoxic treatments for malignant or benign diseases, as well as their parents, should be informed about this risk and the possibility of preserving fertility [4, 5].

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Infertility Risk in Childhood Cancer Survivors

As female germ cells are not able to replicate after birth, the reproductive lifespan is determined by the number of primordial follicles (representing the ovarian reserve) at birth and the rate of follicular depletion through either atresia or ovulation. Ovarian failure occurs when the follicular pool is completely depleted (<1000 follicles) either naturally at menopause or earlier if the follicular reserve is prematurely lost through an accelerated atresia process (e.g. genetic disorders), exposure to gonadotoxic agents (e.g. chemotherapy and radiotherapy), or ovarian surgery. The risk can be evaluated according to the dose and nature of the gonadotoxic agents. The most gonadotoxic agents are alkylating drugs. Based on these criteria, chemotherapy is considered to be highly gonadotoxic when the alkylating agent score (AA score) reaches 3 or when the cyclophosphamide equivalent dose (CED) reaches 7 g/m^2 or more (Table 1).

In a long-term follow-up study of the Childhood Cancer Survivor Study (CCSS) cohort including almost 3000 patients, the authors identified different independent risk factors for nonsurgical premature ovarian failure including exposure to procarbazine ≥4000 mg/m^2 (OR 8.96; 95% CI 5.02–16.00), ovarian radiation (dose ≥500 cGy, OR 8.02; 95% CI 2.81–22.85), and HSCT (OR 6.35; 95% CI 1.19–33.93) [8]. In order to reduce mortality and morbidity, including long-term effects, associated with HSCT, alternative reduced-intensity conditioning (RIC) regimens have been implemented in nonmalignant diseases based on combinations of treosulfan, fludarabine, and thymoglobulin, or alemtuzumab, fludarabine, and melphalan [9]. Limited data are available regarding long-term follow-up on ovarian function after RIC. In one study that included follow-up beyond 2 years of a cohort of 43 children treated with RIC, 1 out of 9 eligible patients for pubertal assessment faced premature ovarian failure [9].

Acute ovarian failure is defined as permanent primary or secondary amenorrhea after a high-risk treatment. Based on the follow-up of the CCSS cohort, it has been reported that 6.3% of female cancer survivors develop acute ovarian failure, defined as the loss of ovarian function within 5 years following diagnosis [10]. When oncological treatment was administered before puberty, children diagnosed with primary amenorrhea may require pubertal induction using hormonal treatment. The major causes of nonsurgical acute ovarian failure include high-dose alkylating agents for lymphoma or sarcoma, conditioning

Table 1 Evaluation of the risk of premature ovarian insufficiency in children according to the dose and type of alkylating agents [6, 7]

Tools	Definition	Scale
Alkylating agent score (AA score)	Total dose per square meter of body surface area—scores are summed over all agents	0 no exposure 1 lower tertile dose 2 middle tertile dose 3 upper tertile dose
Cyclophosphamide equivalent dose (CED)	Cumulative cyclophosphamide dose (mg/m^2) + 0.244 cumulative ifosfamide dose (mg/m^2) + 0.857 cumulative procarbazine dose (mg/m^2) + 14.286 cumulative chlorambucil dose (mg/m^2) + 15.0 cumulative BCNU dose (mg/m^2) + 16.0 Cumulative CCNU dose (mg/m^2) + 40 Cumulative melphalan dose (mg/m^2) + 50 Cumulative thiotepa dose (mg/m^2) + 100 Cumulative nitrogen mustard dose (mg/m^2) + 8.823 cumulative busulfan dose (mg/m^2)	mg/m^2

BCNU carmustine, *CCNU* lomustine, *TEPA N,N′,N″*-triethylenephosphoramide

Table 2 Ovarian function recovery rate after haematopoietic stem cell transplantation in young patients

Authors	Treatment	*n*	Age (range)	Ovarian recovery (%)	Follow-up
Sanders et al. [12]	Cy Bu/Cy Cy + TBI	103 73 532	28 years (13–58) 38 years (14–57) 28 years (11–58)	56 (54.3) 1 (1.3) 53 (10)	12–204 months (median 36)
Sarafoglou et al. [13]	Cy + TBI	16	Prepubertal	9 (56)	
Teinturier et al. [14]	CT CT (Bu)	11 10	5.8 years (2–14.8) 12.7 years (4.7–17.3)	7 (73) 0 (0)	14–156 months (median 84)
Thibaud et al. [15]	CT CT + TBI	8 23	10.3 years (3.2–17.5)	3 (37.5) 3 (13)	14–138 months (median 72)
Bath et al. [16]	CT + TBI	8	11.5 years (5.9–15)	2 (25)	
Couto-Silva et al. [17]	CT+ TBI CT	22 5	7.3 years (1.5–13) 5.3 years (0.6–12.9)	3 (13.6) 2 (40)	
Tauchmanova et al. [18]	Bu/Cy	21	13–45 years	2 (5)	12–62 months (median 38)
Jadoul et al. [19]	CT + TBI CT CT (Bu)	18 6 11	9.8 ± 5.2 years (range 1.2–19.0)	4 (22) 6 (100) 5 (45)	15.5 ± 5.5 years (range 3.3–33.7 years)

Cy cyclophosphamide, *Bu* busulfan, *TBI* total body irradiation, *CT* chemotherapy

regimens before HSCT, and exposure to pelvic or spinal irradiation [11]. Acute premature ovarian failure was observed in the majority of haematological patients treated with HSCT, especially when the conditioning regimen included busulfan or total body irradiation (Table 2). Gonadal failure is also common in cancer survivors treated with high-dose chemotherapy and autologous stem cell rescue for other diseases such as neuroblastoma [20].

When the gonadotoxicity of the therapy and/or the total dose is moderate, ovarian insufficiency may occur later during reproductive life. While most children will experience spontaneous puberty or will recover menstruation after chemotherapy, the ovarian reserve is often impaired by these moderate gonadotoxic treatments. Reduction of the ovarian reserve can induce premature ovarian failure (defined as cessation of menstruation and menopausal FSH levels before 40 years old), infertility, and low response to ovarian stimulation several years or decades after diagnosis. Sklar et al. have shown that childhood cancer survivors with spontaneous menstruation more than 5 years after diagnosis have a 13-fold increased risk of developing premature ovarian failure compared to siblings [21]. Markers of the ovarian reserve in postpubertal women include the measurement of anti-Mullerian hormone (AMH), follicle-stimulating hormone (FSH), and oestradiol (E2) blood levels, as well as follicle counting using ultrasound (antral follicle counting, AFC). While physiological FSH levels remain low until puberty, AMH levels gradually increase to reach a peak at 24.5 years old [22]. Data on the impact of oncological treatment on the ovarian reserve remain limited. One analysis performed in a small cohort of children diagnosed with cancer (n = 22) observed a correlation between AMH levels after 6–12 months and the gonadotoxicity of the chemotherapy regimen. This correlation was not observed with FSH or inhibin B as markers [23]. The recovery rate was also associated with pretreatment AMH levels in young patients (2.6% versus 11.9% per month for basal AMH levels <2 ng/ml or ≥2 ng/ml, respectively) [24, 25]. Compared to a control population, AMH levels were, thus, significantly lower in childhood cancer survivors (n = 10, aged 16–34 years) who spontaneously recovered menstruation, while basal FSH levels were higher (n = 11) [26]. Low AMH has been reported in around 30% of cancer survivors with normal menstruation, and some of these women were still able to achieve pregnancy

[24, 25, 27]. A decline in AMH has also been correlated with increased CED score (cyclophosphamide >7.5 g/m^2), older age at exposure, and pelvic irradiation.

Fertility Preservation

Access to appropriate counselling and fertility preservation procedures remains an important issue in children. In a recent European survey that included 38 centres that developed expertise in HSCT in children and adolescents, the authors reported that only 21 (55%) had a standardized programme for fertility preservation procedures. A total of 39% and 16% of patients treated with HSCT received counselling and had a fertility preservation procedure performed, respectively [28]. However, fertility-related issues are a major concern for this young population and their parents [29].

Fertility preservation counselling remains complex in children and adolescents. In prepubertal girls, the only option available is the cryopreservation of ovarian tissue, which is still considered to be experimental in children. Fertility preservation strategies will be discussed according to pubertal status and disease.

Oocyte Cryopreservation

The established option for fertility preservation in adults is the cryopreservation of mature oocytes or embryos after ovarian stimulation with gonadotropins and transvaginal oocyte collection. Ovarian stimulation (OS) takes around 10 days, but adapted protocols have been implemented that start the treatment irrespective of cycle phase using gonadotropin-releasing hormone (GnRH) antagonists to avoid spontaneous luteinizing hormone (LH) peaks. After around 10 days of OS, regular hormone level assessments, and control of follicular growth by transvaginal ultrasound (or transabdominal, if transvaginal evaluation is not feasible), ovulation can be triggered, either by GnRH agonist or by human chorionic gonadotropin (hCG), in order to collect mature oocytes 36 h later. This protocol is considered standard in adults if there are no contraindications, such as severe haematological disorders or high risk of thrombosis. Moreover, the delay should not compromise the efficacy of the oncological subsequent treatment. This option has also been proposed prior to bone marrow transplantation in teenage girls. In a case series of eight patients aged between 14 and 18 years diagnosed with sickle cell anaemia, the authors reported mature oocyte recovery rates ranging from 1 to 30. Half of the patients had fewer than eight mature oocytes cryopreserved, casting doubt upon the efficiency of using this procedure in adolescents [30]. More recently, data for a cohort of 41 patients aged between 13 and 21 years who underwent ovarian stimulation for fertility preservation were reported [31]. A total of 38 patients completed the cycle, with a median of 10 mature oocytes retrieved per patient (ranging from 0 to 25). Although the procedure is not feasible in prepubertal children, a case report of ovarian stimulation demonstrated the feasibility of the procedure in premenarchal patients who have already initiated puberty. The procedure was offered to a girl with myelodysplastic syndrome aged 13 years at diagnosis. Ovarian stimulation was initiated with 225 IU human menopausal gonadotropin (hMG) using a GnRH antagonist after 1 week to avoid spontaneous LH surge. Follicular development was assessed by transabdominal ultrasound, while oocyte collection was performed transvaginally under general anaesthesia after hCG triggering. Twenty oocytes were obtained of which only 8 were mature. The 12 immature oocytes were cultured for 20 h, and nine additional mature oocytes were cryopreserved after in vitro maturation [32]. In a recent systematic review, the author identified nine publications including oocyte cryopreservation in 20 young patients under 20 years of age, illustrating the paucity of data in the field [33]. There are several barriers limiting the use of this procedure in adolescents, including access to appropriate fertility centres, the delay required for OS, the psychological impact of transvaginal ovarian assessment and oocyte collection, the risk of hyperstimulation, and the burden of daily

subcutaneous injection during ovarian stimulation in this young population. All these limitations make the procedure complex in this population and often not feasible or not accepted. Moreover, data on outcomes and on the success rates of oocyte cryopreservation in the under 18 population are very limited. Successful live birth has been reported using cryopreserved oocytes at the age of 17 years [34]. Recent data have shown that the cumulative live birth rate of oncological adult patients who cryopreserved five and eight oocytes before the age of 35 years reached 15.8% and 32%, respectively. Although the efficiency of the procedure is negatively correlated with age, the success rates of the procedure in premenarchal girls and adolescents are still unclear. Studies in sheep have shown that mature oocytes from prepubertal animals obtained after in vitro maturation (IVM) are less competent than oocytes from adults, mainly due to abnormal cytoplasmic maturation [35, 36]. Incomplete nuclear maturation has been also described in prepubertal lambs, leading to low developmental competence and high rates of pregnancy arrest [37]. The competence of oocytes collected before or shortly after menarche, as well as pregnancy outcomes using these oocytes, needs to be further investigated in humans, and the procedure should be proposed as an experimental approach to these young patients and their parents.

Ovarian Tissue Cryopreservation

Ovarian tissue cryopreservation (OTC) has been proposed as an alternative option to oocyte cryopreservation. It is the only available approach to cryopreservation of gametes in prepubertal girls, and it is the most frequently offered option in the young postpubertal population. Children and adolescents (<18 years old) represent around 25% of the patients who undergo the procedure in our centre. The major indications are benign or malignant haematological diseases such as sickle cell anaemia or leukaemia or lymphoma requiring HSCT or high-dose alkylating agents (Fig. 1).

OTC has the advantage of not requiring prior hormonal treatment or ovarian stimulation and of being performed at any time during the menstrual cycle. The delay between fertility preservation counselling and the procedure can be very short. Moreover, in contrast to oocyte cryopreservation, the procedure can be performed even if chemotherapy has already started. Recent data demonstrated that there were no differences in follicular density after first-line treatment in girls under the age of 18 years, although the ovarian surface area was reduced by 10 and 30% in young girls and adolescents, respectively [38]. The long-term consequences of previous chemotherapy remain to be further explored, but pregnancies have been obtained after transplantation of ovarian tissue collected after first-line therapy [39–41].

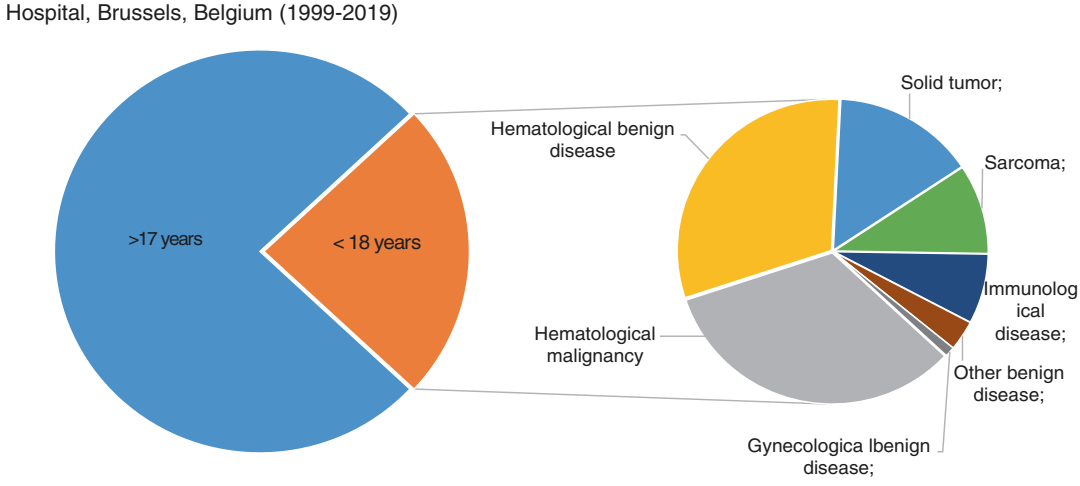

Fig. 1 Proportion and indication of ovarian tissue cryopreservation in children at Erasme Hospital, Brussels, Belgium (1999–2019)

In OTC, the ovarian cortex is collected under general anaesthesia by laparoscopy or mini-laparotomy according to the age of the patient and the surgeon's skill level. The surgery can be performed in the paediatric centre, and the tissue can be transported at 4 °C for up to 24 h before the cryopreservation procedure [42]. In most countries, OTC procedures are centralized in centres with specific expertise and appropriate infrastructure. Therefore, a well-organized network with close collaboration between oncologists, surgeons, and fertility specialists is required to manage fertility preservation in this young population. A recent review reported partial oophorectomy in 43% of the procedures in young patients aged less than 20 years old, although unilateral oophorectomy is usually recommended in all prepubertal children who undergo OTC [33]. Complications are rare but peri- and postoperative bleeding have been described [19, 33]. The safety of anaesthesia in children has also been dramatically improved in the past few decades. Nevertheless, the OTC procedure should always be associated with another intervention such as central venous catheter insertion, biopsy, or tumour resection, when feasible, in order to avoid repeated anaesthesia, especially in very young children [19]. Higher risk of respiratory failure has been observed during anaesthesia in children, and risk factors must be identified during pre-anaesthetic assessment [43]. Chemotherapy treatment can be usually initiated the day after OTC.

The procedure aims to cryopreserve a large number of the non-growing follicles (primordial and primary follicular pool) that constitute the ovarian reserve. Follicular density is negatively correlated with age and is particularly high in children. A predictive model has been established to estimate follicular density and ovarian volume according to age. At 16 years old, the number of non-growing follicles and the ovarian volume were 147,912 and 6358 mm^3, respectively, while these values dropped to 98,106 and 7695 mm^3 at 21 years old, respectively [44]. Poirot et al. [45] evaluated follicular density in a cohort of young patients who underwent OTC. In patients under 7 years of age, the mean number of non-growing follicles was 20.36/mm^2 (n = 6), and this decreased to 4.13/mm^2 for patients between 10 and 15 years old (n = 8) [45]. However, follicular density varied dramatically from one fragment to another. In two patients 12 years of age, ovarian biopsies revealed a follicular pool from 110 to 1138 with 50–93% of primordial follicles within the tissue fragment [46].

The balance between the risks and benefits of the procedure should always be carefully evaluated before surgery, including the additional risk of ovarian insufficiency due to the procedure. A subgroup of 60 girls were evaluated for the risk of premature ovarian failure in a large Danish cohort of 176 patients aged between 12 and 18 years at OTC. When they reached a mean of 21.1 years old, 43% and 10% received hormone replacement therapy and oral contraception, respectively. A total of 45% reported normal regular menstruation [47]. For patients under 12 years of age at OTC, 71% required medical puberty induction. The impact of oophorectomy or ovarian biopsy on the future risk of premature ovarian insufficiency is still unclear. After excluding patients facing acute ovarian failure, childhood cancer survivors who underwent unilateral oophorectomy reached menopause 7 years earlier than those who did not (median, 42 years; 95% CI, 40–46 versus 49 years; 95% CI, 48–50) [48]. These women have a 3.7-fold increased risk of menopause. In patients who underwent fertility-sparing surgery for localized ovarian cancer, younger age at diagnosis was also associated with a higher risk of early menopause [49]. Moreover, unilateral oophorectomy at a young age is associated with an increased risk of surgery-associated menopause during reproductive life in the event of diseases affecting the remaining ovary (e.g. endometrioma, recurrent ovarian cyst, tumour, and torsion). On the other hand, ovarian cortex biopsy is more complex to perform as coagulation should be avoided to prevent damage of the cryopreserved cortex as well as the remaining ovary. Bleeding on the remaining ovary should be managed with caution to maintain an optimal ovarian reserve. For prepubertal girls, this procedure is not feasible, and unilateral oophorectomy is usually performed [40, 41].

Slow freezing is the standard procedure to cryopreserve ovarian tissue in adults and children. After thawing and xenograft into mice, around 80% of the follicles survive [50]. Vitrification has become the standard for oocytes and embryos and has recently emerged as a potential technique for cryopreservation of ovarian tissue. In a meta-analysis comparing both techniques, the authors concluded that the techniques were similar in terms of proportion of intact follicles, but vitrification was associated with less DNA damage and better stromal cell morphology [51]. However, protocols were not standardized, making comparisons difficult, and the number of live births obtained after transplantation remains limited.

Outcomes After Transplantation of Cryopreserved Ovarian Tissue

At present, orthotopic and heterotopic transplantations are the only available options for restoring fertility using cryopreserved ovarian tissue. More than 130 live births have been reported after transplantation of ovarian tissue collected in adults with an overall success rate of around 40% [52]. Data regarding the outcomes of transplantation of cryopreserved ovarian tissue collected during childhood remain much more limited. A first transplantation was reported for pubertal induction in a prepubertal girl aged 10 years at the time of OTC. The procedure was performed before HSCT for sickle cell anaemia, and the patient came back 27 months later to use the cryopreserved tissue for endocrine restoration. Another case of pubertal induction after transplantation of cryopreserved ovarian tissue was reported in a patient aged 9 years at OTC before she was treated for Ewing sarcoma [53]. In both cases, hormonal function was temporally observed with puberty progression, although the indication has raised several concerns. First, the procedure is invasive, and patients can be at risk of malignant cell transmission in cancer survivors, whereas safe hormonal medication is available as an established method for induction of puberty and menarche. Moreover, the duration of restoration of ovarian function after transplantation is limited, and ovarian tissue should be kept for later fertility restoration. Finally, sudden unphysiological rises in oestradiol may induce accelerated puberty, leading to premature growth arrest and overt weight gain [54, 55]. Therefore, autologous transplantation of ovarian tissue is not recommended for this purpose.

Only three cases of successful restoration of fertility after autologous transplantation of ovarian tissue cryopreserved before menarche have been reported. The first live birth was reported in 2015. Unilateral oophorectomy was performed before HSCT for sickle cell anaemia in a patient aged 13 years and 11 months. She had initiated puberty but not menarche. Menstruations were induced 18 months later by hormonal replacement therapy as she was diagnosed with acute premature ovarian failure. Ten years later, she underwent ovarian tissue transplantation to restore her fertility after confirmation of her menopausal status. Spontaneous menstruations occurred after 5 months, and she delivered two healthy babies after natural conceptions 2 and 5 years following the grafting procedure [56]. A second live birth after ovarian tissue transplantation was reported in a woman aged 10 years at OTC [57]. The pregnancy was obtained after an intracytoplasmic sperm injection (ICSI) procedure. Three other patients who underwent ovarian tissue transplantation using tissue cryopreserved during childhood have been reported on in the literature, but no pregnancies were obtained: one faced disease recurrence 4 months after the transplantation procedure, one did not have restoration of endocrine function after a first graft, and one was still being monitored at the time of the report [33, 40, 41]. In the postpubertal population under 21 years at OTC, a total of 15 ovarian tissue transplantations have been reported. Overall, 56% of the patients have had at least one live birth, and 60% of these were naturally conceived [33].

Several issues have to be considered regarding the ovarian tissue transplantation procedure. First, the risk of recurrence of the disease due to potential transmission of the malignant cells must be carefully assessed. This risk depends on the type and the localization of the disease and is considered to be high in leukaemia, neuro-

blastoma, Burkitt lymphoma, and neoplasias with distant metastases or involving the pelvis [58]. Leukaemia represents one of the major indications for OTC in children. The presence of neoplastic cells within the ovarian tissue was observed in more than half of the cases after molecular detection [58]. However, this risk can be reduced when the ovarian tissue is cryopreserved after first-line chemotherapy [59]. A first successful transplantation of ovarian tissue collected at the time of complete remission after first-line chemotherapy was recently reported [60]. Nevertheless, caution should be taken in patients at high risk, and appropriate analysis of the ovarian cortex and residual medulla should be performed using the most sensitive techniques, such as PCR, when available. Second, outcomes after transplantation of ovarian tissue from prepubertal children remain uncertain. Although the tissue contains a large number of non-growing follicles, high rates of abnormal follicles have been described in young children. After analysis of ovarian cortex from 25 patients aged 4–39 years, Westergaard et al. [61] reported an increase in the diameter of the oocytes with age as well as in the number of granulosa cells surrounding primary follicles, while oocyte nuclear diameter seems to be unaffected by age [61]. Another study observed a higher rate of abnormalities in the primordial follicles from prepubertal compared to pubertal girls including the absence of a nucleus, poor vesicle germinal definition, or multinucleated follicles (abnormality rates of 19.4 ± 5.6% of oocytes within non-growing follicles in tissue from the prepubertal group and 4.8 ± 1.6% of non-growing follicles from the pubertal group). After in vitro culture, the follicles from the prepubertal group also exhibited limited growth activation and did not grow at the same rate as follicles from the pubertal group [62]. Finally, animal studies have demonstrated that oocytes from prepubertal growing follicles are less competent, resulting in a lower blastocyst development rate (Table 3). This may be explained by differences in the distribution and density of mitochondria [35].

Table 3 Characteristics of follicles from prepubertal ovaries observed in human or animal studies

Characteristics	Refs
Presence of high density of follicles at all stages (except pre-ovulatory follicles)	[63]
Diameter of primordial/transitory follicles/oocytes increases with woman's age (from age 4 through mid-30s) but nucleus size is unaffected by age	[61]
Number of granulosa cells in small follicles increases with age	[61]
High rate of abnormal follicles	[62, 64]
Growing follicles less sensitive to FSH	[65]
Growing follicles/oocytes obtained after in vitro culture are smaller than those from young adults	[62, 66]
Delay in oocyte maturation Low ATP oocyte concentrations (fewer mitochondria in mature oocytes) Higher spontaneous parthenogenetic activation rate Higher polyspermia rate	[35, 67, 68]
Lower blastocyst development but similar morphology of the embryo Reduction in global methylation High pregnancy loss	[37]

Combined Ovarian Tissue and Oocyte Cryopreservation Procedure

Ovarian tissue cryopreservation can be combined with ex vivo immature oocyte collection as antral follicles are observed even at prepubertal ages [69, 70]. These oocytes can be in vitro matured and cryopreserved for future in vitro fertilization. Although few pregnancies have been obtained using in vitro maturation of immature oocytes collected ex vivo in adult patients, the success rate is probably very low. A recent report of outcomes after thawing 35 embryos and 8 mature oocytes from immature oocytes collected during ovarian tissue cryopreservation procedures in adults showed a survival rate of 82% for the thawed embryos, but only 1 pregnancy was obtained [71]. The success rate may be even lower in children as lower maturation rates have been observed compared to immature oocytes from adults (10.3% versus 28.8%). Moreover, the maturation process was delayed as the majority of the oocytes matured after 48 h and 24 h in prepubertal versus postpubertal patients, respectively [70]. Animal studies have also demonstrated

low and delayed maturation rates when immature oocytes were collected in prepubertal animals (Table 3). Finally, healthy oocytes are usually not found in children aged less than 5 years [70].

Ovarian Transposition

Ovarian transposition is the first procedure to become available for situations when high-dose pelvic irradiation is required in children treated for malignancies, such as pelvic sarcoma or Hodgkin lymphoma. However, only six studies have been reported in this population [33]. It is usually performed after adjuvant therapy concomitantly with tumour resection [72]. The ovaries are relocalized at a distance from the irradiation field to avoid direct damage using laparotomy or laparoscopy. The success rate of the procedure in this population is unclear. A failure rate of 10–14% was previously reported in children [72]. However, long-term follow-up did not demonstrate a real benefit in terms of premature ovarian insufficiency in the St. Jude Lifetime Cohort Study (SJLIFE) treated for Hodgkin lymphoma, most probably due to the association of gonadotoxic chemotherapy [73]. Another long-term study observed ovarian function in all 18 young patients who underwent ovarian transposition after a mean follow-up of 8.6 ± 0.9 years and 2 pregnancies occurred, although 15 out of 18 patients received chemotherapy [74].

Complications were observed including intestinal occlusion, dyspareunia, functional ovarian cysts, and pelvic adhesions with tubal obstruction.

Pharmacoprotective Therapy

Pharmacoprotective therapy aims to reduce the gonadotoxic effects of chemotherapy. Although it appears to be a very attractive approach as it is noninvasive and allows spontaneous restoration of ovarian function after oncological treatment, only one drug has been tested in clinical trials for this indication. Gonadotropin-releasing hormone agonist (GnRHa) has been used in postpubertal patients to mimic the prepubertal status and reduce follicular activation during chemotherapy. Others have suggested that it may act directly on the ovaries through GnRH receptors to reduce apoptosis. However, none of these potential mechanisms of action have been demonstrated by experimental studies [75]. Moreover, there are no rational reasons to offer this option in prepubertal children. All randomized controlled trials including adult patients have demonstrated controversial results. While results obtained in lymphoma patients did not confirm the efficacy of the treatment to prevent premature ovarian insufficiency, data in breast cancer showed a reduction in amenorrhea rates after short-term follow-up [76, 77]. Overall, the recent recommendations stated that this option could be offered in breast cancer patients but should not replace other fertility procedures.

Conclusion

Although counselling regarding the fertility issue in children is complex and options are limited, fertility preservation should be offered to all young patients undergoing highly gonadotoxic treatment. The only available option in prepubertal children is ovarian tissue cryopreservation. Oocyte cryopreservation can be considered in adolescents, but the procedure may be more complex to manage, and better efficacy has not been demonstrated compared to ovarian tissue cryopreservation. Decisions should take into consideration the patient's age and maturity, the disease type and localization, as well as the oncological treatments and imperatives.

Definitions

Ovarian reserve: Number of quiescent follicles (primordial and primary) in the ovary. Ovarian reserve progressively decreases throughout life until menopause occurs. Ovarian reserve can be evaluated using pelvic ultrasound (antral follicle count, AFC) and hormonal levels (anti-Mullerian

hormone, AMH; basal follicle-stimulating hormone, FSH; and estradiol levels measured at the beginning of a natural menstrual cycle).

Premature ovarian insufficiency (POI): POI is a consequence of ovarian reserve depletion. POI is confirmed after at least 4 months of amenorrhea and two FSH values above 25 IU/L (at 1 month interval) based on the ESHRE criteria.

Fertility preservation: Procedures aiming to preserve fertility in patients who need treatments or surgery that can damage the ovary or who have been diagnosed with diseases that can induce premature ovarian insufficiency and infertility. Fertility preservation includes several strategies, such as the reduction of toxicity (ovarian transposition, radiation shield, pharmacoprotection) or cryopreservation of gametes (ovarian tissue, oocytes, sperm, testicular tissue) or embryos.

Take-Home Messages

1. All children treated with gonadotoxic treatment and ovarian surgery or diagnosed with diseases that may accelerate follicular depletion, as well as their parents, should be informed about the risk of premature ovarian insufficiency. Consequences on puberty, menstruation, future contraception, and fertility should be discussed.
2. Fertility preservation strategies should be offered to patients who are at high risk of premature ovarian insufficiency. Close collaboration with fertility specialists should be established to refer them as soon as possible.
3. Oocyte cryopreservation is the first established option for fertility preservation in adults. However, efficacy, success rate, and tolerance of the procedure need to be further investigated in young post-pubertal adolescent.
4. Cryopreservation of ovarian tissue is considered as experimental, but it is the most frequently offered option in young patients. It is the only available option in prepubertal patients, or when oocyte cryopreservation is not feasible due to contraindication, timing, or psychological issues.
5. Knowledge on fertility outcomes after ovarian tissue transplantation using ovarian tissue cryopreservation during childhood is very limited.

Clinical Case and Practical Clinical Tips

A 15-year-old girl was diagnosed with Hodgkin's lymphoma and was treated with two ABVD cycles (doxorubicin, bleomycin, vinblastine, and dacarbazine). The disease rapidly progressed despite the chemotherapy, prompting oncologists to change treatment by using bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone regimen (escalated BEACOPP). She was referred with her parents for fertility preservation counselling.

- Inform them that fertility preservation was not recommended for low gonadotoxic treatment such as ABVD. However, escalated BEACOPP, including high dose of alkylating agents, is an indication of fertility preservation.
- Offer them ovarian tissue cryopreservation option.

Practical Clinical Tips

1. Ovarian tissue cryopreservation is the only option that can be offered when chemotherapy already started or after a low gonadotoxic chemotherapy regimen in children.

A young patient, 18 years old, was diagnosed with AML and had severe pancytopenia, requiring first-line chemotherapy followed by HSCT.

- Inform the patient about the high risk of premature ovarian insufficiency after HSCT.
- Discuss the fertility preservation options.
- Propose ovarian tissue cryopreservation after the first-line chemotherapy.

Practical Clinical Tips

1. Oocyte cryopreservation is not feasible when the oncological treatment should start within a short delay or when health conditions contraindicated ovarian stimulation.
2. Ovarian tissue cryopreservation should be proposed after the first-line chemotherapy in leukaemia patient in order to reduce the risk of disease contamination in the cryopreserved ovarian tissue.

Key Readings

1. Corkum et al. [33]
2. Diesch et al. [28]
3. El Issaoui et al. [38]
4. Demeestere et al. [76]
5. Irtan et al. [72]
6. Sklar et al. [21]

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Elective Egg Freezing

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Introduction

Fertility preservation (FP) has become a specialized branch of reproductive medicine aimed at preserving women's reproductive potential and future genetic motherhood. The American Society for Reproductive Medicine (ASRM) removed the experimental label from oocyte cryopreservation (egg freezing) in October 2012, thereby allowing both medical egg freezing (MEF) and elective egg freezing (EEF) to be practiced (although the latter without official ASRM endorsement) [1, 2]. Today, an increasing number of mostly healthy single women are utilizing this fertility preservation option [3, 4], not only in the United States but in many other countries around the globe [5].

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In this chapter, we begin by describing the role of the laboratory in oocyte cryopreservation. We then turn to women's reasons for pursuing EEF, arguing that "lack of a partner" is the key factor, as shown in a number of quantitative and qualitative studies [6–18]. We explore the EEF difficulties that single women face, thereby emphasizing the need for support and a patient-centered approach to EEF care in in vitro fertilization (IVF) clinics [19].

The Role of the Laboratory: Slow Freezing and Vitrification

The human oocyte is a cell of approximately 120 μm, of spherical shape with a small surface-to-volume ratio. To be cryopreserved, intracellular water needs to be removed by exposure to cryoprotective agents (CPAs). This is a very important step, because residual intracellular water can lead to intracellular ice formation during freezing (or vitrification) and thawing (or warming) [20]. In addition to intracellular ice crystals, oolemma permeability may be impacted by shifts in temperatures, thus playing a role in reduced oocyte survival and viability after cryopreservation and thawing. The osmotic stress occurring during dehydration and rehydration of slow freezing has been noted, impacting the MII spindle structure, with both hypo- and hyperosmotic conditions causing a decrease in the proportion of oocytes with a normal microtubular structure.

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Many protocols of slow freezing have been tried over time, and the one involving a lower sucrose concentration (0.1 M) was associated with a higher rate of normal spindle and chromosome configurations. Still with slow freezing, the survival rate of oocytes varied from a low of 35–40% to a high of 70–75%, based on differences in the sucrose concentration in the freezing solution from 0.1 to 0.3 mol/l. However, improved post-thaw oocyte survival and better fertilization with the 0.3 mol/l sucrose protocol was counterbalanced by a higher embryo implantation rate in the lower (0.1 M) sucrose concentration [21, 22].

The advent of vitrification has thus been a game changer for the efficiency and simplification of the egg freezing procedure. The vitrification process resolved two of the main reasons causing oocyte damage during slow freezing, namely, membrane chilling injury and the lethal ice crystal formation. With vitrification, the chilling injury of the oocytes is avoided by both the fast cooling (rapid immersion of the oocytes in liquid nitrogen) and warming rates of the process, which outruns the time needed for membrane phase transition to occur [23]. Ice crystals are avoided by the high viscosity of the solution with cryoprotectant and most importantly by keeping the biological samples in small volume (so-called minimal drop size, or MDS), which together with the high cooling rate enables the vitrification process to occur [24].

With this new laboratory approach, the egg survival rate after rewarming increased from 70 to 90%, and the pregnancy rates almost approached those seen with the use of fresh eggs [25–29]. Today, egg vitrification/rewarming is the most common method used for both EEF and MEF worldwide.

The Role of (Absent) Partners:Women's Motivations for Elective Egg Freezing (EEF)

Oocyte cryopreservation in healthy women has been called variously "social egg freezing," "nonmedical egg freezing," "elective oocyte cryopreservation," "elective fertility preservation," "oocyte banking for anticipated gamete exhaustion," and "planned oocyte cryopreservation" [8, 17, 27, 30]. Given the ongoing lack of agreement on the best nomenclature, we have proposed that "elective egg freezing" (EEF) be added to the glossary of accepted terms [3, 4], because it most closely mirrors women's preferred usage.

With the laboratory advancements afforded by vitrification as described above, the demand for EEF is steadily growing. For example, in the United States, according to the most recent Society for Assisted Reproductive Technology (SART) statistics, the total number of egg freezing cycles for both MEF and EEF rose from 5000 to 12,390 between 2013 and 2018 [31]. MEF and EEF are also increasingly in frequency around the globe. For example, of 82 countries reporting to the International Federation of Fertility Societies (IFFS) 2019 survey, 68 (83%) allowed egg freezing for medical fertility preservation, 56 (68%) allowed egg freezing for nonmedical indications, and 18 of 42 (43%) countries also reported frequent performance of egg freezing cycles in their clinics [5].

What are the reasons behind this global surge in EEF cycles? Several anonymous surveys have provided unequivocal evidence that partnership problems and advancing reproductive age play major roles in women's EEF decisions. For example, a survey of 183 American women who had completed at least one cycle of EEF during the 2005–2011 years showed that 84% were age 35 or older, and 88% had completed at least one cycle of EEF because they lacked a partner [18]. Another survey of 86 women in Belgium found that women were 36.7 years of age on average, and the overwhelming majority, 81%, lacked partners [8]. Similarly, in Australia, a survey of 96 women who completed EEF between 1999 and 2014 showed that nearly half (48%) were 38 years or older, 90% were unpartnered, and 94% had not returned to use their frozen eggs because they were not interested in becoming single mothers [6, 7].

In our own large-scale, interview-based, qualitative study of 150 women who had completed EEF through 4 American IVF clinics and 3 in Israel, we discovered that 85% of women were pursuing EEF at an average age of 36, because

they lacked a partner with whom to pursue marriage and childbearing [3]. Among these single women, six main pathways to EEF were found (being single, divorced, broken up, deployed overseas, single mother, career planner). Career planning, mentioned by only 2% of women, was the least common pathway. Similarly, among the 15% of women who were partnered at the time of EEF, four pathways to EEF were found (relationship too new or uncertain, partner not ready to have children, partner refusing to have children, or partner having multiple partners), again demonstrating that women's "partnership problems" impeded their marriage and childbearing prospects [4].

In short, our study demonstrated that partnership problems—and not career plans—are the main pathway to EEF among otherwise healthy women. In a few of these cases (six in our study), women who were unable to find partners decided to freeze their eggs on the path to single motherhood. However, whether this EEF-assisted "single motherhood by choice" (SMBC) [32] will continue to grow is uncertain.

In summary, the absence of a committed male partner is the main reason why women are pursuing EEF, generally toward the end of their reproductive lifespans. Even though this "men as reproductive partners" problem has been well defined in public health scholarship [33], it has been insufficiently addressed in the assisted reproduction literature.

The Role of the Clinic: The Need for Patient-Centered EEF

Insufficient attention has also been paid to the specific needs of patients undergoing fertility preservation, as they navigate the various challenges of ovarian testing, stimulation, and retrieval, often on their own. The need for patient-centered clinical care has been well documented over the past decade and is now considered one of six key dimensions of quality care, the others being safety, effectiveness, timeliness, efficiency, and equity of access.

In our recent study of EEF patients, we followed the conceptual framework of "patient-centered infertility care" [19] to identify two broad categories and 11 specific dimensions of patient-centered EEF care, as identified by women in our study. These included (1) *system factors*, i.e., information, competence of clinic and staff, coordination and integration, accessibility, physical comfort, continuity and transition, and cost, and (2) *human factors*, i.e., attitude and relationship with staff, communication, patient involvement and privacy, and emotional support.

As our study showed, EEF patients are fundamentally different from IVF patients, in that the former are generally single, while the latter are generally married (or partnered). Women seeking EEF are affected by their singleness. As noted above, it is the very reason that most women are motivated to seek EEF in the first place. Freezing one's eggs because of the lack of a male partner is a difficult proposition for many women who may experience their partnership problems as difficult, frustrating, and emotionally wrenching. Furthermore, these mostly single EEF patients may feel a sense of isolation and loneliness in the couple-oriented world of IVF, where their lack of male accompaniment adds "insult to injury." Thus, fertility clinics serving EEF patients must be aware of, and highly sensitive to, EEF patients' life circumstances [19]. To the extent they can, clinics should make every effort to provide spaces, materials, and support designed specifically for the largely single EEF clinic population.

On an ethical level, patient-centered EEF care should also entail the protection of EEF patients from non-unified standards of practice and promises of success fueled by entrepreneurs seeking to capitalize on women's fears of losing their reproductive chances if they do not freeze their eggs (e.g., with statements such as "smart women freeze" during martini infomercial cocktail parties) [34]. It is ethically irresponsible not to provide full information and to encourage women to put inordinate faith in techniques that, while exciting and even liberating, have no guaranteed success rates and have very high costs often not covered by insurance.

Doctors and clinics offering egg freezing services should tell patients up front what they need

to know [35]. This includes (1) the number of cycles it might take to yield enough eggs, (2) the cost for each cycle of cryopreservation including the medications, (3) the cost to store the eggs per year, (4) the known and unknown success rates for egg freezing at various ages, (5) the costs associated with the future use of the frozen eggs with intracytoplasmic sperm injection (ICSI), (6) the potential risks like ovarian hyperstimulation syndrome (OHSS) during the ovarian stimulation, and (7) finally, the chances that the procedure may not work and will not produce a frozen egg baby.

Clinicians should also be clear about the disposition of frozen eggs in case of death, severe disability, or occurrence or recurrence of a life-threatening illness or if a decision is made not to use one's frozen eggs. Without absolute confidence and trust, demand for transparency of results, and standardization of care, patients seeking EEF will continue to find themselves confused and, in many cases, disappointed [35].

Future Directions

Success with oocyte vitrification has opened the possibility for healthy single women to preserve their reproductive potential. However, there are still many unanswered questions that need additional research before EEF options can be widened.

First, at what age should EEF be offered? Cost-benefit analysis has demonstrated so far that women at the age 35 are the ones most likely to use their cryopreserved eggs. Thus, freezing eggs in the early 30s may be ideal, whereas women in their 20s should not be encouraged as candidates for this procedure [36].

Second, who should inform reproductive-age women about age-related fertility decline? With whom should these women discuss their reproductive goals and desires? A recent survey of knowledge and attitudes of obstetrician/gynecology residents showed that there is a profound gap in knowledge about reproductive aging, as well as lack of familiarity on how to approach the discussion with women patients without being perceived as intrusive or "pushing" motherhood [37].

Third, should ovarian tissue freezing, alone or in combination with in vitro maturation of germinal vesicle oocytes harvested from the ovarian cortex, be proposed as an alternative to egg freezing [38]? Preliminary studies show an average in vitro maturation rate of around 30%, and so far a number of live births have been described in case reports [39, 40]. However, little is known about the developmental capacity of these oocytes, and more research is needed.

Finally, who will the next generation of EEF users be? As EEF continues to spread around the globe [5], more research will be needed to track this global diffusion of vitrification technology and the reasons why women are pursuing fertility preservation. Will they be "30-something" women without partners? Will they be younger women planning their careers? These global social dimensions of EEF constitute a critical research direction for the 2020s and beyond.

Clinical Cases and Practical Tips

Case 1. A Lack of Patient-Centered EEF Care

Julie was an IT director at a major Silicon Valley tech firm which only offered fertility insurance benefits to married couples. Single at the age of 36 and worried about her fertility, Julie pursued EEF, but without insurance coverage. Julie felt both discrimination and humiliation as a single woman making efforts to preserve her remaining reproductive potential. Her feelings of shame were amplified in her clinical interactions with IVF clinic staff and other patients, all of whom assumed she was married. "I really felt like they didn't even know how to ask the questions," Julie explained. "I mean, everything was about 'your partner'…not only on the insurance side, but even when I was going through the classes to learn how to inject myself and all of that. I mean, I was the only single person in there, you know. And I even

remember one of the women saying to me, 'Well, you know, don't worry; my husband didn't come with me to the first class, either.' And I said: 'Well, I'm actually not married. I don't have a partner. I'm actually going through this because I'm going to be freezing my eggs.' It's already an emotional time and an emotional issue. And, like I said, for a lot of people it can feel like, 'What's wrong with me?' You kind of feel like this is something that you should be able to do. You should be able to reproduce, and this is how it should be happening. And just all of their questions! I mean, they are: 'How often are you having sex?' Really? Is this really necessary? Just the way that they ask the questions and phrase the questions to go through this. It's incredibly invasive at a time when you are already feeling emotional, overwhelmed. It's a very sensitive topic, and there's just no sensitivity in how this is handled."

Clinical Tips: Make sure that all IVF clinic staff, both physicians and nurses, are aware of which patients are pursuing EEF, especially those without partners. Eliminate "partner" language and queries from clinical protocols and documents addressed to EEF patients. To the extent they can, all IVF clinics should make every effort to provide spaces, materials, and support designed specifically for the largely single EEF clinic population.

Case 2. Patient-Centered EEF Care and Outcomes

Janice, a Chinese-American academic physician, was 37 years old when she undertook two successful cycles of egg freezing, ultimately storing 44 mature eggs. Recently partnered but uncertain about the future of her relationship, she undertook EEF on her own with the help of supportive, patient-centered IVF clinic staff. As an unmarried woman attending the IVF clinic, Janice explained that "it's easy to start feeling a little humiliated sometimes, just because you know, you don't want to be [there]. There's so many portrayals of the cougars, the desperateness, and they're always high strung and kind of bossy and Type A. They're always running around trying to catch the man, and being, what's wrong with me? You know? It's this neurotic kind of crazy person. And so you know, it is scary initially, to feel like you might be typecast like that. But at least at [the IVF clinic] the nurses were so nice, the doctors were so nice. They never for a split second made me feel like I was doing something odd or crazy or out of the norm. It really relaxed me about the whole thing…and I'm really glad I did it. I just wish I'd done it sooner. I'm sure you've heard that before, too!"

Clinical Tips: Realize that single women in their late 30s like Janice feel trepidation about entering an IVF clinic alone, where they may be typecast and pitied. Normalize the EEF experience for single patients to reduce stigma and increase patient satisfaction. Make patient-centered EEF the standard of clinical care.

Take-Home Messages

- Vitrification has been a game changer in the world of oocyte cryopreservation, simplifying laboratory procedures, making the egg freezing process more efficient, increasing egg survival rate after rewarming from 70 to 90%, and leading to pregnancy rates almost approaching those seen with fresh eggs.
- Around the world, increasing numbers of women are turning to elective egg freezing (EEF) as a form of fertility preservation; most are in their late 30s and without a male partner committed to marriage and childbearing.
- Single women pursuing EEF without a male partner need patient-centered sup-

port in the couple-oriented world of in vitro fertilization (IVF) clinics.
- Patient-centered EEF care should also entail the protection of EEF patients from non-unified standards of practice and promises of success, especially given the very high costs of EEF often not covered by insurance.

Definitions

Fertility preservation (FP): a specialized branch of reproductive medicine aimed at preserving women's reproductive potential and future genetic motherhood.

Medical egg freezing (MEF): fertility preservation through oocyte cryopreservation (aka egg freezing) techniques undertaken for women with whose medical conditions or treatments (e.g., cancer chemotherapy) threaten their future fertility.

Elective egg freezing (EEF): fertility preservation undertaken electively by otherwise healthy women to preserve their fertility, usually in their late 30s and in the absence of male partners with whom to pursue childbearing; "elective egg freezing" (EEF) most closely mirrors these women's preferred usage.

Vitrification: a process of cryopreservation by which oocytes are first treated with various concentrations of cryoprotectants to prevent ice crystal formation and then rapidly submerged into liquid nitrogen (−196 ° C) to become vitrified. Vitrification has proved to be a "game changer" in the world of oocyte cryopreservation, simplifying the procedure and increasing its efficiency.

Key Readings

See References [1, 3, 19, 27, 29, 30, 35].

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Ovarian Tissue Banking to Postpone Menopause

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Abbreviations

cMHT	cellular hormone replacement therapy
CVD	cardiovascular disease
ER	estrogen receptors
GPER	G protein-coupled estrogen receptor
pMHT	pharmaceutical postmenopausal hormone therapy
PR	progesterone receptors
SERM	selective estrogen receptor modulators
WHI	Women's Health Initiative

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Introduction

The physiological function of the ovaries is to pass on genetic material from one generation to the next. The ovaries undertake this role by providing two major functions: they (1) produce haploid germ cells in the form of mature oocytes that upon fertilization by a sperm can develop into an embryo that can sustain foetal development and (2) secrete a multitude of hormones that facilitate implantation in the uterine endometrium and exert numerous functions elsewhere in the body. Especially, secretion of sex steroids is of importance, including oestrogens and progestins that are mandatory for successful procreation.

The vast majority of oestrogens are produced by the ovaries within the preovulatory follicle producing more than 90% of the oestrogens available in the body during the follicular phase of a specific menstrual cycle. Oestrogens are a class of C_{18} highly conserved steroid hormones, which are produced in all vertebrates with various agonists and antagonists widely found in plants and in food and diets. Four main forms of oestrogen are found in mammals: 17β-oestradiol,

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17α-oestradiol, oestrone and oestriol. Oestrogen mediates its actions via oestrogen receptors (ERs), of which three different types have been identified so far [1].

The main forms of progestins include progesterone, 17-OH-progesterone, 20α-progesterone and various other metabolites of progesterone. Progestins signal through the progesterone receptor (PR), which are one of the master regulators in female reproductive tissues controlling developmental processes including proliferation and differentiation in the reproductive cycle and during pregnancy [2].

The Challenge of the Aging Population

The ovarian reserve (i.e. the number of resting primordial follicles) for each individual woman is determined during foetal life and will define her subsequent reproductive potential. These follicles will support the entire length of the reproductive lifespan. Once the pool of resting follicles is exhausted irrespective of the chronological age of the woman, the ovarian function will be lost, including the inability to reproduce and to secrete the whole armamentarium of hormones and growth factors released from the follicle and the corpus luteum. This invariably leads to menopause, which reflects that no more follicles are present in the ovaries. The age of menopause has remained constant for many years—on average, women enter menopause at the age of 51 years [3].

This constant age of menopause is in sharp contrast to the increased lifespan seen in the industrialized countries where life expectancy today is far beyond any historic records. Today, half of all new born girls are estimated to live more than 100 years in many Western countries [4]. In the USA, people above the age of 85 years are the fasting growing population group. Currently, the average life expectancy for women in Japan has increased from around 50 years right after the Second World War to currently exceeding 87 years and continues to increase, resulting in more than 70,000 women currently being more than 100 years old—a sevenfold increase over the last 20 years. It is estimated that during the next 30 years, the population in the industrialized countries above 65 years will double and constitute more than 20% of the population. This remarkable increase in life expectancy in the industrialized countries is probably the most important societal change occurring during the twentieth century. This means that many women will live three to four decades in menopause or even more and that there will undoubtedly be an increase in the number of women who will experience sequelae from menopause. In fact, a number of diseases develop as a consequence of menopause and the cessation of ovarian sex hormone secretion, including the risk of osteoporosis and cardiovascular disease (CVD) [5].

Postponing Menopause with Autologous Ovarian Tissue: A New Alternative Solution

The increasing life expectancy therefore possesses a serious female health issue primarily due to the lack of sex steroid influence on all parts of the body after menopause (see next paragraph). One aspect of this review is to discuss how these issues may be addressed. As these matters affect large groups of women, it is likely that not only one measure or method is applicable to all, but different and potential new options should be considered. The current review introduces new options that are discussed and which may be more appropriate to some groups of women than to others. The Word Menopause Day in 2014 concluded that individualization and prevention is the focus with the following key points [6]:

1. From a medical economics perspective, preventing disease is much preferred over treatment.
2. Major chronic diseases occur after menopause.
3. Some of these conditions (CVD) accelerate in women after menopause, and others (cancer) increase as a function of age in both men and women.

4. Our goal is to combat all diseases after menopause, with the aim of improving not only the quantity but the quality of life for all postmenopausal women.

It may be argued that preventive measures have already for many years been developed and put in place in the form of pharmaceutical postmenopausal hormone therapy (pMHT) [5]. The MHT indeed has been shown to exert beneficial effects on menopause-related diseases especially CVD [7]; however, it has its shortcomings. pMHT only provides a fraction of the sex steroids secreted from the follicle/corpus luteum, and quite often it contains artificial hormones, which exert some of the effects of the native hormones but not all of them [6]. Furthermore, it is difficult to tailor a hormonal profile that mimics the natural. Equally important, it turns out that many women are reluctant to continue therapy beyond a few years and often stop taking the pills before medical indications suggest termination. Furthermore, the initial data from the Women's Health Initiative (WHI) study, which was published in the early years of this century, reported an increased risk of breast cancer as a result of MHT use, which has since plummeted [8, 9]. Despite several adjustments in the overall outcome as well as in sub-studies covering breast cancer risk and risk of CVD in the WHI population and notably eliminating any risk from oestrogen-only therapy [10], there is still a considerable resistance among women to use pMHT, and an alternative method of protection against menopause-related diseases is warranted.

A radical different solution has been proposed by several different groups [11, 12], which focus on the fact that primordial follicles in the ovaries of women in the earlier reproductive years are available in large numbers. From this pool of follicles, 99% will undergo atresia and will not reach the preovulatory stage and will be of no use to the woman, either fertility-wise or as hormone-secreting structures. It has been argued that these follicles are deselected and are less valid as compared to the dominant preovulatory follicle, which contain an oocyte that may be fertilized and support a pregnancy. However, there is now ample evidence to show that the many follicles not selected for dominancy and ovulation do produce oocytes and hormones that fulfil the criteria for implantation as seen in connection with ovarian stimulation and assisted reproduction. So the proposition is that ovarian cortical tissue with its content of primordial follicles may be harvested during the reproductive active years and stored until menopause, where the tissue may be transplanted back to the woman herself and sustain ovarian function for an additional period of time—in essence, enabling an extended use of the ovarian pool of follicles over a time frame exceeding the age of menopause. This solution has been named cellular hormone replacement therapy (cMHT) and has been tested successful in different mouse and rat studies [13–15].

For cMHT to be an attractive option for women, a number of conditions should be considered, which include (1) the age of menopause, (2) ovarian reserve of the woman during her reproductive years, (3) being technically feasible with good recovery of follicle survival after freezing of the ovarian tissue and (4) being safe with minimal side effects and initially being of interest to some groups of patients, for instance, patients who may run a higher risk of menopause-related diseases like osteoporosis and CVD due to genetic or family-related conditions.

- Ad.1 There is experimental data to support that removal of one entire ovary affects the age of menopause only marginally with around 1 year—so instead of entering menopause on average at the age of 51 years, it may be advanced to around 50 years of age [16].This reflects that the remaining pool of follicles after the removal of ovarian tissue still amounts a huge number of follicles capable of maintaining normal cycles and monthly ovulations in woman until normal age of menopause.
- Ad.2 There is data to suggest that women with one ovary have similar results to those with two during IVF [17]. Interestingly, women with one ovary develop around 80% of the follicles that develop in women with two ovaries, reflecting that the atresia rate is reduced in

women with one ovary probably due to slightly increased levels of FSH caused by a reduced pituitary feedback inhibition.

- Ad.3 Methods to effectively freeze ovarian tissue have now been developed, which, when thawed and transplanted to the woman from where it originated with high efficacy, recreate ovarian function including fertility and menstrual cycles with cycling release of sex steroids and other hormones [18]. While the freezing process has now been documented as quite effective [19], only a fraction of the transplanted follicles survives the transplantation procedure, which obviously is a drawback. However, the transplantation efficacy is likely to become improved during the coming years, and already now many women having frozen/thawed ovarian tissue transplanted now experience several years of ovarian function with some having 5–10 years duration, during which they experience menstrual cycles and sex steroid release [20].
- Ad. 4 Undergoing surgery always presents a risk of in advert mistakes, but the safety of surgically removing ovarian tissue appears to be relatively safe. A recent German study found that from 1302 surgical procedures involving removal of ovarian tissue, only two cases experienced non-serious side effects [21].

The aim of this review is to discuss the *pro* and *cons* of this new solution in relation to the existing mainstream and well-proven pMHT method and to evaluate whether the new cMHT method is of interest to develop further with new studies and clinical trials to provide a broader knowledge on the effects of autologous transplanted ovarian tissue.

To answer this question, we focus on the global body effects of oestrogens with specific emphasis on the risk of breast cancer by continued oestrogen exposure in the form of pMHT.

Effects of Oestrogens

Oestrogens secreted from the ovaries have numerous effects throughout the body (review: [1, 22]). Oestrogens exert their effect through oestrogen receptors (see below). These are expressed in all target organs, where they undertake a multitude of functions. To better understand how oestrogens exert their many different functions, some of the main sites of oestrogen action are presented:

1. *Brain*. In the brain, all three oestrogen receptors are expressed, and oestrogens directly influence brain function. Oestrogens are neuroprotective and, by attenuating inflammation in neurons and glia cells, influence pain directly and influence brain function memory in multiple areas (review: [1]).
2. *Heart*. In the heart, all three receptors are expressed, where oestrogens act to prevent cardiomyocyte dysfunction and where especially GPER recently has received attention as being of special interest. CVD is a major cause of death in women in the developed world, and the risk increases at menopause, and oestrogen probably exerts the protective effect. Epidemiologic evidence further suggests that premature or early natural menopause [22, 23] and a shorter total reproductive period positively correlate with the incident heart [24]. Recently, it has been suggested that the risk of CVD should be evaluated not only by looking at oestrogen levels but at the relative ratios of oestrogens to androgens.
3. *Skeletal muscles*. Skeletal muscles express all three receptor types, resulting in a protective effect of oestrogens on damage and inflammation.
4. *Bone*. In bones, oestrogens regulate turnover and growth of bones and most importantly attenuate development of osteoporosis and possess the possibility of signalling through all three receptors.
5. *Vasculature*. In peripheral vasculature and coronary arteries, oestrogens increase vasodilation, while they inhibit development of atherosclerosis and the response of blood vessels to injury and oestrogen receptor-mediated regulation of microRNA inhibits proliferation of vascular smooth muscle cells.
6. *Breasts*. Both normal and the neoplastic breast epithelium increase proliferation upon stimulation with oestrogens.

7. *Adipose tissue*. Metabolism of adipose tissue is affected by oestrogens.

Many observational studies have shown a benefit of pMHT for the abovementioned organs as well as other organs (review: [1, 22]). Counterbalancing these positive effects, pMHT was considered to significantly increase the risk of breast cancer especially in regimens including progestins. However, in order to understand the wanted and unwanted effects of oestrogens, a closer look at the oestrogen signalling and the target cells is likely to advance understanding.

Oestrogen Receptors

Oestrogen mediates its actions via three identified oestrogen receptors (ERs) [25]. Oestrogen receptor subtypes α (ERα) and β (ERβ) are classical nuclear hormone receptors, which upon binding of oestrogen translocate from the cytosol to the nucleus to initiate expression of target genes [26, 27]. In addition, ERs can be found on the cell membrane via covalent coupling to fatty acids such as palmitic acid (so-called palmitoylation) and coupling to amino acid of membrane-bound proteins. At the cell membrane, ERs facilitate the activation of rapid intracellular signalling cascades that may be different from the classical signal transduction pathways [28, 29].

In addition, a membrane-bound ER distinct from ERα and ERβ has recently been recognized outside the nucleus and was identified as the orphan receptor GPR30 and named G protein-coupled estrogen receptor (GPER) [28, 30, 31]. The GPER is a novel membrane-bound receptor that facilitates non-genomic actions of oestrogens including 17β-oestradiol. While the exact signalling and transduction pathways that GPER initiate are incompletely understood, its actions have been suggested to depend on cell type, site of action and the relative concentration as compared to the other two ERs [32]; however, all three receptors bind oestradiol with similar affinity.

ERα and ERβ exists as monomers in protein complexes in the cytosol. Upon activation with oestrogens, the ER monomers dissociate from these complexes and dimerize with the other free monomers. Thus, different dimers may be formed ERα–ERα or ERβ–ERβ homodimers or the heterodimers ERα–ERβ. These dimers enter the nucleus and cause signal transduction with gene transcription. The specific expression profile induced or inhibited depends on the receptor type, the cell type, the type and concentration of oestrogen and the type of dimer formed plus the presence of different transcriptional cofactors. Further, there is now selective GPER agonists that do not activate the classical ERs at a broad range of concentrations [33], illustrating that oestrogen action is highly complex and may not easily be mimicked by exogenous administration of oestrogens. In addition, several selective estrogen receptor modulators (SERM) have also been identified. These modulators have been developed in order to find compounds with beneficial estrogenic effects on specific tissues, such as bone and the cardiovascular system without the harmful side effects, again illustrating that oestrogen action is indeed multifaceted and difficult to define in detail with current knowledge.

To further complicate the picture of oestrogen actions, single-nucleotide polymorphisms (SNPs) have been shown to exist in both ERα and ERβ with apparent associations to the prevalence of various diseases [33].

Taken together, the precise result of oestrogen stimulation on various cell types in different tissues is very difficult to recapitulate. It is dependent on the concentration and type and oestrogen ligands and the type and concentration of receptors expressed. The effect of how exogenous administered oestrogens administered in the form of hormone replacement therapy is obviously further very difficult to predict, making it a challenge to develop MHT regiments that will work for women in general.

Effect of Progestins and Progesterone Receptors

As the name indicates, progesterone is indispensable for pregnancy establishment and maintenance, and progesterone and its metabolites

exert important functions during the menstrual cycle. Progesterone acts primarily via the well-characterized classical signalling pathway involving a classical member of the nuclear receptor family of ligand-dependent transcription factors (PR), similar to ERα and ERβ, which upon ligand binding results in subsequent activation of genes containing progesterone response elements (PREs) [34, 35]. The classical PR is expressed as two isoforms, PR-A and PR-B. PR-B contains an additional 164 amino acids at the amino terminus [36]. The two isoforms are in humans generally expressed at similar levels in the breast, but mouse studies suggested that PR-B is the functionally important form in the mammary gland, whereas PR-A is important for ovarian function [37]. It has been suggested that PR mediates variable responses to progesterone in a context-dependent manner, and the specific PR isoform expression may be involved in these differences. Further, a recent review concluded that the functional diversity of PR may relate to the conformational flexibility of PR structure and that unique signalling mechanisms contribute to cell-specific actions of progesterone [38].

Taken together, the cellular signals that progesterone as a ligand to PR provides are intricate and complex, being dependent on the target cell and the local environment in which the PR is expressed. On top of this different progestin ligands, concentration of ligands, absolute and relative to one another, makes it further complicated to delineate the response to progestin ligands either in the form of natural secreted ligands or as ligands provided in connection with pMHT.

Breast Cancer and Continued Oestrogen Exposure

Among prescribers and users, breast cancer is perceived as the main risk associated with pMHT as it is a hormone-dependent cancer [39]. In postmenopausal women, about 80% of cases are estradiol receptor-positive. In cohort studies, only estradiol receptor-positive breast cancers are associated with pMHT. Different levels of risk with estrogen-only treatment and combined treatment with oestrogen + progestin are shown in randomized trials and observational studies [21, 40, 41]. The biological mechanism is probably promotion of tumour growth and not induction [42]. At the same time, a variety of positive effects on other vital organ functions are quite well established as mentioned above. Initiating pMHT close to menopause appears to reduce all-cause mortality and cardiac death, with no evidence of an increase in breast cancer mortality [43]. However, pMHT appears to increase the incidence of breast cancer, depending on the type of pMHT [44, 45]. Recently, a new meta-analysis, including prospective studies from 1992 to 2018, was published from the Collaborative Group on Hormonal Factors in Breast Cancer [21]. The overall conclusion was that the effect of pMHT on breast cancer risk may depend on the type of pMHT, dose, duration of use, regimen, route of administration, prior exposure, and individual characteristics.

The prevalence of breast cancer cases for women starting to use pMHT around the time of menopause was compared to women who never used pMHT [21]. The breast cancer risk of women who never used pMHT was 6.3% from age at menopause and 20 years beyond (age range of 50–69 years). Table 1 shows women who used pMHT for 5 or 10 years starting at the age of 50, followed by 15 or 10 years, depending on the type of pMHT used.

All types of pMHT, except vaginal oestrogens, were associated with increased risks of developing breast cancer. The risks were greater for oestrogen–progestogen than for oestrogen-only preparations (RR 2.08; CI 2.02–2.15 vs. RR 1.33; CI 1.28–1.38), particularly if the progestogen was given daily rather than intermittently (Table 2) [41, 45, 46].

The risk of breast cancer was also associated with high and low body weight of the woman with a reduced risk in both obese women and lean women. Further, the associations between breast cancer risk and pMHT were much stronger for oestrogen receptor-positive tumours than for oestrogen receptor-negative tumours [21].

Table 1 Relative risk of being diagnosed with cancer after pMHT use for 5 or 10 years

Type of pMHT	Incidence of breast cancer for nonusers(%)	Incidence of breast cancer for 5 years use	Incidence of breast cancer for 10 years use (%)
E_2+ daily P_4	6.3	8.3% (RR 2·30, CI 2·21–2·40)	10.3
E_2 + intermittent P_4	6.3	7.7% (**RR** 1·93, **CI** 1·84–2·01)	9.2
E_2 only	6.3	6.8% (RR 1·33, CI 1·28–1·37)	7.4

Adapted from the Collaborative Group on Hormonal Factors in Breast Cancer (2019)
pMHT postmenopausal hormone therapy, *E_2* estrogen, *P_4* progesterone

Table 2 Relative breast cancer risk in relation to pMHT duration

Risk according to duration of pMHT use	Relative risk? for current users <1 year	RR for current users of 1–4 years	RR for current users of 5–9 years	RR for current users of 10–14 years	RR for current users ≥15 years
E2 + P4	1.20 (1.01–1.43)	1.60 (1.52–1.69)	1.97 (1.90–2.04)	2.26 (2.16–2.36)	2.51 (2.35–2.68)
E2 only	1.08 (0.86–1.35)	1.17 (1.10–1.26)	1.22 (1.17–1.28)	1.43 (1.37–1.50)	1.58 (1.51–1.66)

Adapted from the Collaborative Group on Hormonal Factors in Breast Cancer (2019)
pMHT postmenopausal hormone therapy, *E_2* estrogen, *P_4* progesterone

Table 3 Relative breast cancer risk in relation to pMHT to the previous use of pHTR

	Past users: < 5 years since last use				Past users: 5–9 years since last use				Past users: ≥ 10 years since last use			
	D < 1 y	D 1–4y	D 5–9 y	D ≥ 10 y	D < 1 y	D 1–4 y	D 5–9 y	D ≥ 10 y	D < 1 y	D 1–4 y	D 5–9 y	D ≥ 10 y
E2 + P4	0.98 (0.85–1.14)	1.18 (1.09–1.29)	1.21 (1.14–1.29)	1.30 (1.25–1.37)	1.00 (0.89–1.14)	1.06 (0.98–1.15)	1.23 (1.15–1.30)	1.28 (1.19–1.38)	1.06 (0.95–1.19)	1.09 (1.00–1.18)	1.19 (1.10–1.28)	1.28 (1.15–1.43)
E2 only	1.12 (0.93–1.36)	1.03 (0.92–1.15)	1.06 (0.97–1.16)	1.21 (1.12–1.30)	1.06 (0.88–1.28)	1.07 (0.96–1.20)	1.06 (0.97–1.16)	1.20 (1.12–1.30)	0.99 (0.87–1.12)	1.04 (0.95–1.13)	1.14 (1.04–1.25)	1.29 (1.16–1.42)

Adapted from the Collaborative Group on Hormonal Factors in Breast Cancer (2019)
D duration of usage, *pMHT* postmenopausal hormone therapy, *E_2* estrogen, *P_4* progesterone

Taken together, these data reinforce that there is significant increased risk of breast cancer by taking pMHT. This risk is dependent on a number of conditions, such as age at pMHT onset, the specific type of pMHT administered and especially the progestin component which appears to be of importance.

However, the data presented from the Collaborative Group on Hormonal Factors in Breast Cancer (Tables 1–3) represents a significant degree of MHT preparations, which are no longer marketed or have higher doses than currently recommended. Also the relative risk figures are discordant/higher compared to a recent Cochrane review [47].

The mechanism by which the risk of breast cancer is increased is only partly understood. It probably is a result of several different effects on cells in the breast, reflecting the complexity and multitude of ways that signal transduction can be modulated and can occur during the different sex steroid receptors. This implies that apart from the unique possibility of using cryopreserved natural sources of endogenous oestrogen, also a tailor-made approach, including the principle of personalized medicine, needs to be further acknowledged [48, 49].

Conclusion

It is clear that an important health challenge is arising as the longevity in the industrialized countries increases. Women live a steadily increasing part of their life in menopause, and the burden of the menopause-related diseases will invariably increase in the coming years. It also appears that existing therapies (i.e. pMHT, lifestyle changes and alternative medications) have their shortcomings and are only to a limited extend capable to solve the problem. New improved solutions are indeed warranted. To what extent the new radical proposal of storing ovarian tissue during the reproductive active years to be used as an autologous source of ovarian activity after natural menopause is obviously controversial and indeed involves surgery to be accomplished. In essence, this proposal will reduce the number of follicles recruited during the fertile years, however, not to a degree that will reduce the fertility potential. The woman will still have normal cycles and one preovulatory follicle ovulated per menstrual cycle. The cryo-stored ovarian tissue with resting follicles can upon transplantation extend the period in which menstrual cycles take place and maintain the natural hormonal fluctuations of a non-menopausal woman.

One advantage of this method is that it provides the natural fluctuations of all the hormones normally secreted from the preovulatory follicle and the subsequent corpus luteum and may therefore represent a continued physiological stimulation with oestrogens and progestins. In combination with the complexity and variability of oestrogen signalling in target cells, especially in different organs and sites, it may be attractive to maintain a natural composition of ligands being released as compared to the rather selective and sometimes unnatural types of hormones as provided with the pMHT options. However, whereas pMHT is a well-proven method with many years of experience, the cMHT option is completely new and needs to be approached with caution. cMHT is unlikely to be suited to more than a fraction of women (e.g. initially women with a known genetic predisposition for POI and women who already have ovarian tissue banked or women who during a caesarean section may choose to have an ovarian biopsy taken)—and indeed we need more data on a number of characteristics for this method, including the risk of breast cancer and other cancers, when menopause is postponed using cMHT. Potential other side effects also need to be studied in more detail. In addition, more precise information on the longevity of frozen/thawed ovarian tissue in relation to age, hormonal parameters and genetic dispositions needs to be evaluated in more detail.

Indeed, both methods possess advantages and disadvantages; how these methods may or may not receive increased attention in the future is too early to predict—probably the personal wish of the women themselves may turn out to be an important parameter.

Practical Clinical Tips (i.e. Within Clinical Cases)

Not applicable

Take-Home Messages

1. Important health challenges to the whole society are developing as life expectancy increased in most parts of the world far beyond the normal menopausal age of 51 years.
2. The incidence of menopause-related diseases will invariably increase including osteoporosis, cardiovascular disease, cognitive decline and many others.
3. It appears that existing therapies (i.e. pMHT, lifestyle changes and alternative medications) have their shortcomings and new alternative therapies are warranted.
4. The present paper suggests that cryostorage of ovarian tissue in the reproductive active years represents a radical solution that may be of interest to certain groups of women, which, however, requires further studies to be implemented at a larger scale.

Clinical Cases

Initially, this new option may be of interest to women with a known genetic predisposition for POI, women who already have banked ovarian tissue or women who, during a caesarean section, choose to have an ovarian biopsy taken.

Key Readings

See refs [1, 2, 4, 6, 8, 11, 13, 18, 21, 33, 45].

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Stem Cell Therapy to Approach Refractory Asherman's Syndrome

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Introduction

The endometrium is a highly regenerative tissue that regenerates every month after each menstrual cycle. Its main function is to enable implantation of the embryo at the right moment. If implantation of the embryo does not occur, the endometrium is partially destroyed and menstruation takes place, producing a new generation of tissue (upper 2/3) in the next menstrual cycle. This endometrial renewal ('self-renewal') is mostly regulated by hormones during 400–500 cycles during a woman's reproductive lifetime. Only tissues with high cellular turnover, such as epidermis, gut epithelium, and bone marrow, have this high cellular turnover. An increasing amount of evidence supports that this process is regulated by endometrium-derived stem cells (EDSCs) [1]. Histologically, the endometrium is divided in two functional layers: the basal and functional layers. The functional layer responds to progesterone and estradiol, and this layer is completely shed during menstruation. The basal layer does not respond to hormones and also does not suffer desquamation, from which it regenerates the mucosa. Recent studies [2] have described the transcriptomic signature of the endometrium at a single cell level, showing that the endometrium is composed of six major cell types including ciliated and non-ciliated epithelia, stromal cells, endothelial cells, lymphocytes, and macrophages. Interestingly, in this study, four major phases of endometrial transformation are described and provide evidence for direct interplay between stromal fibroblasts and lymphocytes during decidualization. Moreover, an abrupt transcriptomic opening of the window of implantation takes place at mid-secretory phase in unciliated epithelial cells.

The biology of the menstrual cycle is a coordinated and complex sequence of events involving the hypothalamus, pituitary, ovary, and finally endometrium. During the menstrual cycle, endometrial breakdown and repair occur simultaneously, side by side, under a carefully regulated balance, which has been termed 'orderly inflammation' [3]. The balance among these processes is critical to understand menstruation and menstrual cycle physiology [4], whose ultimate function of the endometrium is allowing an embryo to implant. However, implantation is not an efficient process, since only 25% of the transfered embryos implant per cycle. It has been estimated that inadequate uterine receptivity is responsible for approximately two-thirds of implantation failures, whereas the embryo itself is responsible for only one-third of these failures [5].

This complex endometrial homeostasis is regulated by stem cells, which is ultimately controlled by the niche. The niche is defined as the

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specific physiological microenvironment in which niche cells secrete molecules to regulate stem cell proliferation and cell fate decisions according to the tissue needs [6]. According to several studies [7, 8], stem cell niche seems to reside in the basal layer of the endometrium. This basalis layer is mostly vascularized by the spiral arteries.

In that sense, several groups have proposed different endometrial stem/progenitor cell candidates, including epithelial, mesenchymal, and endothelial cells, which may contribute to the rapid endometrial regeneration following menstruation [8–11]. Moreover, other groups have managed to prove the capacity of certain endometrial cells to differentiate into cell types from the three different germ layers such as chondrocytes, neurons [12], and insulin-producing cells [13].

Moreover, side population (SP) cells (i.e. cells identified as having the capacity to extrude the DNA-binding dye Hoechst via the ATP-binding cassette transporter G2 (ABCG2) and that generally have a stem cell-like phenotype) contribute to human endometrial regeneration in vitro and in vivo in animal models and human patients [14, 15]. In addition to that, established evidence reported bone marrow-derived stem cells (BMDSCs) to contribute to the repair and regeneration of tissues and organs [16], including murine [17–19] and human endometrium [20–24]. According to these findings, BMDSCs have the capacity to differentiate into fully functional stromal and epithelial endometrial cells.

Finally, certain pathological entities such as Asherman's syndrome (AS) are caused by the absence of functional endometrium that leads to infertility. Therefore, stem cell therapies targeting the endometrial niche with the ultimate aim to improve endometrial function may represent a promising therapeutic approach.

Asherman's Syndrome (AS)

Asherman's syndrome (AS) is an acquired condition defined by the presence of intrauterine adhesions (IUAs), that is, the uterine walls adhere to one another, due to the loss of endometrial stem cells caused by previous curettages or infections (endometritis), and represents a chronically debilitating condition. AS is associated with pelvic pain, recurrent miscarriage, infertility, amenorrhea, and psychological distress associated with these symptoms. AS may also be referred to as intrauterine adhesions/scarring, synechiae, or Fritsch syndrome [25].

The prevalence of AS can vary significantly in different countries, mostly due to different causative factors among countries where gynaecological care is not state of the art (e.g. use of sharp, blunt, or suction curettage for puerperal and postabortal evacuation) or where illegal interventions (e.g. abortions) are performed at a higher frequency and/or to a lesser standard than in industrialized countries. In addition, other causative factors, such as genital tuberculosis, may also have a higher prevalence in some countries. In Europe, the prevalence is difficult to calculate due to the lack of high-quality guidelines for the diagnosis of uterine pathologies in infertile women. However, the European Medicines Agency (EMA) has recently established the prevalence of AS in the EU as 4 in every 10,000 individuals [26]. Notably, this estimated value is below the threshold established by the European Agency for a condition to be considered as a rare disease, i.e. the prevalence is lower than 5 in 10,000. Therefore, based on these criteria, AS is considered to be a 'rare disease' and is included in the Orphanet database under the registry ORPHA137686 [27].

Histologically, AS causes endometrial fibrosis, in which the stroma is largely replaced with fibrous tissue and the glands are replaced by inactive cubocolumnar endometrial epithelium. The functional and basal layers are indistinguishable, with the functional layer replaced by an epithelial monolayer unresponsive to hormonal stimulation and fibrotic synechiae forming across the cavity. The tissue is usually avascular, although thin-walled telangiectatic vessels can be observed [25].

Sometimes different layers of the endometrium, myometrium, or connective tissue may be involved in the resulting intrauterine adhesions.

In the most severe cases, adhesions may be composed of collagen bundles, fibrous strips, or muscle with the same characteristics as normal myometrium [25]. Biopsy specimens from patients with intrauterine adhesions contain 50–80% more fibrous tissue than patients without intrauterine adhesions, limiting uterine myometrial activity and reducing the perfusion of sex steroids, resulting in atrophy [28].

Hysteroscopy is considered as the gold standard for the diagnosis of AS and provides a real-time view of the uterine cavity, allowing for a meticulous definition of the site, extent, and character of any adhesions, and it is the optimum tool for assessing the endometrium. Currently, this technique can be performed in ambulatory setting with less discomfort than a blind HSG and also makes immediate treatment possible in some favourable cases [29]. Moreover, the magnification and the direct view of the adhesions allow for a more precise and safer treatment.

It is paramount that AS be treated by experienced surgeons who use hysteroscopy to remove scarred tissue. However, adhesions have a tendency to reform, especially in more severe cases. Several different methods to prevent re-scarring after surgery for AS have been proposed. In this sense, hysteroscopic treatment of AS offers good results and resolves menstrual disturbance in the majority of cases [30]. Fertility restoration after hysteroscopic treatment seems to be influenced by several factors, such as menstrual pattern before and after the surgery, severity of adhesions, and adhesion recurrence rate after treatment. Data regarding reproductive outcome came, in the majority of cases, from non-randomized or prospective studies. An overall pregnancy rate from 40 to 63% was previously described [29, 30]. According to two prospective trials, with a total of almost 750 patients, pregnancy rates were 66% [31] and 30–35% [32] in moderate to severe cases of AS. Additionally, other retrospective studies have also assessed reproductive outcomes after hysteroscopy, achieving similar rates for moderate and severe cases [33]. Moreover, other reproductive consequences of AS, such as recurrent miscarriage, intrauterine growth restriction, placenta accreta, and others, are also well known [34].

Due to the possibility of adhesion recurrence, all patients undergoing surgery for intrauterine endometrial adhesions or endometrial fibrosis should be counselled about the possibility of a second surgery. This is most likely to occur when the adhesions are severe since these patients often require several repeated procedures because of the difficult nature of the procedure and the high rate of reformation of adhesions [35]. Other additional therapies, such as estrogen supplementation, insertion of intrauterine device (IUD) and intrauterine balloon, and hyaluronic acid among others, have also been proposed to prevent the recurrence of intrauterine adhesions [36] as suggested by certain studies.

Stem Cell Therapies

Many adult tissues contain populations of stem cells that have the capacity for renewal after trauma, disease, or aging. These cells may be found within the tissue or in other tissues that serve as stem cell reservoirs [16] and have the ability to proliferate into undifferentiated cells both in vitro and in vivo (self-renewal) and to differentiate into mature specialized cells [37].

Adult bone marrow stem cells represent an important reservoir of vascular progenitor cells that contribute to neoangiogenesis for different physiological and pathophysiological processes, such as wound healing and limb ischemia, post-myocardial infarction, endothelialization of vascular grafts, atherosclerosis, retinal and lymphoid organ neovascularization, and vascularization during neonatal growth and tumour growth [38]. Cells derived from the bone marrow express different biomarkers, including CD133 with endothelial progenitor capacity, and are known as endothelial progenitor cells (EPCs) [39]. Furthermore, these cells can be mobilized to the peripheral circulation as circulating endothelial progenitor cells (CEPCs) and improve neoangiogenesis afforded by pre-existing endothelium. In this sense, CD133+ and CD34+ differentiate into non-haematopoietic cell lineages and long-term

repopulation potential in SCID mice [40]. Moreover, there is also evidence that bone marrow-derived stem cells can engraft and fully differentiate into the endometrium [22, 24] in women who have previously undergone bone marrow transplantation.

Based on this evidence and previous physiological background, stem cell biology seems to be relevant to understand endometrial physiology, and, therefore, cell therapy offers an attractive alternative to the treatment of AS, since endometrial tissue had an intrinsic capacity of regeneration.

As previously explained, there is substantial evidence in literature that adult endometrial tissue contains epithelial progenitor cells and mesenchymal/stromal cells (MSC) [11] that may be the target of a specific therapy in order to regenerate the endometrial tissue in cases of dysfunctional or damaged endometrium.

A case report of a severe AS treated with autologous stem cells isolated from the women's own bone marrow has been described [41]. Briefly, a woman suffering from infertility and hypomenorrhea was treated hysteroscopically for severe intrauterine adhesions with an IUD placed inside the uterus for 6 months. During this time, she also received therapy with combined oestrogen and progesterone (ethinylestradiol and medroxyprogesterone). Finally, after failure of hormonal therapy in restoring endometrium, bone marrow stem cells were implanted inside the uterus after curettage on the second day of menstrual cycle. A clinical pregnancy was obtained after a heterologous embryo transfer. These pioneering discoveries could open a new scenario in the management of AS, although more evidences are mandatory. In this line, our group published a proof of concept study using CD133+ BMDSCs, demonstrating that in the first 3 months, autologous cell therapy, using CD133+ BMDSCs in conjunction with hormonal replacement therapy, increased the volume and duration of menses as well as the thickness and angiogenesis processes of the endometrium while decreasing intrauterine adhesion scores [42], demonstrating the potential of these cellular therapies.

Clinical Background on Stem Cell Therapy

Current cell therapeutics for AS employ numerous sources of stromal and haematopoietic cell populations, including menstrual blood-derived stromal cells (menSCs), umbilical cord (UC)-derived mesenchymal stromal cells (MSCs), bone marrow-derived mononuclear cells (BMMNCs), and peripheral blood-derived mobilized populations [43].

Certain pilot clinical trials with endometrial-derived stromal cell sources have been proven to be safe and potentially effective [44] in a cohort of seven women with severe AS, where in vitro expanded autologous menSCs were delivered demonstrating increased endometrial thickness as measured by ultrasound and three conceptions after one or more rounds of cell therapy. There were no significant adverse events reported, although this was a study with a limited study population.

On the other hand, our group has performed research on haematopoietic stem cells, precisely autologous bone marrow-derived CD133+ stem cell tested in clinical trials for patients suffering from AS and endometrial atrophy (EA).

CD133+ Bone Marrow-Derived Stem Cells

Autologous CD133+ BMDSCs represent a subpopulation of cells known as endothelial progenitor cells (EPCs) with endothelial progenitor capacity [39] that can be mobilized to the circulation and can improve neoangiogenesis of pre-existing endothelium. CD133+ BMDSCs have been previously used in clinical trials for regenerative medicine in non-haematological applications and have functionally contributed to neoangiogenesis during wound healing and limb ischemia, post-myocardial infarction, endothelialization of vascular grafts, atherosclerosis, retinal and lymphoid organ neovascularization, and tumour growth [38]. A large body of preclinical evidence has also shown that CD133+ cells (a

subset of CD34+ progenitors) act by directly differentiating into newly forming vessels and, predominantly, by indirectly activating proangiogenic signalling through indirect paracrine mechanisms (such as bone morphogenetic protein 6, platelet-derived growth factor β, thrombospondin 1, TNF-α, and IGF-1) [45]. Through this combined mechanism of action, these cells are capable of inducing proliferation of the neighbouring endometrial cells in the damaged endometrium mainly in the epithelial compartment.

Moreover, CD133+ cells have been able to enhance angiogenesis, astrogliosis, axon growth, and functional recovery in a mouse spinal cord injury model [46]. In another study, when CD133+ cells were embedded in atelocollagen gel within a silicone tube that was used to bridge a 15 mm defect in the sciatic nerve of athymic rats, the sciatic nerves were structurally and functionally able to regenerate within 8 weeks, and the transplanted CD133+ cells differentiated into Schwann cells [47]. Finally, in a muscle injury rat model, G-CSF-mobilized peripheral blood CD133+ cells showed the potential to enhance histologically and functionally recovery from skeletal muscle injury via indirect contributions to the environment conducive for muscular regeneration [48].

In this sense, when cells or tissues are damaged by chemical, physical, or mutational causes, CD133+ progenitor or stem cells are activated to self-renew, proliferate, and differentiate in order to repair this damage in response to the changes of cell microenvironment [49]. Importantly, these CD133+ cells have been isolated from bone marrow, cord blood, and peripheral blood as well as tissues and tumours, and they have been tested in animal models and human clinical trials in an attempt to repair injured tissues.

In a preclinical study of our group [50], we observed a statistically significant increase of Ki67+ cells in horns treated after CD133+ BMDSC treatment, regardless of the administration route. After intrauterine injection, the proliferation rate increased from 14% ± 10.37% to 23.15% ± 10.89% ($P < 0.01$) at 90 days after administration, whereas, after tail vein injection, Ki67+ cells increased from 6.92% ± 7.03% to 20.55% ± 10.89% ($P < 0.05$) 12 days after administration. Additionally, the expression of Thbs1 was upregulated (2.065 vs. 0.752 fold change; $P < 0.05$), while the expression of IGF-1 (0.651 vs. 0.995 fold change; $P < 0.05$) was downregulated in the treated damaged horns compared to non-treated damaged horns when cells were administered intrauterine, suggesting that human CD133+ BMDSCs induced indirect proliferation of the neighbouring endometrial cells in the damaged endometrium mainly at the epithelial compartment.

On the clinical side, the endometrial stem cell niche is located at the endothelium of the spiral arterioles in the basal layer [51]. In this regard, there are two published case reports, suggesting the therapeutic utility of stem cells from bone marrow in the treatment of certain endometrial pathologies [41]. Our group designed a prospective, experimental, non-controlled, open labelled study including 18 patients aged 30–45 years with refractory AS or EA, and 16 of these completed the study [42]. The end point was assessing the use of CD133+ BMDSC as a potential therapy to refractory cases of AS and EA. Prior to the enrolment, 8 out of 11 patients diagnosed with AS and all the patients diagnosed of EA had previously undergone a mean of 2 (range 1–9) hysteroscopies and 2 subsequent cycles of HRT after each hysteroscopy with no clinical neither sonographic improvements.

The study is summarized in Fig. 1. Briefly, once patients were included in the study, an initial ecographical and hysteroscopic diagnosis was performed in proliferative phase. Uterine cavities were assessed following the American Fertility Society classification. Once assessed, adhesions were removed in AS with sharp and blunt scissors. All patients received hormone replacement therapy (HRT) immediately after the surgery for 2 months.

Several days after hysteroscopy was performed, mobilization of BMDSCs was induced in all the enrolled patients by pharmacological administration of recombinant human granulocyte colony-stimulating factor (G-CSF) at a dose of 10 μg/kg/day for 5 days. G-CSF is a cytokine extensively used for this purpose in both autolo-

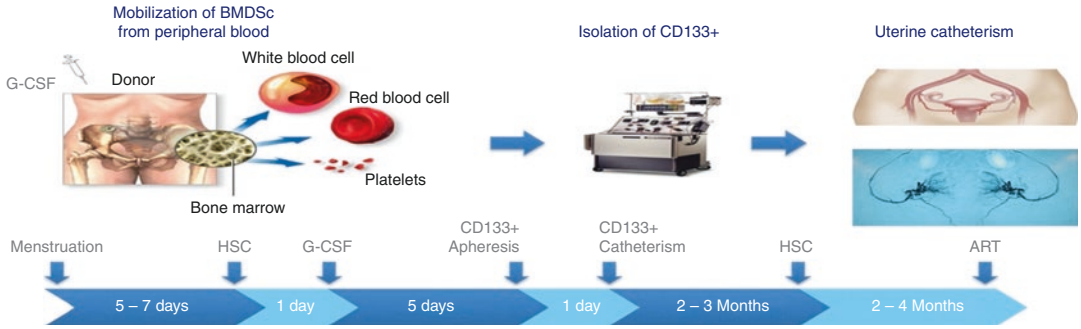

Fig. 1 Summary of the study. Once patients were included in the study, an initial ecographical and hysteroscopic diagnosis was performed in proliferative phase. Then, mobilization of BMDSCs was induced in all the enrolled patients by pharmacological administration of recombinant human granulocyte colony-stimulating factor (G-CSF). After a second-look hysteroscopy, ART was performed, and reproductive outcomes as well as endometrial thickness were assessed

gous and allogeneic donors. One day after the last injection, isolation of the PBMCs was performed via apheresis through peripheral venous access, and then, CD133+ cells were isolated. A mean of 124.39 million cells (range 42–236) were delivered into the spiral arterioles through catheterization adjacent to the endometrial stem cell niche, representing an effective minimally invasive approach to homogenously instil these cells next to the endometrial niche.

Three months after the stem cell instillation, a second-look hysteroscopy was performed in the proliferative phase of the cycle, and the endometrial cavity was re-scored according to the American Fertility Society classification. Some patients required a third-look hysteroscopy that was performed 5–6 months after stem cell instillation. An improvement in the scores and stages was observed in all the patients, although cavity was not completely normalized in all the cases. Specifically, all patients diagnosed with stage III AS improved to stage I, while one of the two patients affected with stage II showed a completely normalized endometrial cavity, and the other improved to stage I. The remaining patients initially with stage I improved in qualifying score.

However, the duration and intensity of menstruation decreased progressively from a mean of 5.06 days (ranging from 3 to 7) in the first month to 3.25 days (ranging from 1 to 3) in the third month after cell therapy. Menstrual volume assessed by number of pads used also decreased from a mean of 2.69 (ranging from 1 to 5) to 1.75 (ranging from 1 to 4) pads per day in the third month.

Moreover, a statistically significant improvement in endometrial thickness was observed in AS patients from 4.3 mm (range from 2.7 to 5.0 mm) to 6.7 mm (range 3.1–12 mm; $p = 0.004$). In the EA group, endometrial thickness improved from 4.2 mm (range 2.7–5.0 mm) to 5.7 mm (range 5–12 mm; $p = 0.03$).

Angiogenesis in the endometrium was also assessed since CD133+ BMDSCs are described as circulating endothelial progenitor cells (CEPCs) with the capacity to improve neoangiogenesis. A statistical significant increase in the number of blood vessels in the endometrium was demonstrated 3 months after stem cell instillation together with a histological improvement of the stromal and epithelial layers of the endometrium was clearly visible (Fig. 2). However, this process seemed to decrease 6 months after the cellular therapy as observed with the duration and intensity of menses. Within the pilot study, each patient acted as its own control. Patients had gone through several negative rounds of reproductive treatments, and none of the patients had previously been pregnant after hysteroscopy or echography diagnostic [42]. All the patients underwent assisted reproductive techniques after observing

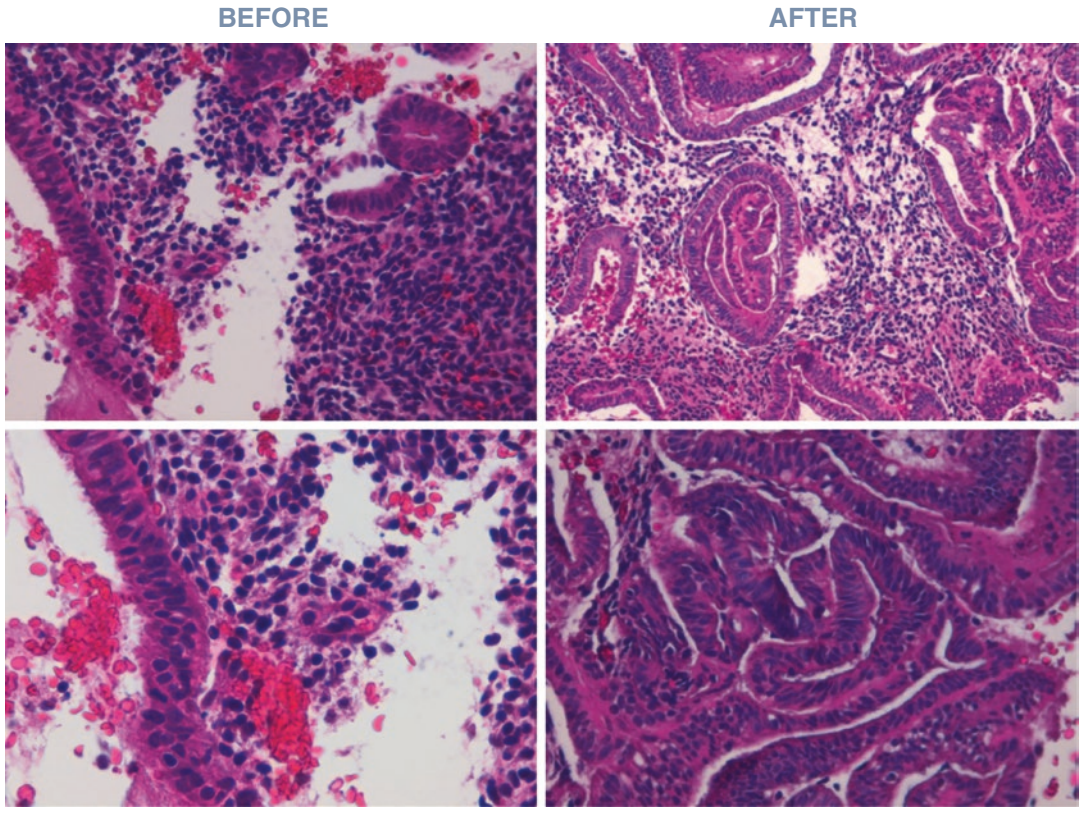

Fig. 2 The endometrium has a unique capacity to reconstitute stomal and epithelial compartment during the menstrual cycle. Intrauterine adhesions can be formed by endometrial tissue, connective tissue, or muscular tissue. Hysteroscopy is considered the gold standard to diagnose intrauterine adhesions. In severe cases, the cavity can practically be occluded by fibrotic and extensive lesions. This picture displays histological improvement in a patient after stem cell therapy

improvement in the second-look hysteroscopy, and no side effects related to treatment were observed during the study.

Functionality of the treated endometrium was assessed with ART results after stem cell therapy. Three patients became pregnant spontaneously, 2, 4, and 19 months after cell therapy, resulting in two healthy babies born and a miscarriage at the 17th week due to a premature rupture of membranes. Seven positive pregnancies were obtained after 14 embryo transfers, resulting in 3 biochemical pregnancies, 1 miscarriage at the ninth week due to a chromosomally abnormal embryo identified after the analysis of the products of conception, 1 ectopic pregnancy, and 3 healthy newborns out of two patients since one was a twin pregnancy. In one case, embryo transfer was cancelled due to chromosomal abnormalities in all of the embryos, and, in another case, transfer was not performed due to failure of cell therapy.

Moreover, other studies have also suggested [41, 52] positive results in treating AS with autologous stem cells isolated from bone marrow. A case report [41] showed positive results in treating AS with autologous stem cells isolated from bone marrow by CD9, CD40, and CD90 expression and placed into the endometrial cavity, while another series of cases described the direct placement of non-characterized mononuclear stem cells into the subendometrial zone with a needle. In this latter study, six 25–35-year-old women with refractory Asherman's syndrome were treated with mononuclear cells (MNCs), and a statistically significant restora-

tion of the endometrial thickness (ET) was reported compared to baseline measures ($P < 0.05$) with menstruation reported in 5/6 women [52]. MNCs were isolated by a Ficoll density separation method, and a volume of 3 mL of MNC was implanted in the sub-endometrial zone transmyometrial at two to three sites (fundus, anterior, and posterior part) of the myometrium. However, no reproductive outcomes were assessed in this study.

On the whole, all these findings suggest that autologous cell therapy may be useful in treating patients with refractory AS and EA suffering infertility. As a matter of fact the European Medicines Agency (EMA) as well as the Food and Drug Administration (FDA) has acknowledged this results and has issued a positive opinion to consider CD133+ cells as the first orphan drug-designed (ODD) therapy for the treatment of AS, categorizing these cells as advanced therapy medicinal product (ATMP), and supervised phase I/II and III trials are being performed [26]. Obviously, the eventual integration of this therapy into health systems involves many steps, such as determining the best dosage, a long-term follow-up, and a randomized trial, but this can be considered as the first focused proof-of-principle study. Currently, a phase I/II trial is being conducted in order to assess long-term safety, optimal dosage, as well as determination of different mechanisms of action.

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Uterus Transplantation in the Context of Fertility Preservation

Mats Brännström

Introduction

Uterus transplantation (UTx) is a procedure to transplant a uterus into a woman with absolute uterine factor infertility (AUFI). This type of infertility is caused by the absence of a uterus from birth or after hysterectomy. It can also exist in the presence of a uterus without capacity to carry a pregnancy. So far, the absolute majority of patients undergoing UTx have had the Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome, which is a condition with aplasia of the uterus and upper vagina.

Uterus transplantation can also be applied in patients who have undergone treatment for uterine/cervical malignancy and who are cleared from the risk of recurrence or immunosuppression activating the disease. Only one patient with a prior malignancy diagnosis has undergone UTx, and this patient participated in the initial Swedish UTx trial, with surgeries mainly during the first half of 2013 [1]. The patient had cervical cancer stage 1b at age 25 and underwent radical hysterectomy with lymphnode dissection. At age 32, she volunteered to be the first patient to undergo surgery in the initial Swedish UTx trial, with UTx from her 50-year-old mother, who then was still regularly menstruating. She and her partner had, prior to UTx, two IVF stimulations to have ten embryos cryopreserved before transplantation. The patient had one initial mild rejection episode [2], which was reversed by corticosteroid treatment, but apart from that, the post-transplantation period was uneventful. Since 8 months after transplantation, she was only on low-dose tacrolimus for single immunosuppression. She underwent two normal pregnancies with elective cesarean sections, first in week 35 to deliver a boy in 2014 and then in week 37 to deliver a girl in 2016 [2], with hysterectomy performed during the same surgical session as the last cesarean section.

In this context, it should be emphasized that UTx should only be considered in a woman where risk of recurrent cervical cancer or a uterine malignancy is considered almost none. The woman receiving a uterine allograft will be on immunosuppression for 2–5 years, and it cannot be excluded that the medication will affect immune mechanisms that may be operative to prevent recurrence. A meta-analysis, including studies of solid organ transplant recipients, demonstrated that pre-transplant malignancy is associated with an increased risk of cancer-specific mortality and de novo malignancies compared to no prior history of malignancy [3]. A nationwide population-based study in Sweden including all (more than 10,000) solid organ transplants

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between 1970 and 2008 found a 30% increased mortality risk in patients with previous malignancy, and this increased risk was driven by cancer-specific death [4]. However, gynecological cancers were classified as low risk for recurrence. It was also found that a delay in transplantation of more than 5 years post-malignancy reduces risk further. This is in line with our recommendations of a waiting period of at least 5 years since hysterectomy because of cancer.

Modern research in the UTx field started in a few places in the late 1990s, around the same time that the first types of transplantations with vascularized composite allografts (VCA), such as the hand [5] and larynx [6], were introduced. These types of transplantations, with initial cases performed in 1998, will naturally not save the life of a person, such as with transplantations of vital organs (heart, liver), but will provide increased quality of life. Uterus transplantation is developed as a quality of life-enhancing and life-giving transplantation procedure.

Preclinical research activities in UTx have involved studies in several animal species including rodents (mouse, rat) and nonhuman primate species (baboon, macaque). These efforts have been instrumental in optimizing the UTx procedure before clinical introduction. The preclinical research studies with high impact will be mentioned in the section below.

There are some special features with UTx as compared to other types of transplantations in the VCA group, where UTx is also grouped. Specific for UTx, not only deceased donor (DD) grafts but also live donor (LD) grafts can be utilized. The possibility to also use organs/tissues from LDs offers many benefits, such as low histocompatibility mismatch between relatives, minimal cold ischemia, sufficient time to investigate a graft concerning normality and absence of premalignant/pathological conditions, and ability for an elective transplantation procedure. However, the major disadvantage is that a great surgical risk, with associated morbidity and mortality, will be imposed on an individual who has no direct benefit of the procedure. All non-uterus types of VCAs and solid organ transplants are meant for the entire life, but UTx represents an ephemeral transplant that will only be used for a few pregnancies. The uterus can then be removed with cessation of immunosuppression, which will minimize the risk of long-term side effects of immunosuppression, such as nephrotoxicity and malignancy risk. Importantly, chronic rejection, which is a major problem in traditional VCA, would most likely not apply to UTx.

Animal Research

The animal UTx research activities were undertaken in several animal species and followed a systematic approach to develop a safe and efficient procedure appropriate for human use. Although several animal species have been used in this research, results from studies in rodents (mouse, rat), to initially investigate basic aspects of the UTx procedure, and in nonhuman primate species (baboon, macaque), to develop surgery and optimal immunosuppression in a near human-like situation, have been essential in the development of UTx.

Concerning rodent studies, exclusively DD models were used, due to the necessity of harvesting parts of aorta/caval vein (mouse) or common iliacs (rat) to achieve vessels of a size that anastomoses could be performed. In the mouse, the graft was placed and combined with the native uterus with end-to-side anastomoses of aorta-aorta and cava-cava [7]. In the initial method, the cervix was hanging free in the abdomen, but uterine drainage was suboptimal [7]. The method was modified to exteriorize the cervix on the abdominal wall [8]. Fertility after UTx was demonstrated in a syngeneic mouse model already in 2002 [7] and in a follow up-study the year after showing offspring of normal birth weight and growth trajectory [8]. Moreover, in the syngeneic mouse model, offspring were born after the uterus had been ex vivo under cold conditions for 24 h before transplantation, showing a great tolerability to cold ischemic conditions [9]. In the rat UTx model, the common iliacs of the graft were anastomosed end-to-side to common iliacs of the recipient [10]. Tolerability to warm

ischemic conditions was found to be at least 5 h in the rat UTx model [11]. This is a long duration of warm ischemic tolerability, compared to other types of organ transplantations, thus showing that the uterus is a tough organ concerning ischemic damage. Fertility after UTx in the rat was first shown in the syngeneic model [12]. The first UTx model to test pregnancy potential in a non-syngeneic/autologous UTx situation was the rat allogeneic UTx model with the added strains of immunosuppression and rejection also tested. It was first tested in a non-pregnant model that single therapy with the calcineurin inhibitor, tacrolimus, prevented uterine rejection [13] and that pregnancies up to midterm could be accomplished [14]. In a follow-up study, it was shown that pregnancies were uneventful and that the offspring from the allogeneic rat UTx model had normal growth trajectory [15].

Uterus transplantation has also been extensively explored in nonhuman primate species, with initial experiments in the baboon LD UTx model by auto-transplantation, using end-to-side anastomosis of internal iliac arteries and ovarian veins of the graft to the external iliac vessels [16]. This LD UTx model in the baboon was later implemented for allogeneic grafts [17] and later extended to allogeneic UTx in a DD model, using a macrovascular patch technique of the aorta and vena cava [18]. The smaller cynomolgus macaque species was also tested in experiments of autologous UTx, with uterine vessels anastomosed end-to-side to the external iliacs [19] and also with outflow being ovarian veins [20]. Allogeneic UTx was recently applied in the cynomolgus macaque with similar surgical technique [21]. A series of experiments showed that the nonhuman primate allogeneic UTx models need induction immunosuppression, followed by triple immunosuppressive therapy to prevent rejection [17, 18, 21], indicating the primate uterus is more demanding in relation to suppression of rejection by immunomodulation, as compared to rodents (see above). Live birth after UTx in nonhuman primate species has only been demonstrated in the autologous UTx model of the macaque [22].

Human Live Donor (LD) Uterus Transplantation by Laparotomy

The first human UTx attempt ever was a LD procedure attempted in Saudi Arabia in year 2000, and this involved classical laparotomy technique in both donor and recipient [23]. A 46-year-old donor, planned for elective surgery because of ovarian cyst, altruistically donated her uterus to a woman in her mid-20s who had undergone emergency peripartum hysterectomy because of atonic uterine bleeding, when giving birth to her first child. The surgeries of donor and recipient were mainly uneventful apart from a ureteric laceration in the donor, and interposing saphenous grafts had to be used to reach the external iliacs in the recipient. However, possibly due to poor blood flow, a necrotic uterus had to be removed 3 months after UTx [23].

In 2013, the Swedish team initiated the first clinical LD UTx trial (1) by traditional laparotomy technique, and the trial included nine pairs of recipients (27–38 years) and donor (37–62 years), with seven donors being genetically related (five mothers, sister, and maternal aunt) and two donors not genetically related (mother-in-law and family friend). All but one recipient had the MRKH syndrome, and one had undergone hysterectomy because of cervical cancer as described in the Introduction section. Donor surgery (10–13-h duration) through laparotomy involved retrieval of the uterus with deep uterine vessels connected to the segments of the internal iliac vessels. The long surgical duration was mostly due to the very complicated dissection of the deep uterine veins that are firmly attached to the surrounding tissue with several small branches in between the separate veins. All nine procedures of LD organ procurement were without perioperative complications. However, one donor acquired a ureteric-vaginal fistula 2 weeks after organ donation, which was later repaired by reimplantation in the bladder. Recipient surgery (duration of 4–6 h) was also by laparotomy and involved dissection of the vaginal vault, end-to-side anastomosis of the segments of the internal iliac vessels to the external iliac vessels, vaginal opening with anastomosis, as well as fixation of

the graft to ligaments (1). One uterus was removed within the first postoperative week because of vessel thrombosis, and another uterus was taken out after 3 months because of intrauterine infection, which developed into an abscess (1). Thus, seven out of nine recipients had grafts with long-term survival, and they had regular menstruations from around 2 months after transplantation with their first embryo transfer (ET) scheduled 1 year after the UTx procedure.

In 2014, a second LD UTx trial was initiated in the Czech Republic, with initial results available for five procedures, including only MRKH patients (age 23–30 years) as recipients [24] and mothers/mother's sister (age 47–58 years) as donors. The surgery had been modified somewhat so that venous outflows were routed through utero-ovarian veins in several cases, instead of deep uterine veins, and surgery by laparotomy was now considerably shorter (5.5–7 h) than in the initial trial from Sweden (1). Recipient surgery was around 4–5 h and included end-to-side anastomoses of the vessels of the graft to the external iliacs in four cases and to the internal iliacs in the first case [24]. One graft removal was done after 2 weeks because of uterine vessel thrombosis, and the other four resumed menstruations within 2 months.

Two LD UTx procedures were reported from a trial in Germany, with two MRKH recipients and donating mothers [25]. The surgical technique and procured veins were similar to that of the Swedish trial (1). Durations of donor plus recipient surgery in the first and second cases were 9 h plus 4.5 h and 12 h plus 6 h, respectively. Menstruations were reported 6 weeks after UTx in both recipients [25]. No complications of donors were reported.

A total of 13 donor surgeries have now been reported from the initial LD UTx trial in the USA [26], and data relating to recipient surgery and early outcome is available in five of these procedures [27]. The age of the donors were 32–56 years, with seven (< 40 years) of them being of clearly fertile age [26]. One donor (48 years) was postmenopausal, and the number of deliveries spanned between one and seven, with two of the donors having two cesarean sections each [26]. Surgery was by infra-umbilical midline incision, and the surgery included harvesting of arterial vascular pedicles, including uterine arteries and patches of the internal iliac artery, and venous vascular pedicles of the deep uterine veins and/or the utero-ovarian veins. Surgical durations were between 5.5 and 7.5 h. The intra- and postoperative complications were reported with a follow-up time between 6 and 24 months. Two donors had serious intraoperative complications related to acute blood loss, and two donors had serious postoperative complications (fecal impaction and vaginal cuff dehiscence), which necessitated new procedures during general anesthesia [26]. The profiles and surgeries of the five initial UTx procedures have also been described [27]. There were some variations in anastomosis technique of the arteries. The first three procedures included a patch of the internal iliac arteries of the graft sutured end-to-side to the external iliac arteries, and in the following two cases, segments of internal-iliac arteries of grafts were used. Venous outflow was by a combination of deep uterine veins and parts of utero-ovarian veins in the first three cases, while the proximal parts of the utero-ovarian veins were the exclusive venous outflow in the last two cases. The first three UTx procedures resulted in early hysterectomy, since vascular thrombosis and uterine necrosis developed shortly after UTx. The authors stated that technical causes of the three failures were primarily related to harvesting and anastomoses of the venous outflow sections [27]. The last two recipients presented with uterine function and spontaneous menstruations during the follow-up of initial months.

Human Live Donor (LD) Uterus Transplantation by Minimal Invasive Surgery

Minimal invasive surgery (MIS) in donor surgery may have benefits in regard to surgical tissue trauma, blood loss, surgical duration, and length of hospital stay. The first case of MIS in UTx was a case in China, including a fully robotic-assisted laparoscopy procedure of the donor with a dura-

tion of 6 h [28]. Vascular pedicles were of uterine/anterior internal iliac arteries and complete utero-ovarian veins, necessitating oophorectomy in the premenopausal uterus-donating mother. The recipient surgery was by conventional laparotomy but had a long (9 h) duration, possibly because of the difficult anastomosis of the thin-walled utero-ovarian veins to the external iliacs. The transplanted daughter had regular intervals of menstruation during the initial post-UTx year [28]. There is however some controversy of the case since bilateral oophorectomy was performed in the premenopausal donor-mother, and in general, this will substantially increase cardiovascular morbidity and overall mortality [29].

Classical laparoscopy, but with retrieval by laparotomy, was used in four donor surgeries involving three MRKH patients (21–30 years old) and one patient with Asherman's syndrome (26 years old) as recipients [30, 31]. All received uteri from their premenopausal (42–48 years) mothers. The surgeries of the LDs were greatly simplified by only harvesting the utero-ovarian veins, instead of deep uterine veins, as outflow sections. However, this necessitated oophorectomy of the premenopausal donors. Similar controversy exists as in the case in China [28], with increasing risk of cardiovascular morbidity and mortality [29]. The donor surgery was in all four cases converted to laparotomy for final vascular dissection with organ harvesting. Surgical duration of the combined laparoscopy-laparotomy donor surgery (3–4 h) was comparable to that of open surgery of the recipient (4–4.5 h). Recipient surgery included anastomosis to the external iliac vessels in the initial two cases [30] and to internal iliac vessels in the subsequent two cases [31]. Menstruations resumed within 2 months in all cases.

Human Deceased Donor (DD) Uterus Transplantation

The first DD UTx case in the world took place in Turkey in 2011, and it comprised a 21-year-old MRKH woman and a 22-year old, nulliparous, multiorgan donor with no previous pregnancy [32]. Retrieval surgery and recipient surgery, including end-to-side anastomoses of the internal iliac vessels to the external iliacs, took 2 h and 6 h, respectively. Menstruations started within 2 months. Any clinical pregnancy was never reported and it is unknown when the graft was removed.

Some years later, the first series of four DD UTx attempts, involving recipients (25–33 years) with MRKH and donors (20–57 years old), were reported from a trial in Prague [24]. Recipient surgery took 4–5 h. The transplants of the two oldest uteri failed with a 57-year-old uterus removed after 1 week because of vascular thrombosis, and a 56-year-old uterus developed herpes infection and did not acquire cyclic endometrial growth. Hysterectomy was performed 6 months post-UTx in the latter case. The two uteri of younger age (20 and 24 years) showed regular menstruations within the first months post-UTx [24].

In 2016, another DD UTx procedure was performed in Brazil, when a 32-year-old MRKH woman received a uterus from a 3-parous, 45-year-old brain-dead woman [33]. The uterus was harvested with arterial inflows through the bilateral internal iliac arteries, extending from the uterine artery, and four venous outflow sections, via bilateral deep uterine veins and the utero-ovarian veins. Cold ischemia was as long as 6.5 h since the organ was transplanted between hospitals. Recipient surgery included end-to-side anastomosis of two arteries and four veins to the external iliac vessels of the recipient [33], with duration of uterine implantation of 2 h and total recipient surgery of 10.5 h. This time difference implies that the organ had several leakage points that had to be sutured to achieve acceptable hemostasis. Menstruations resumed at postoperative day 37.

Live Births After Human Live Donor (LD) Uterus Transplantation

The first live birth after UTx [34] took place in September, 4, 2014, within the original Swedish LD UTx trial (1) and in this specific case was

after UTx in February 2013. The mother, with MRKH, was 35 years old at UTx when she received a uterus was from a 61-year-old family friend. The first frozen ET was with a day 2 embryo, with three out of four blastomeres surviving thawing. Transfer was in the natural cycle but with an endometrium of suboptimal thickness (6 mm). The patient had a mild rejection episode at gestational week 18, and this was reversed with temporary increase in the dose of corticosteroids. Ultrasound monitoring during pregnancy showed normal growth of the fetus, with no signs of cervical shortening [34]. At gestational week 31 + 5, preeclampsia became apparent, and cesarean section was performed the following day. A healthy boy of normal weight (1775 g) for gestational age and with Apgar scores of 9-9-10 was delivered [34]. The uterus was removed 3 months after childbirth.

The second live birth [35] in the world came in November 2014 from a 28-year-old woman with MRKH of the original Swedish trial (1), who had been transplanted with a uterus from her 50-year-old mother. The transplanted patient became pregnant at her first ET, which was with a blastocyst. The pregnancy proceeded normally until gestational week 34, when intrahepatic cholestasis of pregnancy developed. Cesarean section was performed week 34 + 4, with delivery of a healthy boy (2335 g; Apgar 9–10-10). Hysterectomy was performed 3 months after delivery [35].

Additionally, a total of seven healthy babies were born between 2014 and 2020 from four additional women of the cohort of seven patients of the Swedish trial who underwent the full treatment of IVF, UTx, and ET (2).

There also exists one published report of live birth from the LD UTx trial of the USA [36]. Uterus transplantation had been performed to a 23-year old woman with MRKH from an altruistic uterus donor in her mid-30s. The uterus recipient became pregnant at her first ET attempt, which was performed 6 months after UTx and with a euploid blastocyst, who had undergone preimplantation genetic testing for aneuploidy. Elective cesarean section was performed remarkably early at gestational week 33 + 1 and with a birth of a healthy boy (1995 g). Hysterectomy was performed in conjunction with delivery.

Live Birth After Human Deceased Donor (LD) Uterus Transplantation

The only scientifically reported live birth after DD UTx was after UTx performed in Brazil in September of 2016 [33]. The 32-year-old MRKH woman who had undergone UTx had her first menstruation after 37 days. Afterward, 7 months after UTx, a single blastocyst ET was performed in the natural cycle, and this resulted in a clinical pregnancy. Elective cesarean section was performed at week 35 + 3 with delivery of a healthy girl (2550 g; Apgar 9–10-10). Hysterectomy was planned and performed immediately subsequent to delivery.

Conclusion

In conclusion, UTx is the only available procedure for fertility restoration/preservation in women hysterectomized for cervical/uterine malignancy. The process has so far only proved to be successful in one case, involving a cervical cancer patient. The vast majority of patients who successfully have undergone UTx have been women with MRKH, and in this group of women, UTx should be viewed as a pure infertility treatment. Although UTx is still an experimental clinical fertility preservation procedure, it is likely that the UTx field will be expanded to new patient groups for fertility preservation purposes.

Definitions

Absolute uterine factor infertility (AUFI): irreversible infertility due to anatomical absence of the uterus or presence of a uterus that is nonfunctional in regard to implantation or pregnancy.

Uterus transplantation (UTx): transplantation, by vascular anastomosis, of a uterus from one person to another.

Live donor uterus transplantation (LD UTx): transplantation of a uterus from a healthy woman, often a family member or friend for directed donation but exists also as altruistic and non-directed anonymous donation.

Deceased donor uterus transplantation (DD UTx): transplantation of a uterus from a woman who has been declared dead, so far only performed after brain death but in the future also possibly after cardiac death.

Take-Home Messages

1. Uterus transplantation has proved to be a successful infertility treatment with organs both from live donor (LD) and deceased donor (DD).
2. A vast majority of patients who so far have undergone uterus transplantation have Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome, but there are also successful cases after radical hysterectomy for cervical cancer.
3. Uterus transplantation is the first ephemeral type of transplantation, with the transplanted graft remaining in the recipient for a limited time after a desired number of children have been born. This will reduce the exposure period for immunosuppression, with the associated long-term side effects of nephrotoxicity and increased risk of certain malignancies.
4. Uterus transplantation is still an experimental clinical procedure, and any new attempts should be performed within clinical trials, with all results to be published in scientific journals.
5. Surgery of both donor and recipient in uterus transplantation has traditionally been performed through laparotomy surgery, but there is a transition toward the use of minimal invasive surgery, especially robotic-assisted laparoscopy, for live donor uterus transplantation surgery.
6. It is quite possible that uterus transplantation will be a clinical infertility treatment in several countries within a period of 5 years.

Clinical Case

A 16-year-old woman is investigated for primary amenorrhea. Gynecological examination finds absence of vagina, and further diagnostic imaging/laparoscopy discovers the absence of a proper uterus but existence of normal ovaries. Karyotype is normal. The patient is diagnosed with the Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome. The message concerning this diagnosis to the young women will naturally create psychological distress, and she should be offered psychological support. The focus at this age should be to create a functional vagina so that she can experience a normal sexual life during the years to come. A vagina can be created either by self-dilation or by one of several surgical methods. Important is also to give her advice that she will have a good chance to become pregnant and to give birth to children after uterus transplantation in the future. Current research shows that more than 80% of women with MRKH who undergo a surgically successful uterus transplantation will give birth within a 5-year-period after transplantation. It is highly likely that efficiency will be even better in the future. The woman with MRKH should start to plan for pregnancy at a relatively young age in order to increase the chances that there will be a suitable and healthy donor, such as the mother, available in her close family. She could also be recommended to cryopreserve unfertilized oocytes or embryos, at an age before 35 years, if she has to postpone transplantation because of lack of suitable donor or other circumstances.

Practical Clinical Tips

The possibility of uterus transplantation for fertility restoration in cases of hysterectomy at fertile age, such as because of cervical cancer, and as a fertility treatment for women with MRKH should be brought up early after diagnosing the disease.

Cancer victims with cervical/uterine cancer should wait at least 5 years, and with no evidence of recurrence, from hysterectomy to possibly undergo uterus transplantation.

Programs for uterus transplantation should only be initiated at large tertiary medical centers with expertise in transplantation surgery, gynecology, assisted reproduction, and high-risk obstetrics.

Key Readings

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Surrogacy

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Introduction

The definition of "surrogacy" is derived from the Latin word *surrogatus*, meaning "substitute" or "appointed to act in place of."

Surrogacy can be broadly divided into two types: (a) *traditional surrogacy* when the woman carrying a pregnancy for someone else is also genetically related to the baby since she is providing her own eggs and (b) *gestational surrogacy* when the woman carrying the pregnancy is not genetically related to the baby since the egg(s) are either from the intended biological mother, who generally for medical reasons cannot carry a pregnancy herself, or from an egg donor. Gestational surrogacy is the most common form accounting for 95% of all surrogate pregnancies in the USA. Women acting as gestational surrogate may be recruited and paid for their service (the most common arrangement) or may be providing their service pro bono, such as when a family member or a friend volunteers. In the last four decades, thanks to in vitro fertilization (IVF) and later to oocyte donation, surrogacy has become an integral part of reproductive options when other treatments are not possible. Historically, the first commercial, traditional, surrogacy events capturing wide attention occurred in the 1980s. The first documented case of a *traditional surrogate* being paid occurred when Elizabeth Kane was inseminated and gave birth to a son for the compensation of about $11,000. She was considered a good candidate as she was married, had children, and had also given up her first child for adoption prior to marriage. However, after relinquishing the child after birth and giving up parental rights, she spoke out against the practice of surrogacy (she even wrote a book *Birth Mother: The Story of America's First Legal Surrogate Mother*). She felt completely unprepared for the distress surrounding this type of reproductive arrangement. The same year another case of *traditional surrogacy* caught the public attention, that of baby M. Here Elizabeth Stern did not want to risk a pregnancy since she suffered from a medical condition (multiple sclerosis). Through a newspaper ad, she (and her husband) sought the help of Mary Beth Whitehead, who agreed to carry a baby for them by providing both an egg and the uterus. But after giving birth, Whitehead decided to keep the child, and the case went to court in New Jersey where the Sterns were awarded custody of Baby M, but

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the court also banned all such future surrogacy contracts [1–5].

The development of in vitro fertilization (IVF) opened the way to a different type of surrogacy where a woman could carry a pregnancy with a child not genetically related to her. The first successful case of a *gestational surrogacy* occurred in 1985, after the biological mother (also called intended or biological parent) had a hysterectomy. Technological advances have allowed for possibilities few would have imagined: grandmothers giving birth to their own grandchildren, sisters giving birth to their own niece and nephew, gay male couples having babies, and the creation of an industry that some critics refer to as "rent a womb" [1, 2] and the demand for strict regulations.

In the USA, there are States that are considered surrogacy-friendly granting pre-birth orders for the intended parents, thus not needing adoption, and others that are not so friendly. At the time of this writing, the very "friendly" States are California, New York, Connecticut, Delaware, Maine, District of Columbia, New Hampshire, Nevada, Oregon, Rhode Island, and Washington. These States allow compensation of the surrogates and allow surrogacy regardless of the marital status and sexual orientation of the intended parents. In other States, surrogacy can be practiced, but laws may offer varying or uncertain levels of protection for both surrogates and intended parents. The most "unfriendly" State is Michigan, where surrogacy is not allowed. Statistics on the number of gestational carrier cycles and births in the USA are compiled by the ASRM-SART Registry reports since the 1980s. Between 2004 and 2009, the number of initiated gestational cycles grew by almost 70% (1508–2566), while the number of births more than doubled (530–1013), with almost 6600 live born babies as of 2013 (SART Registry) and comprising about 2.5% of all assisted reproduction cycles [2]. In the years 2014–2017, the number of gestational carrier cycles using their own eggs went from 4276 to 6396, an increase of about 50% and the live birth rates per cycle assessed across all ages of about 46% [2]. It must be mentioned, however, that while surrogacy is regulated in the USA, globally there are many countries in which it is banned (see Table 1). Because of these prohi-

Table 1 Gestational surrogacy across countries. In countries not listed, it is allowed

Completely illegal
• Italy
• Germany
• Cambodia
• Denmark
• Austria
• Spain
• France
• Japan
• Malaysia
• South Korea
• Sweden
• Switzerland
Legal only if there is no compensation
• Canada
– Compensation to a woman acting as surrogate is "prohibited under law and subject to a serious penalty"
• New Zealand
– Surrogates cannot be paid, must be altruistic
• UK
– Cannot be paid or commercialized
• Australia
– Cannot be paid or commercialized
However, northern territory has differing laws
• Iceland
– Only altruistic and no commercial
• the Netherlands
– Only altruistic and no commercial
• South Africa
– Surrogates cannot be compensated, but the biological parents are responsible for the surrogate mothers' medical expenses
Out-of-ordinary surrogacy laws
• Nepal
– The supreme Court's final verdict as of December 12, 2016
For infertile Nepali married couples – Surrogacy is legal
For single men, women, transgender couples, and foreign nationals – Surrogacy is illegal
• India
– A "willing" woman can be a surrogate
• Portugal
– For single men and homosexual couples – Surrogacy is illegal
– A surrogate mother cannot receive compensation
In between
• Bulgaria
– Currently moving forward with the legalization of surrogacy
• Thailand

Table 1 (continued)

– Prior to 2015, used to be a destination place for surrogacy, but now only Thai residents of opposite sex and married couples can have commercial surrogacy. Since July 30, 2015, in response to the controversial Baby Gammy (2014), Thailand has banned foreign people travelling there to have commercial surrogacy contract arrangement, under the Protection for Children Born Through Assisted Reproductive Technologies Act

bitions, cross-border reproductive travel has become a known phenomenon whose implications will be discussed later in this chapter as well [6]. Alternatively, uterine transplants have recently been proposed in place of surrogacy for cases of absolute uterine infertility in places where surrogacy is banned. Successful births have been reported by teams who have been able to efficiently address the complexity of the surgeries and the ethical/moral ramifications [7–9].

Indications

The main medical indications requiring the use of gestational surrogacy for parenthood and the main users are as follows:

1. Women born with congenital absence of the uterus and the upper third of the vagina (Mayer-Rokitansky-Küster-Hauser syndrome), women with non-repairable uterine malformations (e.g., small unicornuate uterus), women with extensive uterine scarring like in the Asherman's syndrome, patients treated with endometrial ablation for severe menometrorrhagia, or women after hysterectomy due to benign medical emergencies.
2. Any woman suffering from severe medical conditions incompatible with pregnancy (e.g., severe heart disease, severe pulmonary hypertension, kidney failure requiring dialysis, post-organ transplantation status (liver, pancreas, lungs, heart)), severe clotting disorders, or extremely severe hyperemesis.
3. Patients with recurrent pregnancy loss strongly suspected due to uterine factor (severe adenomyosis) or patients with multiple and unexplained implantation failures.
4. Male same-sex couples.

The Process

Medical Evaluation and Screening of the Gestational Carrier and Intended Parents

Ideal requisites for women desiring to be commercial gestational carriers are as follows: (a) age between 21 and 38, (b) healthy and BMI between 19 and 32, (c) having previously carried a pregnancy to term without complications, (d) no more than four cesarean sections or five normal vaginal deliveries, (e) no smoking or use of illicit drugs, (f) no history of child abuse and no felony convictions, and (g) not being on welfare assistance programs [10–12].

In the USA, women desiring to be gestational carriers are identified either directly by the intended parents (particularly when family members or friends are involved) or through various surrogate agencies. Subsequently, a thorough medical evaluation, including a review of the past medical and surgical history, family and social history, and a complete physical exam, establishes whether a surrogate is deemed as a suitable candidate for pregnancy. The uterine cavity is assessed with a sonohysterogram, and if the chosen gestational carrier is 40 years or older, a mammogram and a maternofetal consultation are also required. A specific set of screening tests must be performed in non-FDA-approved laboratories for the carrier and her male partner, if applicable (see list in Table 2).

Laboratory Testing for the Intended Parents

The intended genetic parents require screening tests performed in FDA-approved laboratories (summarized in Table 3). To be FDA-compliant, the screening test for the intended parents needs to be done twice: the first time before the gestational carrier begins her medical evaluation and the second set of the same tests no more than 30 days before the oocyte retrieval or at the

Table 2 Testing requirement for the *gestational carrier and her partner* (if applicable)

CBC, blood type Rh, antibody screening, TSH, PRL, vit D (only for the carrier);
HIV 1 and 2; hepatitis B (HbsAg, anti-HBc); hepatitis C (anti-HCV); RPR; CMV (IgG and IgM) (for both, if applicable)
Rubella, varicella (only for the carrier)
Urine drug screen (only for the carrier)
Urine culture screening for gonorrhea and chlamydia

Table 3 Laboratory testing for the *intended parents* (those indicated by the asterisk need to be performed in an FDA-approved laboratory)

For the female partner
(A) CBC, blood type and Rh, TSH, PRL, AMH; day 3 FSH and E2
(*B) HIV 1 and 2; hepatitis B (HbsAg, anti-HBc); hepatitis C (anti-HCV); RPR
(*D) urine culture for gonorrhea and chlamydia
(*E) West Nile virus
For the male partner
(A) Blood type and Rh, semen analysis
(*B) HTLV 1 and 2, HIV 1 and 2; hepatitis B (HbsAg, anti-HBc); hepatitis C (anti-HCV); RPR and CMV (total)
(*C) urine culture for gonorrhea and chlamydia
(*D) West Nile virus

baseline ultrasound appointment. For the intended genetic father, the second set of testing must be no more than 7 days prior to the egg retrieval. If the intended parents are using either donor oocytes or donor sperm, then the FDA-required set of laboratory screening applies to the gamete donors.

Ovarian Stimulation for the Intended Genetic Mother and Hormonal Synchronization of the Gestational Carrier

Protocols for ovarian stimulation vary according to age of the patient, her body mass index (BMI), her ovarian reserve (AMH and AFC), and the ovarian response to previous cycles, if any. The most common protocol for ovarian stimulation is the use of either recombinant FSH alone or in combination with hMG and flexible start of GnRH antagonists (ganirelix or cetrotide) to prevent premature ovulation. Importantly, the menstrual cycle of the gestational carrier is often synchronized with the use of leuprolide acetate (GnRH agonist) started in the midluteal phase of the previous menstrual cycle. Generally, the menses of the surrogate are manipulated to start ahead (about 5 days) of the menses of the intended mother. Two days before the intended mother starts ovarian stimulation, the gestational surrogate, in addition to leuprolide acetate, begins estradiol tablets at fixed incremental doses (2 mg per 5 days, followed by 4 mg for 4 days and then 6 mg from cycle day 10 at which time the leuprolide acetate is stopped), while the intended parent starts the gonadotropin stimulation (rFSH and/or hMG). The hCG is administered when an appropriate number of follicles have reached a mean diameter between 18 and 20 mm. The day before oocyte retrieval, the gestational carrier is instructed to start progesterone (vaginal preparations). On the day of oocyte harvesting, the gestational carrier increases the vaginal progesterone to twice daily and begins also the use of daily intramuscular progesterone injections (50 mg) [4]. Embryo transfers are generally performed 5 days after the egg retrieval unless there are only one or two cleaving embryos available calling for a day three transfer.

If a pregnancy is achieved, estradiol doses are progressively decreased from gestational week 6–7, until the completion of the tenth gestational week.

Psychological Assessment and Preparation

The psychological assessment of intended parents (couples, partners, or individuals) and of the gestational surrogates (and their partners, if applicable) is an important step to assess the coping skills of the parties involved from the emotional impact and from potential legal risks of the "arranged" reproductive process. The most commonly used strategy for an appropriate psychological evaluation involves separate counseling sessions of the surrogate (and partner, if applicable) and of the intended parents, culminating with a group meeting [4].

Often the parties involved have already met and established that they want to work together. However, they may have little concept of what is entailed in a successful surrogacy relationship and must be informed and prepared for a successful surrogacy relationship [13].

Assessment of the Gestational Carrier

The gestational carrier has a right to be fully informed of the risks of the gestational arrangement and of pregnancy. These should include known physical, psychological, and social risks that may occur. Common motivations for becoming a surrogate include financial gain; enjoyment of pregnancy; self-fulfillment, value, and worth; and wanting to help others [10, 12]. It takes a special woman to be a surrogate: she must be able to work with the intended parents before, during, and after the pregnancy; she must be able to relinquish the baby after giving birth; and she will have to handle these relationships and experiences while caring for her own family and her own feelings. Although research indicates that overall women do not experience psychological problems from being surrogates [4, 10, 11], the challenges and the potential for problems exist.

As reported earlier, there are personality and characteristics which serve as positive indicators of a woman's appropriateness to be a surrogate [10–12]. Having given birth already not only will provide important information about the dynamics of pregnancy but will be also informative of her psychological adjustment after delivery. Gestational carriers who are dealing with job loss or stress, health and family problems, or marital difficulties may not be appropriate choices due to risk of emotional instability. It is also important to conduct a criminal and financial background check prior to working with a gestational carrier.

Gestational surrogates need to have a personality that can deal with ambiguity and stress, as well as being empathic, adaptive, and resilient [4, 10]. To identify these psychological characteristics, all potential gestational carriers are given standard personality psychological testing with the Minnesota Multiphasic Personality Inventory −2 (MMPI-2), which has been used in psychiatric, employment, and forensic settings for over 80 years, or the Personality Assessment Inventory (PAI) [13–15]. These tests will indicate not only the presence of difficult personality characteristics but also whether the surrogate is honest in her test-taking attitude and approach to the assessment. Recent research on the use of MMPI-2 with surrogates has found that majority of applicants are within normal clinical limits [15].

The clinical interview of the gestational carrier and, if applicable, her husband/partner begins with a discussion of motivations for becoming a surrogate, the general support system, and description of the quality of the interactions with the commissioning parents. A discussion of expectations and fantasies/wishes about the relationship during pregnancy and after birth should be addressed. Discussions should also cover feeling and decisions about abortion, multiple pregnancy, and circumstances for potential selective fetal reduction. What contact does she desire and imagine will occur while pregnant, during birth, and after relinquishing the baby? How does she see her relationship and contact changing with the IPs and child after birth and in the future? What issues does she see occurring within her own family during this time, and how will she deal with it? These questions and discussion will help in preparing for what is ahead [4]. (A list of positive and negative indications for being a gestational carrier can be found on Table 4.)

Assessment of the Intended Parents

Intended parents, whether heterosexual, gay, or single, need similar personality and character assessments. They need to be adaptive, trusting, resilient, and able to tolerate lack of control [11, 13].

The psychological clinical interview will assess decision-making and relationship expectations with the gestational carrier and, if applicable, discuss how past experiences of losses and failures may have impacted their marriage. Expectations regarding contacts with the carrier during the pregnancy and after birth should also be reviewed. If they will be using an egg and/or sperm donor, the issues related to raising a non-genetically related child will need to be addressed. In addition, it is important to discuss and confirm

Table 4 Psychological Evaluations of Gestational Carriers [Adapted from 4]

Positive indicators
History of at least one healthy full-term pregnancy
Experience and competence with motherhood
Motivations that reflect empathy
Spousal support if applicable
Stable lifestyle
No major conflicts or transitions in the next 2 years
Cognitive ability to provide informed consent and conceptualize risks
Absence of psychopathology
History of making successful decisions for herself
Financial stability
Able to express and articulate concerns and questions
Negative indicators
Poor obstetric outcome, poor nutrition in pregnancy, severe postpartum blues/psychosis
Lack of social/spouse support; severe financial distress or coercion
Defensive psychological testing with unrealistic expectations about time involved
Chaotic lifestyle, impulsivity or high anxiety, or lack of empathy
History of anti-authority behavior, drug/alcohol addiction/abuse
Unresolved or untreated history of child or sexual abuse
Unresolved issue concerning prior abortion or reproductive loss issues
Inability to communicate in her native language with medical professionals

that legal consultation and contracts for both the IP and surrogate have been obtained prior to treatment [16].

Time should also be given to talking about the future plan as to whether and what they will be disclosing to their child about the origins of his or her birth. It is comforting to note that despite the difficult road to become a parent, ongoing research is indicating that families conceived via surrogacy are doing well and adapting normally [4, 17].

Surrogacy Agencies and the Legal Contracts

With the growth of technology allowing for gestational surrogacy, a whole industry has developed that identifies and brings together potential surrogates and intended parents. The Internet has created a means for people to meet and pursue these arrangements with numerous national and international websites devoted to surrogacy [4]. It is also worthy to mention that finding a gestational carrier and the right matching is the most time-consuming step of the treatment.

At times, intended parents may try to find a gestational surrogate on their own, either through the web or friends, sometimes because of financial concerns. However, this may open patients up to exploitation, and working with a reputable surrogacy agency or agent can alleviate many potential problems.

1. There is wide variation in screening and services offered by surrogacy agencies and lawyer/agents. Some act as a "matchmaking" service, while others provide full legal and psychological services throughout the process. Matchmaking agencies/agents will search and find women interested in being a gestational carrier but often do only minimal prescreening, usually outsourced to independent practitioners, before matching the intended parents with the gestational carrier. It is crucial to understand, however, that without an adequate agency prescreening of "want-to-be" gestational carriers, intended parents are more vulnerable to economic loss, disappointment, and sadness (if the proposed surrogate carrier is found to be not a suitable candidate at the time of the medical exam for the matching). Reputable surrogacy agencies can provide full service to both biological parents and surrogates and will also have legal, psychological, and medical staff in-house to assess, facilitate, and support both parties before treatment, during a pregnancy, at birth, and after relinquishment so to ensure the best interests of everyone involved. These agencies will have potential carriers undergo medical screening; criminal, credit, legal, and driving background checks; psychological testing; and legal consultation. It is only after both the intended parents and the carriers have been fully screened and accepted by the agency and by the treating physician that a match takes place. While each clinic will have their own requirements for medical and psychological screening, there are many questions intended parents should consider when

choosing a surrogacy agency. For example, how long has the agency been in business? What legal problems, if any, has the agency incurred with their arrangements? Does the agency complete credit checks, criminal/legal backgrounds, and driving records prior to selecting a surrogate? Has the potential surrogate been a surrogate before, and what was this experience for her and the commissioning parents? Does the agency/agent utilize an independent escrow agency? Is the entire agency fee due if a pregnancy doesn't occur, or is it broken into installments? As for legal contract, it is important to know the legalities of the States in which surrogates are recruited [16]. It is crucial to know if the surrogate lives in a surrogacy-friendly State (as reported in Table 1) or will the GC have to travel to give birth and whose names will go on the birth certificate. Generally, the intended parents and the gestational carrier should have their own legal representation (often provided by surrogacy agencies/agents), and the contract should also specify whether the carrier has health insurance or the agency will have to obtain it for her. If the carrier has health insurance, it must be checked to see if it excludes surrogacy pregnancy care. Finally, the contract should also include notes on the number of embryos for transfer (preferably single embryo transfer) and about the acceptance or rejection of prenatal testing and the consideration for termination of pregnancy in the event of a genetic condition. In the event of a disagreement, the legal contract should help to reach possible resolutions. Gestational carriers should receive fair and reasonable economic compensation and healthcare coverage for pregnancy care and potential complications. Payment to the carrier, however, should not create undue inducement or risks of exploitation.

Cross-Border Surrogacy

International travels have proliferated for intended parents requiring the service of gestational surrogacy. The growing interest in "reproductive tourism" and "reproductive outsourcing," including a dramatic rise in Indian gestational surrogacy, has generated both legal and ethical concerns [6, 18, 19].

There are a number of factors that may promote cross-border surrogacy: (1) individual countries may prohibit the service for religious, ethical, or legal reasons; (2) the specific service may be unavailable because of lack of expertise or lack of affordability and supply of donor gametes and surrogates; (3) the service may be unavailable because it is not considered sufficiently safe; (4) certain categories of individuals may not receive a service in their countries, especially at public expense, on the basis of age, marital status, or sexual orientation; (5) individuals patients may fear lack of medical privacy and confidentiality and thus travel abroad; and finally, (6) services may simply be cheaper in other countries [18, 19]. For gestational surrogacy, the economic motivation is the most cited reason for Americans travelling abroad (Eastern European countries have now replaced India as a surrogacy destination). The Government of India passed a law on surrogacy in December 2018, which banned commercial surrogacy and curtailed the freedom of foreigners to apply for surrogacy. A key reason for passing this law was the unjust treatment of the women who provided surrogacy services [20]. The entire process can cost $25,000 (inclusive of airfare, accommodations, and the surrogate's fee), which is significantly lower than the costs for the same service in USA (approximately $80,000).

Take-Home Messages

The use of gestational surrogacy and its acceptance has been slowly growing over the last two decades. There are a variety of indications for resorting to gestational surrogacy, with the most common being cases of absolute uterine infertility due to absent or nonfunctional uterus (like congenital absence of the uterus or irreparable uterine scarring) or same-sex male couples. Even in the USA where surrogacy is generally

accepted, there are unfriendly States where it is not allowed. Patients requiring gestational surrogacy are called intended parents and together with the gestational surrogate must undergo a complete medical and psychological screening prior to being accepted into a program. Identifying a gestational carrier and the right matching with intended parents is the most time-consuming step of the entire treatment, which takes about 4–5 months. Many surrogate agencies are available to bring together potential surrogates and intended parents, and the Internet has also created a means for people to meet and pursue these arrangements with numerous national and international websites devoted to surrogacy. However, it is critical to work with reputable agencies. The prohibition or unavailability of gestational surrogacy in many countries has promoted the phenomenon of cross-border surrogacy; however, it is important to be familiar with the legal frameworks required even in countries where surrogacy is allowed.

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Medical Treatments for Ovarian Protection

Charlotte Sonigo, Isabelle Beau, Nadine Binart, and Michael Grynberg

Introduction

Nowadays, several techniques aiming to preserve some gametes from chemotherapy-induced ovarian damages should be proposed to young patients with cancer prior to the beginning of treatment [1]. The application of these fertility preservation (FP) techniques, such as oocyte or embryo cryopreservation with or without ovarian stimulation or cryopreservation of the ovarian cortex, may be limited by age, pubertal status, disease, and emergency. In addition, these methods may be difficult to perform, and the future utilization of cryopreserved germ cells remains uncertain. Therefore, improving upon the FP strategies currently available and developing new FP approaches represent major challenges in oncofertility.

Chemotherapeutic agents exert a direct toxicity on the ovaries, and it is important to distinguish between the short- and long-term effects of these drugs. On the one hand, chemotherapy induces apoptosis of growing follicles rapidly after the beginning of treatment, leading to temporary amenorrhea. On the other hand, chemotherapy may lead to infertility years after the treatment. The impact of drugs on fertility after healing concerns the effects on the primordial follicular reserve as these treatments may lead to a premature loss and, at worst, primary ovarian insufficiency (POI). The extent of ovarian damages depends upon several factors, of which the most important are the type of drug, its dosage, and protocol and the ovarian reserve before the beginning of treatment [2]. Chemotherapeutic agents can be divided into five categories, including alkylating agents, platinum-based drugs, antitumor antibiotics, antimetabolites, and taxanes. Mechanisms implicated in the gonadotoxicity of these molecules have been explored in various experimental models, such as analysis of histological female ovary section after chemotherapy, animal models treated with injections, xenograft models, or cell cultures in the presence of active metabolites of chemical

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agents [3]. Nevertheless, these mechanisms are not yet fully understood; several hypotheses have been proposed and could coexist. Firstly, it was proposed that drugs exert a direct toxicity effect on primordial follicles, inducing DNA damages and subsequent apoptosis. More recently, it has been suggested that these drugs could induce depletion of ovarian reserve through primordial follicle over-recruitment. Increasing knowledge of the possible mechanisms implicated in chemotherapy-induced ovarian damages had facilitated the development of new therapies, called fertoprotective agents [4], aimed at decreasing the impact of chemotherapy on the ovarian reserve [5].

Reminder

Follicular Ovarian Reserve and Its Regulation

The follicular ovarian reserve, constituted by primordial follicles, is established early in life and then keeps declining regularly throughout the reproductive period. Each primordial follicle can remain quiescent for years, be activated and enter the growing process, or undergo atresia directly from the dormant stage [6]. The maintenance of female reproductive function implies the presence of a vast majority of quiescent primordial follicles and a continuous repression of primordial follicle activation into early growing follicles. This activation, starting during fetal life in human, is controlled by inhibitory and stimulatory factors. Numerous factors, such as growth factors, hormones, transcription factors, or cytokines, produced by oocytes and/or granulosa cells, can act in an autocrine, paracrine, or endocrine manner [7]. The quiescence of primordial follicles is maintained by several molecules, including phosphatase and tensin homolog deleted on chromosome 10 (Pten), tuberous sclerosis complexes 1–2 (Tsc1–Tsc2), Forkhead box protein O3A (Foxo3A), p27, anti-Müllerian hormone (AMH), and Forkhead box L2 (FoxL2) [6]. Many studies have highlighted the crucial roles of the phosphatidylinositol 3-kinase (PI3K) signaling pathway in oocytes in controlling follicular activation [8]. The transcription factor FoxO3A, mainly expressed in the oocytes of resting follicles, acts downstream of the PI3K signaling pathway and appears to be the main actor involved in follicular activation [7]. At the same time, the survival of primordial follicles is maintained by other mechanisms involving PDK1 signaling, rpS6, or the autophagy process. In the same manner, primordial follicle survival or apoptosis results from a balance between the expression of survival (antiapoptotic) and proapoptotic factors. Among these factors, the proteins B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (BAX) likely play a critical role. Therefore, a coordinated suppression of follicular activation, provided by multiple inhibitory and activator molecules, is required to preserve the primordial follicular stockpile in association with the process maintaining dormancy. Any disorder in these mechanisms might lead to a premature loss of the follicular reserve [9].

Pathophysiology of Chemotherapy-Induced Ovarian Damages

The assessment of the gonadotoxicity of chemotherapy is often based on organotypic or cell culture models in vitro. Moreover, in vivo studies in rodents as well as models of human ovarian xenograft are also commonly used to investigate the impact of chemotherapy on primordial follicles. The main chemotherapies used in these fundamental studies were cisplatin, cyclophosphamide, or doxorubicin. Thus, during the past decades, several hypotheses concerning chemotherapy-induced ovarian damages had been raised.

DNA Alteration, Follicular Atresia, and Apoptosis

Molecules used in chemotherapy induce alterations in the DNA and mainly double-stranded breaks (DSB). DSB can, in turn, lead to either cell death by apoptosis or DNA repair pathways

allowing cell survival [10]. DNA repair pathways differ according to several factors such as chemotherapeutic agent and may involve pATM, RAD51, or PARP1 proteins, for example [10]. When the repair pathways are not sufficiently activated, DNA damages induce cellular apoptosis. This mechanism is mainly mediated by p63 protein (and, more specifically, the TAp63 isoform), which activates Bcl2-associated X (BAX) protein and the Bcl-2 antagonist killer (BAK) protein [11]. These mechanisms are particularly complex within the ovaries and differ according to the type of chemotherapy molecule. A recent and extensive review discusses the induction and repair of DNA damages in the ovaries [10].

The DNA damage induced by drugs within growing follicles has been well-documented [5, 12]. Almost all classes of chemotherapeutic agents induce DNA alteration of granulosa cells and/or oocytes, leading to either apoptosis of growing follicles or the survival of mutagenic oocytes. Clinically, this phenomenon is revealed by temporary amenorrhea rapidly after the beginning of treatments [13]. More rarely, it can lead to spontaneous abortion or congenital abnormalities in the offspring if fertilization occurs during drug exposure [14]. Nevertheless, fertilization months or years after the end of protocol seem to be safe for offspring as these pregnancies are achieved from oocytes exposed in a dormant state, which remained genetically undamaged [15].

While apoptosis and atresia in growing follicles in response to chemotherapeutic agents have been well-investigated, the nature of these mechanisms in quiescent follicles is still under debate [10]. According to several studies, chemotherapeutic agents induce follicular depletion by directly affecting the primordial follicles entering massively into atresia [10]. Overall, in vitro ovary cultures, rodent models, as well as models of human ovarian xenograft were used to investigate the impact of chemotherapy on primordial follicles. Cyclophosphamide is a widely used alkylating agent, recognized as one of the most gonadotoxic drugs. It has been shown to induce DNA DSB and subsequent DNA damage response in a human ovarian xenograft model [16]. These results were confirmed in in vitro ovarian cultures with cyclophosphamide active metabolite [17–19] or after in vivo cyclophosphamide injection [20]. Same results were found with cisplatin, in in vitro analysis of newborn mouse ovaries [21, 22] or after in vivo cisplatin injection in newborn or adult mice [11, 20]. Recently, a model of a xenograft of human cortex ovaries in nude mice revealed the same results [23]. At least, similar effects with similar models were also found secondary to doxorubicin exposition [24, 25].

Follicular Activation

A more recent theory, called the "burnout effect," suggests that chemotherapeutic agents induce follicular depletion through the massive growth of dormant follicles, occurring simultaneously with the apoptosis of growing follicles [26]. Recruitment of primordial follicles would be secondary to the activation of the PI3K signaling pathway, whose role in follicle quiescence has been well-established by many knockout mouse models as well as in vitro studies on human ovarian cortex fragments [7, 27]. In addition, as mentioned above, cytotoxic agents destroy growing follicles, resulting in AMH secretion decrease. As this hormone is supposed to inhibit primordial follicles' recruitment, its decrease amplifies follicular activation and subsequent depletion of the follicular reserve. Moreover, no primordial follicles showed apoptosis signs [26]. Other studies, using the same mouse model, were in accordance with this burnout effect [3, 28] secondary to cyclophosphamide or cisplatin treatment [28]. In consideration of this theory, Lande et al. showed that, in vitro, phosphoramide mustard, a cyclophosphamide metabolite, enhances human primordial follicle activation in developing follicles [29]. This hypothesis could also explain the depletion of the follicular reserve observed in the presence of an ovarian endometrioma [30] or the massive follicular loss secondary to ovarian cortex transplantation [31, 32]. Nevertheless, the molecular mechanism by which chemotherapy activates the PI3K pathway within primordial follicles remains unclear.

Vascular Damage

Modifications in the ovarian stroma and vascularization are another mechanism potentially implicated in chemotherapy-induced follicle depletion [3, 13]. Indeed, vascular damages, revealed by decreased ovarian blood flow and reduction in ovarian size, have been demonstrated in women [33]. Moreover, the histological analysis of human ovaries previously exposed to chemotherapy revealed thickening and hyalinization of cortical stromal blood vessels in association with the disorganization of blood vessels in the ovarian cortex and cortical fibrosis [34]. Same results were observed in mice ovaries following doxorubicin administration [35].

Fertoprotective Agents

Improving the knowledge of the molecular mechanisms involved in chemotherapy-induced ovarian damage can lead to the development of treatments to limit follicular depletion in vivo [3, 13, 28, 36]. Molecular mechanisms implicated in the protective role of these different agents are more or less clear. Table 1 summarizes the main fertoprotective agents that have been evaluated in a mouse model, their mechanism(s) of action, and the proposed mechanism(s) to explain ovarian protection.

Molecules Inhibiting Primordial Follicular Apoptosis

The improvement of knowledge of the specific apoptotic and DNA repair pathways involved in chemotherapy-induced ovarian damages had revealed targets for protective agents to reduce or prevent follicular depletion [3].

Sphingosine-1-Phosphate and Ceramide-1-Phosphate

Sphingosine-1-phoshate (S1P) is a membrane sphingolipid involved in several physiological processes, including apoptosis of ovarian follicles. It was demonstrated that the sphingomyelin pathway regulates the developmental death of oocytes and S1P protects the ovarian follicular stockpile from radiation injuries [37]. Furthermore, S1P seems to reduce the atresia of primordial follicles occurring during slow freezing and thawing of human ovarian cortical strips [38].

Table 1 Main molecules evaluated, in in vivo rodent model, to limit chemotherapy-induced follicular depletion

Fertoprotective mechanism	Fertoprotective agent and mechanism of action	
Inhibition of primordial follicle recruitment	Rapamycin	mTOR inhibitor
	Everolimus (and INK128)	mTORC1/mTORC2 inhibitor
	Melatonin	Pineal hormone
	AS101	PI3K modulator
	AMH	Ovarian hormone
Inhibition of primordial follicular apoptosis	GNF2	c-Abl kinase inhibitor
	LH	Gonadotropin
	Imatinib	Competitive tyrosine kinase inhibitor (c-Abl kinase inhibitor)
	Sphingosine-1-phosphate Ceramide-1phosphate	Membrane sphingolipid
Several mechanisms proposed: – vascular effect – follicular recruitment inhibition, etc.	GnRH analogs	Inhibition of the pituitary-gonadal axis
Vascular effect	G-CSF	Granulocyte colony-stimulating factor
Prevention of chemotherapy nuclear activation	Bortezomib	Proteasome inhibitor

Several studies demonstrated that S1P was able to protect chemotherapy-induced ovarian damages. S1P injection directly into mice ovaries was shown to decrease primordial follicle apoptosis and to protect fertility after cyclophosphamide treatment [39, 40]. In a human ovarian xenograft model, S1P can block human apoptotic follicle death induced by doxorubicin and cyclophosphamide, preserving the ovarian reserve [41, 42]. Recently, ceramide-1-phosphate (C1P), another sphingolipid, was also found to be a potential ovarian protective agent as ovarian administration of this drug reduces follicular depletion induced by cyclophosphamide via the inhibition of follicle apoptosis and improvement of stromal vasculature [43]. However, one study provides conflicting results in a rat model treated by intraperitoneal cyclophosphamide injection [44]. One of the major limitations of these treatments is that they must be administered directly into the ovaries or by continuous administration. Nevertheless, recently, a long-acting oral form of an S1P analog has been developed, making these molecules potentially appropriate to human use [45].

Imatinib

Imatinib is a competitive tyrosine kinase inhibitor and, more specifically, a c-Abl kinase inhibitor. It is implicated in the apoptotic pathway induced by DNA damages in activating TAP63 transcriptional activity. Clinically, it is used in the treatment of cancer and especially in hemopathies. Based on its role as a c-Abl kinase inhibitor, imatinib was evaluated as a fertoprotective agent from cisplatin-induced primordial follicle loss. Indeed, this drug was shown to induce DNA damages and subsequent apoptosis in primordial follicles via TAP63 activation. Thus, it was hypothesized that imatinib could prevent TAP63 accumulation and activation induced by cisplatin and impede follicle apoptosis. Gonfloni et al. were the first to evaluate this theory in 2009 [21]. They observed the occurrence of massive primordial and primary follicle depletion in cisplatin-treated mice, whereas they noted a significant rescue of these follicles in ovaries of mice simultaneously treated with cisplatin and imatinib. Furthermore, they showed that this treatment had a long-term impact on fertility and reproductive outcomes. The same team confirmed these results in 2012 [46], and others found similar effects using in vitro newborn ovary cultures [47] and in vitro culture and subrenal grafting of mouse ovaries [48]. Nevertheless, two studies found that imatinib did not protect primordial follicle from cisplatin-induced apoptosis and did not prevent impaired fertility [23, 49]. Thus, due to the existence of conflicting results, additional studies are needed to evaluate whether imatinib could be a new treatment to limit cisplatin gonadotoxicity. Moreover, as imatinib interferes with the apoptotic pathway, it will be crucial to show that imatinib does not interfere with the antitumor activity of cisplatin.

Molecules Interfering with the DNA Repair Pathway

The efficiency of the DNA repair pathway is a critical determinant of a cell's survival following DNA damage occurring spontaneously or induced by chemotherapy. Thus, several studies have tried to develop molecules aiming to induce the DNA repair instead of apoptosis pathway in order to preserve follicle survival and limit follicular depletion.

For example, RAD51 is a protein implicated in DNA repair after DSBs. It was shown that, in an in vitro oocyte culture model, oocytes possess the machinery and capability for repairing DNA damage induced by doxorubicin through RAD51 activation [50]. Therefore, strategies manipulating RAD51 could be potential candidates to limit follicle depletion due to chemotherapy.

Recently, Rossi et al. reported a protective effect of luteinizing hormone (LH) on the primordial follicle pool of prepubertal mouse ovaries against cisplatin-induced follicular depletion [22]. These authors conducted an in vitro analysis and showed that LH treatment of prepubertal ovarian fragments generated antiapoptotic signals, reducing the oocyte level of proapoptotic TAp63 protein and favoring the DNA repair pathway in the oocytes. Thereafter, they showed that the administration of a single dose of LH to prepubertal female mice, concomitantly to cisplatin injection, limited the depletion of the primordial follicle pool.

Molecules Inhibiting Follicular Over-Recruitment

In accordance with the burnout effect, several investigations have been carried out to develop new molecules that would preserve the ovarian reserve by inhibiting the PI3K pathway and follicular activation [3, 36].

AS101

AS101 [ammonium trichloro(dioxoethylene-o,o′)tellurate] is an immunomodulatory compound that modulates the PI3K-Pten-Akt pathway [51]. This molecule was tested to prevent cyclophosphamide-induced follicle loss as this drug was found to activate the PI3K pathway, inducing primordial follicle recruitment and subsequent follicular depletion of ovarian reserve [26]. In vivo treatment of mice with AS101 was found to reduce cyclophosphamide-induced follicular depletion. Moreover, no increase in fetal malformation was observed in mice previously treated with AS101, indicating the safety of this treatment for offspring.

Anti-Müllerian Hormone

Anti-Müllerian hormone (AMH) is a glycoprotein hormone expressed by granulosa cells surrounding the oocytes. It is produced by follicles from the primary stage of development until selection for dominance. It has been shown to limit the activation of primordial follicles in in vivo or in vitro mouse models [52–54]. Recently, it was suggested in three studies that this hormone could be an option to limit chemotherapy-induced gonadotoxicity [55–57]. As AMH is produced only by ovaries and acts through a specific receptor expressed mainly by the ovaries, this hormone might be of particular interest since it could act as a targeted therapy without interfering with physiologic mechanisms or the efficacy of chemotherapy.

First, Kano et al. reported that superphysiologic doses of AMH delivered either by a recombinant protein via osmotic pumps or gene therapy could limit primordial follicle loss induced by cyclophosphamide, doxorubicin, or cisplatin in mice [55]. In this study, the protective effects of AMH vary between drugs, suggesting that mechanisms for chemotherapy-induced ovarian damage differed between drugs. Recently, Sonigo et al. assessed the protective effect of AMH in pubertal mice treated with cyclophosphamide [56]. In this model, the ovaries of cyclophosphamide-treated mice were depleted of primordial follicles, whereas the number of primordial and early-growing follicles was similar to controls in mice treated with concomitant injections of cyclophosphamide and AMH. At least, the number of ovulated eggs after ovarian stimulation was significantly reduced in cyclophosphamide-treated mice and rescued by AMH co-administration. Molecular mechanisms underlying these effects were explored. That study also provided data suggesting that AMH regulates FOXO3a phosphorylation and induces autophagy in ovaries. These results are in accordance with those of other studies suggesting the involvement of autophagy in the regulation of follicular ovarian reserve [58, 59]. Later, Roness et al. confirmed the fertoprotective role of recombinant AMH in the same mouse model as pharmacological administration of AMH during chemotherapy treatment reduced follicle activation and primordial follicle loss and significantly improved reproductive outcomes [57]. Interestingly, they also showed that AMH does not interfere with the therapeutic actions of chemotherapy.

Melatonin

Primarily revealed as a secretory product of the pineal gland, melatonin is commonly used in various biological processes, such as treating insomnia. Moreover, it can be used as a potential therapeutic adjuvant during chemotherapy as it has been shown to reduce some adverse effects of drugs [60]. Interestingly, melatonin is also produced in various tissues including reproductive tissues such as ovaries [61], and melatonin receptors are present in the oocytes and granulosa cells

of various species including humans [62, 63]. Recently, it was suggested as a new fertoprotective agent option against ovarian damages induced by chemotherapy [36, 64, 65].

Jang et al. evaluated the protection effect of melatonin on cisplatin-treated ovaries in a mouse model [64]. They demonstrated that combined treatment with melatonin and cisplatin significantly prevented primordial follicle loss in cisplatin-treated ovaries. The molecular mechanisms implicated were also analyzed, and the authors showed that the protection effect of melatonin was mediated by suppressing follicular recruitment through activation of the PI3K-Akt-FoxO3a signaling pathway. The same authors confirmed recently these results and revealed that ghrelin enhances the protective effect of melatonin against cisplatin-induced ovarian failure [65].

mTOR Inhibitors

mTOR is a serine/threonine kinase implicated in several critical processes, such as cell growth, proliferation, autophagy, and cell survival [66]. In animal model, mTOR stimulators increase the activation of primordial follicles, and mTOR inhibitors block the primordial-to-primary follicle transition [67, 68]. According to these data and after having confirmed the burnout theory, recent studies used mTOR inhibitors to preserve ovarian reserve from cyclophosphamide-induced follicular depletion in mice [69–71]. Goldman et al. explored the use of the clinically approved drug everolimus (RAD001) or the experimental drug INK128, showing that mTOR inhibition preserves the ovarian reserve measured through primordial follicle counts and serum AMH levels [69]. Moreover, cotreatment of cyclophosphamide with mTOR inhibitors preserved normal fertility. A protective effect of everolimus was also demonstrated against cisplatin-induced gonadotoxicity in an in vivo mouse model [71]. As everolimus can be used in the treatment of some breast cancers, this approach represents a very interesting option for fertility preservation during conventional chemotherapy. On the other hand, Zhou et al. observed that concomitant administration of chemotherapy with rapamycin, another mTOR inhibitor, significantly reduced primordial follicle loss [70].

Other Molecule Candidate as Fertoprotective Agent

G-CSF

In light of the vascular damages induced by chemotherapy, granulocyte colony-stimulating factor (G-CSF) was tested as a fertoprotective agent. It was shown that treatment with G-CSF decreased chemotherapy-induced ovarian follicle loss and extended the time to POI in female mice treated with cyclophosphamide and busulfan [72]. Later, follicle counts and serum AMH levels were found to be significantly increased in mice treated with cisplatin and G-CSF as compared with mice treated with cisplatin alone, confirming this fertoprotective effect [73].

GnRH Analogs

Tested in 1995 in rhesus monkeys, gonadotropin-releasing hormone (GnRH) analogs were the first agents considered as possible chemoprotective molecules against cyclophosphamide ovarian damages [74]. Later, several studies had evaluated the possibly protective effects of this treatment in a rodent model, and conflicting results were observed [75–80]. In a more recent study, it was proven that ovarian damages occurred even in the absence of FSH, suggesting that the inhibition of the pituitary-gonadal axis is not involved in ovarian protection during GnRH agonist treatment [80]. Nevertheless, other mechanisms, potentially implicated in this protective influence, were suggested as vascular effects or the upregulation of antiapoptotic molecules [81–83].

Several clinical studies have been performed to assess, in women, the efficacy of this treatment to protect ovaries from chemotherapy ovarian damages. POI incidence, chemotherapy-induced amenorrhea, menses recovery, or pregnancy rates were evaluated in cancer patients who received GnRH analogs or not at the time of chemother-

apy treatment. Conflicting results were reported [82, 84], and meta-analysis of randomized clinical trials revealed diverse conclusions about the ability of GnRH analogs to preserve fertility [85–88]. Nevertheless, while clinical evidence for the efficacy of this treatment is still under debate, the safety of this strategy has been already clearly demonstrated. Thus, this treatment could be proposed for all young women requiring chemotherapy, although gamete cryopreservation should be performed, if possible, for women who want to preserve fertility.

Tamoxifen

Tamoxifen is an estrogen receptor antagonist currently used as an adjuvant therapy for hormone-sensitive breast cancer. In a rodent model, the administration of tamoxifen significantly decreased doxorubicin- or cyclophosphamide-induced follicle loss [89]. Comparable results were found in cultured rat ovaries [90]. Nevertheless, the molecular mechanism of this protective effect during chemotherapy remains unclear.

Other Molecules

In the past few years, several other molecules have been reviewed to decrease chemotherapy-induced ovarian damages in order to preserve fertility, including Chinese herbal medicine, [91], fennel [92], sildenafil citrate [93], tocotrienol [94], genistein [95], and erythropoietin [96].

Conclusion

New therapies, aimed at limiting follicular loss and protecting ovaries, would be of great interest for young women facing with cancer. They could be used in combination with currently available fertility preservation techniques and administered regardless of age, pathology, or proposed treatment. Moreover, they would also prevent hormonal deficiencies and their consequences (e.g., pubertal delay, osteoporosis). Moreover, these treatments may be of particular interest among women with altered ovarian reserve parameters in whom no fertility preservation method could be proposed**.**

Whereas several molecules had been tested to limit chemotherapy-induced ovarian damage in mice, their application in clinical practice is complicated for several reasons. Firstly, the applied protocols incorporate a combination of several drugs. Therefore, the results obtained should be carefully extrapolated to women, and caution should be observed when interpreting the clinical relevance of such findings. Indeed, it is difficult to mimic doses and protocols used, and ovarian physiology and responses to treatments can differ Secondly, for these molecules to be used in clinical practice and studied in women, it is essential that they do not interfere with the therapeutic action of chemotherapy or important physiological processes. However, as apoptosis represents the main mechanism of anticancer action, apoptosis inhibitors could reduce the anticancer effect of chemotherapy. In addition, by blocking the death of oocytes with DNA alterations, some molecules could facilitate the survival of damaged germ cells and thus promote infertility, an increased risk of spontaneous miscarriages, or fetal malformations. Finally, the PI3K pathway is a ubiquitous pathway, and molecules modulating this pathway could interfere with various physiological processes.

Supplemental Elements

Definitions

Fertoprotective agents: molecules aiming at decreasing the impact of chemotherapy on the ovarian reserve.

Practical Clinical Tips (i.e., within Clinical Cases)

If these strategies seem promising, several studies should be performed before a possible current application for women.

Take-Home Messages

Several theories regarding chemotherapy gonadotoxicity have been raised: (1) direct detrimental effect on the DNA of primordial follicles and subsequent apoptosis, (2) massive growth of dormant follicles which are then destroyed, or (3) vascular ovarian damage. The improvements in the understanding of the mechanisms involved allowed the development of molecules aiming to limit the negative impact of chemotherapy on the ovaries.

Clinical Cases

With the exception of GnRH agonists, there is no treatment currently used for women undergoing chemotherapy.

Key Readings

1. Roness et al. [3]
2. Woodruff [4]
3. Roness et al. [5]
4. Winship et al. [10]
5. Blumenfeld [82]

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Transplantation of Isolated Follicles and the Engineered Ovary

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Introduction

Cancer is one of the major health issues worldwide and the first or second leading cause of death depending on the world area investigated. Indeed, the International Agency for Research on Cancer estimated that in 2018, more than 276,000 people under 44 years were diagnosed with cancer in Europe [1]. Incidence rates are significantly higher in females (65%) than males (35%) in these younger age groups (Fig. 1). However, thanks to vast improvements in cancer treatments, 5-year survival rates are now 65% in adults and as high as 83% in children [2]. Taking into account the known gonadotoxicity of some cancer treatments and increasing numbers of cancer survivors, fertility preservation is becoming a clinical and moral duty in oncological practice.

While most cancer patients can choose among different fertility preservation strategies, prepubertal patients and women who cannot delay the start of their chemo- or radiotherapy have had to rely on ovarian tissue cryopreservation, followed by orthotopic reimplantation once cured [3]. Despite successful outcomes reported with this approach, including more than 130 live births to date [3], it cannot be universally applied. Indeed, in certain types of cancer, malignant cells may be present in the ovaries, so transplantation of frozen-thawed ovarian tissue fragments is not advised [4]. Although most malignant tumors in reproductive-age subjects do not metastasize to the ovaries, hematological malignancies like leukemia and Burkitt's lymphoma, as well as neuroblastoma, carry a high risk of ovarian metastasis [5]. Leukemia is the most common cancer in Europe among young girls up to 19 years of age and the seventh most prevalent in women of reproductive age (20–44 years old), representing 5% of all cancer cases in women. Thanks to early diagnosis and improved treatments, almost 50% of patients survive [1]. Burkitt's lymphoma accounts for around half of all malignant non-Hodgkin's lymphomas in children [6], with an estimated annual incidence of 3000 girls aged between 0 and 14 years [1]. Fortunately, around 85% of patients survive after treatment [6]. Neuroblastoma represents around 10% of solid tumors in children under 15 years of age, affecting 1 in every 70,000 [7]. In patients aged over 1 year, 5-year survival rates can reach 95% for some localized tumors [7].

Since studies have shown that cancer cells are able to resist the freeze-thawing procedure and spread in the host after xenotransplantation [8], autografting of cryopreserved tissue can potentially

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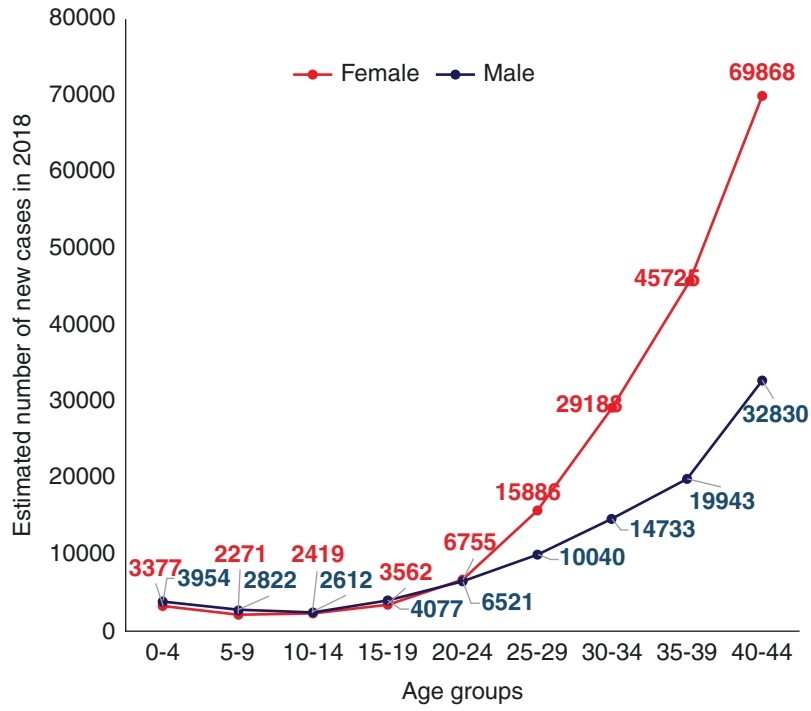

Fig. 1 Estimated number of new cancer cases and age-specific incidence rates [1]

lead to recurrence of the disease in affected patients. Sadly, there are no currently available options for fertility restoration in these women. However, there are two main approaches under development: in vitro growth of preantral follicles, followed by in vitro maturation of their oocytes (see Chap. 29), or transplantation of isolated preantral follicles, also known as the engineered ovary. In the last decade, the idea of engineering an ovary has been gaining ground, and increasing numbers of research teams worldwide have been concentrating their efforts on developing this strategy. The aim of this chapter is therefore to discuss the latest advances in the field and future directions of this emerging fertility restoration approach.

Transplantable Engineered Ovary Concept

By definition, the transplantable engineered ovary has the essential role of replacing the natural organ, thereby allowing complete folliculogenesis, with production of fertilizable mature oocytes and secretion of sex hormones. In order to construct a tissue-engineered ovary, we need to consider three essential points: (1) safe isolation of a large number of preantral follicles, (2) presence of different populations of ovarian cells, and (3) creation of a three-dimensional (3D), biodegradable matrix to encapsulate isolated follicles and cells and protect them during and after grafting.

In practice, after patient remission, fragments of her frozen ovarian tissue would be thawed for further follicle isolation. In the meantime, a second ovarian biopsy would be taken from the patient after her cancer treatment for cell isolation. In this way, we could ensure that only ovarian cells would be added to the transplantable engineered ovary, as all malignant cells would have been destroyed by chemo- or radiotherapy. Isolated preantral follicles and ovarian cells would then be encapsulated inside the matrix and transplanted back to the patient.

Are Isolated Follicles Able to Survive and Resume Folliculogenesis After Transplantation?

The follicle is the functional unit of the ovary and has two main functions: it is responsible for hormone production and providing the ideal environment for oocyte growth and development. Follicles are present in the ovary at different stages: primordial, primary, secondary, and antral. Preantral follicles (primordial, primary, and secondary) represent 90–95% of all follicles, but, of this number, only 0.1% will go on to develop to the antral stage, while the rest will undergo atresia. Why so few follicles are destined to ovulate remains a mystery. However, it is known that not only follicle themselves but also surrounding ovarian cells and extracellular matrix (ECM) play a role in the process.

Isolation of preantral follicles potentially allows us to safely use these structures in the transplantable engineered ovary, as they are enclosed inside a basement membrane that prevents their granulosa cells and oocyte from coming into contact with capillaries, white blood cells, and nerve processes [9]. However, they cannot survive alone, as demonstrated in some in vitro culture studies. Indeed, while isolated mouse follicles enclosed in a collagen gel matrix were able to survive in vitro for at least 2 weeks, when they grew to multilayer stages, they showed signs of degeneration and no antrum or theca cell formation [10, 11]. On the other hand, when isolated mouse follicles were transplanted together with ovarian cells, they successfully reached the antral stage in 3 days, with blood vessels appearing after 5 days and theca cells 6 days post-grafting [11]. After 10 days, 16 oocytes were recovered for in vitro fertilization, and 12 developed up to the two-cell embryo stage [11].

After incorporating mouse ovarian tissue digests (isolated follicles and ovarian cells) in plasma clots, Gosden [12] transplanted them to the ovarian bursa of sterile mice. These constructs were able to restore fertility in their hosts, as pregnancies and offspring were obtained after mating. Following graft removal, this author found ovary-like structures, with follicles at different stages of development. Carroll and Gosden [13] subsequently repeated the experiment using frozen-thawed ovaries and obtained similar results.

After isolation of follicles from human tissue, encapsulation of preantral follicles in plasma clots, and xenotransplantation to immunodeficient mice, Dolmans et al. [14, 15] also reported follicle survival and growth. Moreover, stroma-like tissue of human origin was formed, suggesting the presence of isolated human stromal cells in the constructs. Indeed, when retrieving isolated follicles, we may inadvertently pick up isolated cells too [16]. The constructs were found to be well vascularized already 1 week after xenografting [14], and, like in mice [12, 13], ovary-like tissue was observed [14, 15].

Based on these pioneering studies, we can confirm that preantral follicles are able to survive and resume growth after isolation and transplantation, but they need to be transplanted along with their accompanying ovarian cells and encapsulated in a 3D matrix.

How Can we Improve Survival and Growth of Isolated Follicles After Transplantation?

The pioneering studies mentioned above revealed the basic requirements for survival of isolated follicles after transplantation. In recent years, numerous experiments have investigated matrix materials and cell populations for the development of the transplantable engineered ovary.

Matrix Materials: From Polymers to Decellularized Ovarian Tissue

One of the key steps in the development of the transplantable engineered ovary is finding the right matrix material to encapsulate isolated ovarian preantral follicles. Different hydrogels have been used for transplantation of isolated follicles in an attempt to enhance efficacy [11, 12, 17–22]. Despite great advances, we have yet to

identify the best matrix material to support complete human folliculogenesis. Indeed, our knowledge of human ovarian tissue remains incomplete, and great differences emerged when murine and human follicles were transplanted inside an engineered ovary prototype [23]. Therefore, in this section, we will first briefly mention the general features of a 3D matrix for isolated preantral follicles and then discuss the main findings obtained from murine and human ovarian follicles in various in vivo studies. Irrespective of the nature of the matrix (biological, synthetic, or hybrid), there are other crucial points to consider before selecting one material over another. First of all, we must look at biocompatibility and biodegradability, features that are normally closely interconnected. Greater biocompatibility and biodegradability have been demonstrated with natural polymers (collagen, gelatin, alginate, fibrin, decellularized ECM) than synthetic ones (poly(ethylene glycol) [PEG]) because cell metalloproteinases cleave them without producing toxic compounds that may affect cell survival [24, 25]. Indeed, broader application of biological matrices rather than synthetic scaffolds is documented in the literature [26]. In addition, natural hydrogels, like collagen or fibrin, provide better cell adhesion than their synthetic counterparts, thanks to the presence of peptides containing arginine-glycine-aspartate (RGD) sequences involved in integrin binding on cell surfaces. The architecture of the hydrogel's structure and its viscoelastic properties and biochemical constitution are also key elements. The composition and porosity of the network will determine not only the rate at which nutrients, gas, and metabolic waste move through the hydrogel but also how follicle survival and development are affected. A recent study [27] showed how the controlled microporous geometry of the applied scaffold impacted mouse follicle survival, growth, and maturation in vitro. The ovarian environment is indeed highly dynamic. Unlike any other cellular complex in the body, human ovarian follicles, once activated, increase in size up to 600X during growth and release various proteases essential to modifying their surrounding ovarian ECM and generating a more acquiescent milieu for their complete development. A hydrogel's viscoelastic properties are therefore key parameters to consider in the choice of matrix material. In this context, the ideal matrix should be rigid enough to mimic the stiffness of the collagen-rich ovarian cortex in the first few days post-transplantation but then provide enough elasticity to allow complete follicle growth and protease degradation. A more challenging goal is mimicking the native biochemical ovarian environment. A recent study [23] was able to distinguish 1508 different proteins in the human ovary, with more than 80 of them derived from the ovarian ECM. Although our knowledge of the biochemical composition of ovarian tissue is still in its infancy, complete characterization of the ovarian follicle microenvironment is vital to design a bioengineered ovary resembling the native tissue.

Animal Studies

After groundbreaking studies on transplantation of isolated mouse follicles by Gosden's group [11–13], several decades passed before new attempts were made to graft these structures. Laronda et al. [27] developed a gelatin-based matrix using a 3D bioprinter, selecting specific architecture to maximize follicle-matrix interaction. When mouse ovarian follicles were grafted inside this matrix, restoration of fertility was achieved, demonstrating the importance of the architecture of the supporting scaffold for complete follicle development in mice.

Alginate-based matrices showed encouraging results with murine and nonhuman primate follicles after in vitro culture [28], but efficiency was diminished in in vivo studies. Vanacker et al. [18, 29] were the first to encapsulate and transplant isolated mouse preantral follicles in alginate. Although these follicles were able to grow to the antral stage, poor matrix degradation and revascularization were noted 1 week after autotransplantation. Both softer and more rigid alginate remain a good choice for culture of isolated murine [30] and human [31–33] follicles, respectively, but new hydrogels are needed for follicle transplantation. In this context, fibrin matrices have found broader application. Unlike the previ-

ously mentioned natural matrix materials, this protein has shown numerous advantages. First of all, it was already being applied in diverse surgical settings. Moreover, thanks to the combination of its two main constituents, namely, thrombin and fibrinogen, fibrin formulations and architecture could be adjusted according to the desired application [34]. Indeed, softer fibrin formulations (made from lower fibrinogen and thrombin concentrations) were found to be optimal for mouse ovarian follicle transplantation [17, 35, 36], also leading to restoration of endocrine and reproductive functions when vascular endothelial growth factor (VEGF) was cross-linked to the matrix and gradually released after transplantation [19].

To date, only one synthetic hydrogel has been tested as a material for the engineered ovary. A combined PEG-vinyl sulfone (PEG-VS) matrix, with integrin-binding peptides like RGD cross-linking PEG-VS with protease-sensitive peptides, was used to encapsulate partially isolated mouse preantral follicles before transplantation to mice [37]. After 30 days, multiple antral follicles, corpora lutea, and reduced follicle-stimulating hormone levels were observed, demonstrating that PEG-VS was able to support folliculogenesis and steroidogenesis. Furthermore, on day 60, around 60% of primordial and primary follicles were still detected in the graft, proving selective follicle recruitment over time [37].

An emerging new generation of matrices is now being derived from decellularized ovarian ECM. Ideally, allogeneic [22, 38] or even xenogeneic cell-free matrices retain the natural architecture of native tissue and, to a variable extent, most of the physiological components inside the native ECM. A few studies have reported decellularization of murine [39], porcine [40], bovine [38], and human [22, 38, 41] ovarian tissue. It is important to stress that decellularization protocols have been shown to affect matrix composition differently, changing concentrations of collagen, glycosaminoglycans (GAGs), and elastic fibers [39]. When rodent ovarian cells were seeded in such a matrix, they were able to survive, proliferate, synthesize estradiol [38, 40], and even induce puberty in mice after grafting [38]. This matrix was also successfully tested in the context of isolated mouse follicle transplantation [22].

Human Studies

While fertility restoration using an engineered ovary prototype has already been proved in mice [12, 13, 19, 27], studies in humans have been rather more challenging [14, 20–22, 31]. One of the main reasons is the physiological difference in folliculogenesis and the ovaries between rodents and humans. Extrapolating data from murine models to humans is therefore unreliable and disappointing.

To transplant isolated human preantral follicles, plasma clots [14, 15] were replaced by a fibrin matrix, as it was shown to have highly variable composition and fast degradation rates, but it led to follicle loss and irregular results after transplantation. Moreover, due to its very delicate structure, it could potentially be damaged during laparoscopic surgery.

Although softer fibrin formulations have been successfully used to transplant isolated mouse follicles [17, 35], our experience has shown that isolated human follicles require a stiffer fibrin matrix. After xenografting them inside a fibrin matrix containing increased fibrinogen and thrombin concentrations, follicle recovery rates were 20–35% [20, 21], comparable to the results of xenografting of human ovarian tissue [42].

As in rodents, decellularized ovarian ECM has also been tested as an alternative to transplant isolated human preantral follicles. However, when a whole ovary or fragments of ovarian tissue are decellularized [38, 41], we obtained a heterogeneous tissue architecture with small and very large pores that were once occupied by different-sized follicles (Fig. 2). For this reason, routine cell seeding protocols to repopulate the matrix [43] cannot be applied. Instead, it is necessary to resort to different strategies that may actually defeat the purpose of one of the best features of decellularized matrices, namely, structure. Indeed, when Pors et al. [22] created scalpel-made pockets in decellularized human ovarian ECM to insert isolated human follicles,

Fig. 2 Decellularized human ovary [41]

they did not find any follicles after transplantation to mice. Positive results were achieved only when isolated follicles were embedded in Matrigel before being poured onto the constructs [22], which demonstrates the need for another material to be used alongside decellularized ovarian ECM. It is, however, important to bear in mind that the use of Matrigel, which is not approved in clinical practice, may also have positively influenced these findings.

Another strategy to take advantage of the biochemical cues of ovarian ECM is its application in the form of decellularized hydrogels. Studies have revealed that these hydrogels can promote de novo formation of functional tissue [44], as they retain their biological characteristics and encourage tissue regeneration [45]. After the decellularization process, numerous ECM proteins from native tissue can be found in the hydrogel [45], as well as cytokines stored in tissues or cells [46], which may promote tissue growth as they are released into regenerating tissue during hydrogel remodeling [45]. We recently developed a decellularized bovine ovarian ECM hydrogel [47], in which ovarian follicles and cells could easily be seeded. Our preliminary results showed that after 10 days of in vitro culture, preantral follicles remained viable in the hydrogels [47].

Ovarian Cells and their Role in the Transplantable Engineered Ovary

While the main players in the creation of a transplantable engineered ovary are isolated preantral follicles, the co-presence of cells may be fundamental to achieving complete folliculogenesis and improving ovarian regeneration. Indeed, several studies have pointed out that the number and spatial distribution of ovarian cells in an engineered ovary appear to play a key role in follicle development [48–50].

Ovarian Stromal Cells

Initial studies by Gosden's group [11–13] provided the first evidence of the role of ovarian stromal cells in the transplantable engineered ovary. These cells were needed for ovarian tissue reconstitution and possibly had an impact on follicle development. Indeed, stromal cells can synthesize numerous factors that regulate primordial follicle activation [51]. In turn, granulosa cells and oocytes from growing primary follicles will secrete growth factors to recruit and differentiate cells from the ovarian stromal compartment into theca cells [52, 53] essential for further follicle development.

Autologous stromal cells can be easily isolated from fresh or frozen-thawed ovarian tissue biopsies [54]. The safest option is to harvest them from ovarian samples collected after patient disease remission, namely, after cancer treatment, so there is no risk of malignant cell contamination of the engineered ovary. However, it is possible that some patients may not have any remaining ovarian tissue to collect these cells. In such cases, stromal cells can be retrieved from tissue samples cryopreserved before chemo- or radiotherapy. Cells can be isolated after follicle pick-up [20, 21], but a purging step is required to avoid any possible malignant cell contamination [55, 56]. Soares [57] compared stromal cells collected from fresh ovarian tissue procured from patients after cancer treatment to frozen-thawed ovarian samples. This author did not observe any difference in the cells from the two groups in terms of survival [57]. Moreover, isolated ovarian stromal cells can successfully proliferate after transplantation inside different matrices [17, 18, 20, 21, 29, 35, 57–59] and have been shown to contribute to matrix degradation and ECM synthesis (Fig. 3).

Ovarian Endothelial Cells

Our first evidence that endothelial cells isolated from ovarian tissue play a significant role in the

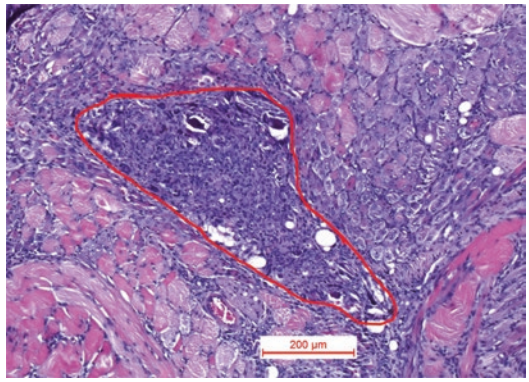

Fig. 3 Isolated human ovarian stromal cells after encapsulation in a fibrin matrix (outlined in red) and 7 days of xenotransplantation to immunodeficient mouse

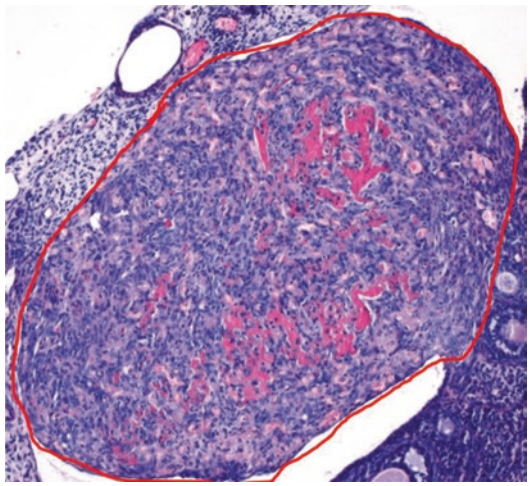

Fig. 4 Isolated human ovarian stromal and endothelial cells after encapsulation in plasma clots (outlined in red) and 7 days of xenotransplantation to immunodeficient mouse

engineered ovary came from the study by Dath et al. [59]. After comparing grafts containing isolated stromal cells with or without endothelial cells, they observed a large grafted area showing an impressive number of vessels as early as 1 week after transplantation [59] (Fig. 4). Soares et al. [54] subsequently assessed the influence of endothelial cell concentrations on vessel area and demonstrated a positive correlation between the number of grafted isolated cells and vascularization after 7 days of xenografting to immunodeficient mice [54]. As endothelial cell concentrations are quite low in ovarian cortex, we could consider isolating these cells from the medullary layer, as suggested by Soares et al. [54].

While vascularization in the transplantable engineered ovary can initially be induced by the presence of isolated endothelial cells encapsulated in the matrix, it is important to stress that the number of vessels may also be influenced by growing follicles [60]. Indeed, when isolated mouse secondary follicles were transplanted separately from small primordial and primary follicles, the grafts exhibited a greater vessel surface area and more vessels [60], possibly due to expression of angiogenic factors like VEGF by these growing follicles [61].

Deconstructing the Native Human Ovary to Construct an Engineered Ovary

Ovarian Matrisome

Ovarian follicles and cells are inherently surrounded by a complex meshwork of proteins forming the ECM, which is destroyed by isolation procedures. Therefore, in order to develop a transplantable bioengineered ovary that offers an environment in which isolated human follicles can survive and grow, it is necessary to provide them with a biomimetic ECM. While numerous attempts by various research teams have demonstrated the feasibility of this concept [17, 18, 29, 34], they also revealed that developing an engineered ovary requires more than the simple use of biomaterials and application of tissue engineering strategies. Indeed, an essential requirement for replicating the complexity of a functional ovary is a comprehensive understanding of the composition, architecture, and plasticity of the human ovarian ECM, something we do not yet have. Thus, deconstructing the microenvironment of ovarian cells and follicles is a prerequisite to constructing a bioinspired engineered ovary.

Proteomics is a powerful tool that can be used to scrutinize the composition of the ovarian ECM without a priori knowledge, in contrast to targeted studies using specific antibodies [62]. Very

recently, proteomics was used to characterize the composition of human ovarian tissue in reproductive-age subjects and develop the first draft map of the human ovarian matrisome [23]. The matrisome is essentially an extended description of the ECM [63], including not only all structural ECM components but also proteins able to regulate and remodel the ECM. It can be categorized as follows:

- Core matrisome proteins:
  - ECM glycoproteins
  - Collagens
  - Proteoglycans
- ECM-associated proteins:
  - ECM-affiliated proteins, namely, proteins showing biochemical and architectural analogy with ECM proteins or known to be associated
  - ECM regulators, namely, proteins responsible for ECM turnover
  - Secreted factors, namely, secreted proteins interacting with core ECM proteins

Ouni et al.'s study shows the human ovarian matrisome to be composed of 49% collagen, 7% proteoglycans, 15% glycoproteins, 18% ECM-affiliated proteins, 10% ECM regulators, and 1% secreted factors [23]. Indeed, around 100 proteins make up the ovarian matrisome, so the task of developing a bioinspired engineered matrix is quite challenging. Selection criteria need to focus on key proteins whose presence is absolutely required in the engineered ovary. Only comparative studies between different age groups can elucidate specific features of the reproductive-age ovary that make it more inclined to follicle survival and development than its younger and older counterparts.

Understanding the composition of the ovarian matrisome is not enough to be able to construct a biomimetic ovary. It is also crucial to bear in mind that ovarian follicles are exceptional in that they can grow to around 600X their size during folliculogenesis (e.g., human follicles grow from 30 μm at the primordial stage to 18–24 mm when they are ready to ovulate). It is therefore important to explore the intrinsic elasticity of the human ovary. In contrast to proteomics, very few studies have investigated the elasticity of human ovarian tissue [64, 65]. However, only recently, Ouni et al. (unpublished results) revealed the true elastic system components that confer intrinsic mechanical features to biological tissues, including collagen, known as the provider of tensile strength to tissue. On the other hand, elastin, elastin microfibril interface-located protein 1 (EMILIN-1), fibrillin-1, and GAGs are known to temper the rigidity of collagen by providing recoil and elasticity. This study revealed some interesting spatiotemporal changes to elastic ECM deposition and turnover, dictating the mechanical features of the human ovary in the course of a woman's lifetime. This is consistent with the emerging hypothesis related to the mechanobiology of the human ovary and dynamic reciprocity that exists between ovarian cells and their microenvironment [66–68].

Ouni et al.'s study also shed light on follicle-specific ECM composition, dependent on follicle stage and age. These data will prove very useful in developing the bioengineered ovary. Indeed, they emphasize the importance of encapsulating each type of isolated follicle in an appropriate biomaterial that must replicate the corresponding functional perifollicular ECM and respect ovarian tissue heterogeneity [69] to guarantee its biomimicry.

Another facet of the ovarian matrisome that needs to be investigated is its microarchitecture and ultrastructure. Indeed, it is essential to respect native tissue porosity and fiber organization because they are involved in nutrient and oxygen transport [70]. Moreover, fibrous matrisome proteins provide cells with external stimuli and instructive cues to influence gene expression, cell behavior, and morphology, as evidenced by emerging publications on this subject [71–73]. Proof of the involvement of matrisome ultrastructure in dictating cell phenotype is how collagen alignment has been used as a prognostic signature for survival in human breast carcinoma [74]. However, in the context of the ovary, these data are still lacking. Novel analytical techniques must therefore be applied to conduct in-depth studies of the ovarian microarchitecture and pro-

duce a map of the ultrastructural features of reproductive-age tissue and follicle stage niche.

Increasing evidence today points to different roles of the matrisome, particularly the ovarian matrisome, beyond its apparent primary function of physical support. Knowing that biodegradable matrices cannot replicate the complexity of a given microenvironment, decellularization has emerged as the ultimate means of creating more efficient biomimetic scaffolds [75]. However, it is worth mentioning that decellularization methods rely on the use of harsh physical, enzymatic, and chemical procedures (e.g., sodium dodecyl sulfate) [22, 76] that can deplete the decellularized scaffold and erase key matrisome elements. Therefore, without comparing the properties of such matrices against a reference of the native human ovarian matrisome, it is impossible to monitor their reliability to reproduce the ovarian cell microenvironment.

In contrast to most tissue engineering strategies relying on the presence of generic ECM components (collagen, fibrin, laminin, etc.) as indicators of the success of their biomimetic constructs, deconstructing the natural human ovary seeks to map the ovarian matrisome with its multiple facets. The aim is to build a reliable reference of its unique features, which will serve as a roadmap for developing a more closely biomimetic matrix tailored to human ovarian cells and follicles, and applicable in the field of fertility preservation.

Subpopulations of Ovarian Cells

While it is known that human ovaries contain a vast majority of stromal cells, followed by endothelial cells [77], it was only recently that in-depth characterization of cell populations was undertaken in human ovarian tissue [78, 79]. In their study analyzing the ovarian cortical layer, Wagner et al. [78] not only identified other subpopulations of cells, but also reported their proportions; stromal, endothelial, and perivascular cells correspond to around 83%, 5%, and 10% of cells in the ovarian cortex, respectively, while macrophages and T-cells account for around 0.5%. These findings are extremely important for development of an engineered ovary. For instance, macrophages play a role in matrix remodeling and synthesize growth factors and cytokines [80]. Moreover, M1 macrophages appear to be implicated in folliculogenesis in mouse ovaries [81], while M2 macrophages are involved in tissue repair through synthesis of anti-inflammatory cytokines, such as VEGF and transforming growth factor beta [82]. Interestingly, these authors did not find a population of germline stem cells, which was a rather contentious finding by Tilly's group [83, 84]. Analysis of the inner cortical layer of human ovaries revealed clusters of stromal, endothelial, theca, immune, smooth muscle, and granulosa cells [79]. Most importantly, Fan et al. [79] reported several types of these cells.

As these cell populations are essential to folliculogenesis, ECM synthesis, tissue remodeling, vascularization, and other such processes, it is vital to ensure that they are present in the transplantable engineered ovary. Information generated by such studies is also crucial to evaluating the constructs after transplantation and understanding our findings.

Theca Cells

Theca cells have been poorly investigated compared to granulosa cells and oocytes, but their role in folliculogenesis is undeniable. Indeed, if they were not included in engineered ovaries, follicles could not develop beyond the secondary stage, and, consequently, ovarian function and fertility would never be restored.

Theca cells start to appear at the later stage of secondary follicles and form a layer around granulosa-oocyte structures [85]. They play an indispensable role in folliculogenesis by providing structural support for follicles and androstenedione as a substrate for granulosa cells to convert to estradiol [86–88]. Besides such key tasks for theca cells, we have limited knowledge about their recruitment, growth, and differentiation due to a lack of information concerning their origin [53, 89]. A few studies using murine models have shown the presence of a subpopulation of ovarian cells, called theca progenitor stem

cells, which are recruited to differentiate into theca cells under the influence of factors secreted by oocytes and granulosa cells [53, 89]. Honda et al. [53] studied neonatal mouse ovaries and reported that theca progenitor stem cells express GLI family zinc finger 3 (GLI3) and protein patched homolog 1 (Ptch1) as markers and can differentiate into theca cells in the presence of factors issuing from oocytes and granulosa cells. After differentiation, these cells express luteinizing hormone receptor (LHR), GLI2, and Ptch2. Liu et al. [89] found that there are two origins of theca progenitor cells in embryonic mouse gonads: (1) Wilms tumor 1-positive mesenchymal cells intrinsic to the ovary, which differentiate into theca cells after birth, giving rise to theca fibroblasts, perivascular smooth muscle cells, and interstitial ovarian cells, and (2) GLI1-positive cells, which migrate from the mesonephros to the ovary and become steroidogenic androgen-producing cells inside the ovary. In large animal models, like cows [52] and goats [90], ovarian cortical stromal cells can differentiate into theca cells in the presence of granulosa cells by expressing cytochrome P450 17A1 (CYP17A1) and LHR as markers, as well as and androstenedione [52, 91], but there are no studies confirming the presence of a subpopulation of theca precursor cells in human ovaries. However, Fan et al. [79] detected a theca cell population in human ovaries that they termed common progenitor theca cells, which progress to theca interna or externa cells, but these findings still need to be confirmed. Moreover, these cells were found surrounding small antral follicles, in the inner cortical layer, while recruitment and differentiation of theca cells take place during the early primary/secondary stage of follicle development. One may therefore wonder if these cells surround follicles right from the start or are spread throughout the ovarian cortex.

In a study using isolated bovine cortical stromal cells, Orisaka et al. [52] reported that cells can differentiate into theca cells under stimuli provided by granulosa cells in vitro, as evidenced by the steroidogenic markers they assessed. Subsequent to these intriguing results, Asiabi et al. (unpublished data) isolated stromal cells from the cortical layer of postmenopausal ovaries and in vitro-cultured them using a medium supplemented with growth factors presumed to be involved in theca cell differentiation. After 8 days of in vitro culture, up to 43% of cells differentiated into theca interna cells. Moreover, a modest proportion of small luteal cells was also identified. These findings indicate the presence of theca precursor cells spread throughout the cortical layer, which remain in the human ovary throughout its lifetime. In vivo, factors used by Asiabi et al. (unpublished data) are synthesized by granulosa cells and oocytes from growing follicles. We can therefore hypothesize that once isolated follicles and ovarian cells are transplanted along with the 3D matrix, these follicles can induce recruitment and differentiation of theca cells.

Conclusions

Pioneering studies into murine and human follicles [11–15] have demonstrated the feasibility of grafting isolated follicles, but recent findings indicate that this approach can be improved through utilization of a transplantable engineered ovary. Over the last years, we have shown that human preantral follicles can be safely isolated in large numbers by adapting the isolation protocol according to ovarian tissue type [16, 21, 92, 93]. We have also learned that in order to restore ovarian function and fertility, ovarian cells need to be incorporated into the engineered ovary. Indeed, we witnessed their impact on the formation of ovary-like tissue, with vascularization and synthesis of the ECM [21, 35, 54, 59, 60]. Under the influence of growing follicles, a subpopulation of these cells can be recruited to differentiate into theca cells (Asiabi et al., unpublished data). Taking advantage of the insights acquired from single-cell analysis of human ovarian cortex [78, 79], we know it is possible to enrich the engineered ovary with specific subpopulations of ovarian cells to enhance tissue remodeling or release growth factors that can positively affect folliculogenesis or synthesis of elastic proteins, for instance. Regarding

the 3D matrix to be used to encapsulate and transplant isolated follicles and cells, numerous biomaterials have been tested [11, 14, 27, 28, 34, 36, 37]. While they offer good mechanical support for follicles, they do not possess the biomechanical and biochemical cues normally present in the ovarian ECM. In recent years, investigations have shed new light on the composition and structure of human ovaries [23, 65], showing that the complexity of the ovarian ECM is impossible to replicate with a polymer. On the other hand, we may get closer to our goal with the use of decellularized ovarian matrices [22, 38–41, 43, 47]. However, studies are still in their infancy, and it is not yet possible to determine if this type of structure will be the matrix of choice for the engineered ovary.

Definitions

- Decellularization: a procedure first developed by Prof. Stephen F. Badylak, where cells from a given tissue or organ are destroyed and removed, leaving only the extracellular matrix scaffold. The purpose of this procedure is to create matrices to be used in tissue engineering and regenerative medicine approaches.
- Folliculogenesis: a process describing the activation and growth of ovarian primordial follicles up to preovulatory stage.
- Tissue engineering: an interdisciplinary field that uses the combination of cells, materials, biochemical and physicochemical factors, and engineering with the goal of replacing, repairing, or improving tissues and organs in the body.

Take-Home Messages

- Human preantral follicles can be safely isolated in large numbers.
- Survival and development of isolated ovarian preantral follicles are feasible.
- Ovarian cells have an important role in transplantable engineered ovary, as they aid in the scaffold degradation, synthesis of extracellular matrix, and vascularization.
- A subpopulation of ovarian cells is able to differentiate into theca cells, which are essential for follicle development.
- Current biomaterials used to transplant isolated follicles and cells do not possess the biomechanical and biochemical cues normally present in the ovarian ECM.
- New studies have start revealing the complexity of the ovarian ECM in terms of composition and microarchitecture, which are crucial for the development of a 3D matrix for the transplantable engineered ovary.

Key Readings

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In Vitro Perfusion of the Whole Ovary

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Introduction

For many types of systemic cancers (particularly leukemias), the options available for fertility preservation (oocyte, embryo, or ovarian tissue freezing) are not recommended either for the lack of time (oocyte or embryo cryopreservation) or for the risk that cancer cells might be present in the ovarian tissue and could later be reintroduced into the patient at the time of re-transplant (ovarian tissue freezing). Similarly, neither ovarian tissue freezing nor from ovarian stimulation (not to induce puberty) can help prepubertal girls with systemic cancer. In these cases, systems for in vitro follicle culture of isolated follicles or those that support follicle growth to allow retrieval of mature oocytes via whole ovary perfusion could be alternative methods for fertility preservation. In vitro follicular growth from ovarian cortical tissue is a work in progress; however, at the time of this writing, obtaining human mature oocytes has proven to be very difficult.

In this chapter, we describe the development of an ex vivo ovarian perfusion system able to sustain ovarian and follicular survival as well as oocyte production during the whole perfusion period.

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In Vitro Perfusion: Initial Experiments and the Setup

In vitro perfusion of whole organs enables to study their function ex vivo, and it has been used as experimental models since the 1960s [1]. Specifically related to the ovary, the in vitro perfusion apparatus utilizes a closed circuit system where medium is continuously perfused through a peristaltic pump connected to the ovarian vascular tree, at predetermined pressures, thus supplying cells and tissue with specific solutes and at the same time being able to collect effluent for endocrine analysis. The advantage of this in vitro system, in comparison with the ovarian cortical

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tissue culture, is that the entire organ can be studied, keeping the tissue structure (stromal and epithelial scaffold) and cell communications intact. Perfusion of whole organs have demonstrated different responses to external stimuli than isolated tissue [2] since nutrients are delivered to the whole organ through its intact vasculature and cells are supported in a manner resembling the in vivo conditions. The setup of the in vitro perfusion apparatus consists of a perfusion chamber, circulation system, roller pump, oxygenator reservoir, gas humidifier, bubble trap, and a manometer (see Fig. 1) [3]. The ex vivo perfusion is enclosed in a bioreactor, enabling the maintenance of a sterile condition and giving the possibility to monitor and regulate temperature, pH, oxygenation, perfusion pressure, and flow during the various perfusion times. Specifically concerning the whole ovary, this organ is connected to perfusion apparatus through an isolated ovarian artery, as described for sheep and humans [4, 5], or through a dissected aorta in small animals [6, 7]. The perfusion medium has been modified many times during the development of the in vitro perfusion procedure. The medium mostly utilized consists of M199 with Earle's salts, 26 mmol/l $NaHCO_3$, and 0.68 mmol/l l-glutamine and is supplemented with 2% bovine serum albumin (BSA) and equilibrated with 5% CO_2 and 95% O_2 [5, 6, 8]. Antibiotics (piperacillin/tazobactam) and antimycotics must be added to perfusion medium both to reduce the risk for contamination and to ensure longer perfusion time, keeping normalcy of the tissue [6, 7]. Due to the hypo-osmolality of fluid medium that could induce tissue edema, BSA or dextran must be added to perfusion fluid [8]. Most of the studies utilizing in vitro perfusion techniques were developed to study ovarian function and physiology [9–11]. Ovarian morphology after perfusion has been studied using light and electron microscopy after perfusion of rabbit ovaries for up to 15 h (mean 11.5 h) [7], and normal histology and ultrastructure has been demonstrated. In 2 out of 11 rabbit ovaries, bacterial contamination was observed, but interestingly bacteria were not seen within the follicles, indicating that basement membrane around the granulosa cells is an efficient barrier [7] in blocking contamination.

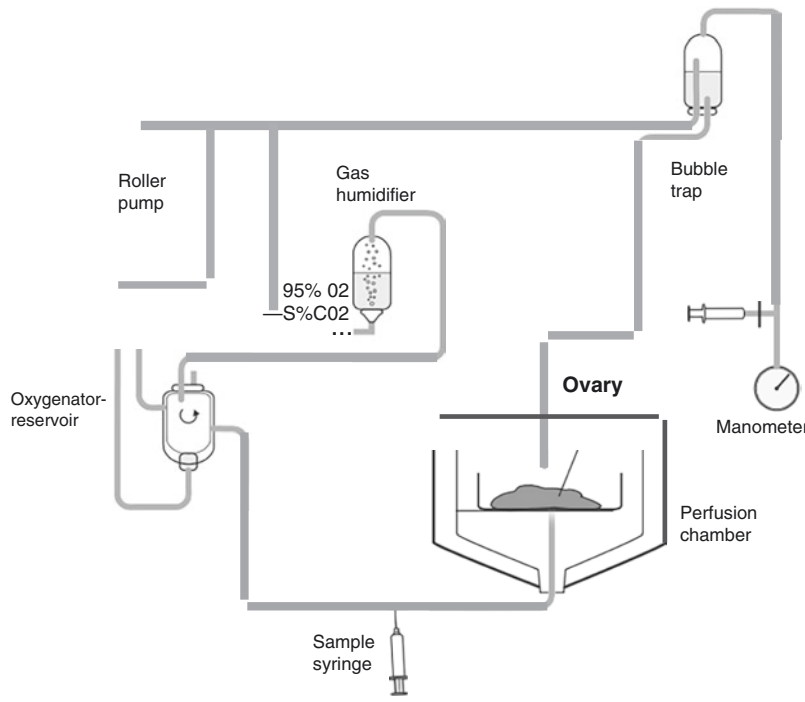

Fig. 1 Schematic drawing of the in vitro perfusion system

Ovulation and steroidogenesis were demonstrated in one study, where isolated rabbit ovaries were perfused for 12–24 h [3]. The animals were injected in vivo with human chorionic gonadotropin (hCG), 5–6 h before the start of the perfusion or with luteinizing hormone (LH) added in the perfusion medium. Ovulation occurred after hCG and LH with subsequent estradiol and progesterone secretion assessed in the effluent media. Hormonal secretions and ovulation were not observed in the control group not receiving hCG and LH [3]. Furthermore, oocytes that were collected after inducing in vitro ovulation of rabbit ovaries were fertilized in vitro and then transferred by laparotomy in oviducts [12]. Pregnancies were achieved and resulted in 3.8% term delivery rate as compared with 18% when eggs were obtained after in vivo ovulation [12]. Despite low rate of live birth, this was an important step in research and propelled the use of this technique in other animal models in preparation to the application in humans. Indeed, rat and mouse whole ovaries have also been used as a model to study ovarian function ex vivo [6, 11, 13].

Ovarian in Vitro Perfusion Models to Study Chemotherapy Effects and Organ Cryopreservation

The understanding about the effect of chemotherapy on ovarian function is not fully elucidated; however, initial research is starting to appear using in vitro perfusion apparatus [2]. These authors perfused bovine ovaries and analyzed apoptosis by TUNEL method after 24 and 48 h of perfusion. The ovaries that were perfused with doxorubicin showed an increase in apoptotic cells percentage from 11.4 ± 0.5-fold at 24 h to 17.2 ± 3.4-fold at 48 h compared to the control group. Before perfusion with doxorubicin, the ovaries were perfused with trypan blue dye, demonstrating an equal distribution of stained fluid across the various anatomical sites of the ovaries.

Regarding cryopreservation, some studies analyzed ovarian function after freezing and thawing of whole sheep [4, 14] and human ovaries [5]. Sheep ovaries have similar structure as the human ovary with collagen dense stroma that contains primordial follicles [15], although in the premenopausal ovary, the size is about 20% of the human ovary [16].

In studies using sheep, the ovaries were obtained by experimental surgeries under sterile condition and then cryopreserved by a slow freezing protocol [17] using dimethylsulfoxide (DMSO) [14] and propanediol [4]. After thawing, the sheep ovaries were connected to an in vitro perfusion apparatus and perfused for 120 minutes using the perfusion medium described before [3]. Post-thawing functions of the whole ovaries were analyzed by assessing the hormonal content of the effluent medium. Higher progesterone and cyclic adenosine 3′5’-monophosphate (AMP) secretion and better cell survival and preserved histological structure were demonstrated after freezing using both DMSO and propanediol, in comparison with control groups [4, 14]. Whole human postmenopausal ovaries have also been used for cryopreservation research using DMSO as a cryoprotectant [5]. After thawing, the ovaries were connected to in vitro perfusion system for 4 h. Androstenedione and testosterone production was demonstrated in increasing manner, and electron and light microscopy after 4 h of perfusion revealed well-preserved ovarian tissue morphology [5]. Another study used fresh human postmenopausal ovaries obtained after bilateral oophorectomy for benign conditions demonstrated estradiol, progesterone, and testosterone production measured in the effluent, perfusate fluid [18].

Summary and Conclusions

In vitro perfusion systems of the whole intact ovary can potentially be used to support follicle development and to produce mature eggs. These systems are suitable for perfusing ovaries retrieved before gonadotoxic treatment in women with oncological diseases, where the option of ovarian cortical tissue freezing and future re-transplant is not feasible due to a risk of

reintroduction of malignant cells. Likewise, these systems can be offered to preserve future fertility in prepubertal girls. Preliminary work using in vitro culture of ovarian cortical strips has shown that human oocytes can start the process of in vitro maturation [19], but fertilization has not yet been reported. Another option described in this chapter is the establishment of whole ovary perfusion ex vivo. One ovary could be removed from patients with leukemias or from prepubertal girls before starting gonadotoxic treatments, connected to the in vitro perfusion apparatus and stimulated by gonadotropins to achieve superovulation. After the addition of hCG to perfusion fluid, oocytes could be aspirated, cryopreserved, or fertilized by partner or donor spermatozoa, followed by embryo freezing. This method can be combined with cryopreservation of part of the ovarian cortex before the perfusion starts and after in vitro follicle growth and oocyte maturation have been obtained.

Future studies should expand the utilization of in vitro whole-organ perfusion by studying the effect on follicular growth, viability, and oocyte maturation with gonadotropins perhaps in combination with other molecules, such as growth factors activating signaling pathways and/or block growth suppressive pathways. In conclusion, ex vivo perfusion systems may have several applications: (a) to study follicle development in vitro, (b) to stimulate growth and maturation of oocytes by using known molecules and new agents, (c) to test gonadotoxic effects of chemotherapy drugs, and (d) to assess ovarian colonization by cancer cells and methods to "purge" these cells from the ovarian cortex prior to ovarian tissue cryopreservation.

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Culture of Human Ovarian Follicles from Primordial Stages to Maturity

Evelyn E. Telfer

Introduction

Storage of cryopreserved ovarian tissue for subsequent reimplantation is now routinely offered to safeguard the fertility of girls and young women at high risk of sterility or premature ovarian insufficiency (POI) [1]. Fragments of ovarian cortex containing mainly immature follicles can be removed, cryopreserved and subsequently thawed and autografted to an orthotopic or heterotopic site to restore fertility [2, 3]. Whilst this technique has been successful for patients worldwide and has resulted in the birth of over 130 babies [4], for girls and women with blood-borne leukaemias or cancers with a high risk of ovarian metastasis transplantation of ovarian tissue carries risks of reintroducing malignant cells [5]. For this patient group, the possibility of restoring fertility could only be safely achieved by growing the immature oocytes, contained within the stored tissue, entirely in the laboratory using culture techniques that support the complete in vitro growth and maturation (IVGM) of cryopreserved primordial oocytes into fertile metaphase II (MII) oocytes [6, 7]. IVGM would be beneficial for fertility restoration in girls and young women with cancer who have a medium to high risk of malignant cells in their cryopreserved ovarian tissue. The therapeutic potential of IVGM would be great, but the technology is extremely challenging. This chapter will outline the advances that have been made in this area and will consider the steps that need to be taken to progress this field to clinical application.

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Developing Culture Systems

Fragments of ovarian cortex removed and cryopreserved for fertility preservation contain mainly the most immature stages of female gametes, primordial follicles. These follicles consist of an oocyte arrested at the dictyate stage of prophase 1 of meiosis enclosed within flattened somatic (granulosa) cells and are formed prenatally, leading to the production of a non-proliferating pool of germ cells from which recruitment for growth will take place throughout the female's reproductive life [8] (Fig. 1). Primordial follicles are continuously activated to grow and begin the process of follicle/oocyte development, which will lead to the production of an ovulatory follicle and a mature oocyte (Fig. 2). This process can be defined as a sequence of developmental stages as follows: (1) initiation/activation of primordial follicle growth; (2) growth of the activated primordial stage to form multilaminar preantral follicles; (3) formation of a fluid-filled cavity (antrum) with differentiation of somatic granulosa cells to mural and cumulus cells; (4)

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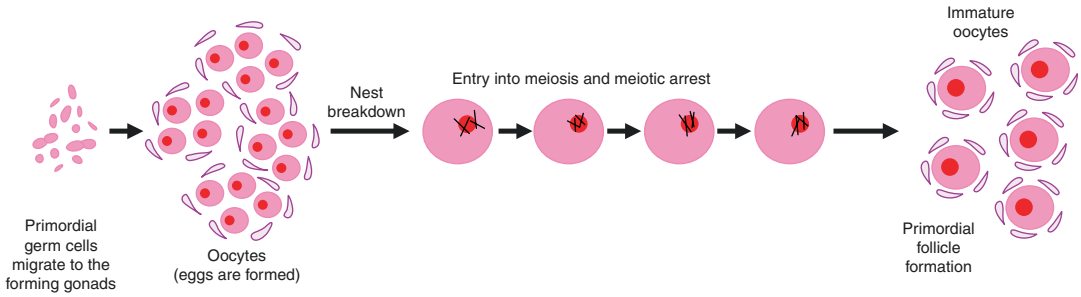

Fig. 1 Formation of primordial follicles: Oocytes are formed prenatally from primordial germ cells that migrate to the gonadal ridge. Oocytes form nests intermingled with somatic cells, and as these break down, oocytes enter the process of meiosis reaching the dictyate stage of prophase I of meiosis at which stage meiosis is arrested. Oocytes form associations with somatic cells (pregranulosa cells) and form primordial follicles. These follicles represent the store of germ cells that must last the female throughout life

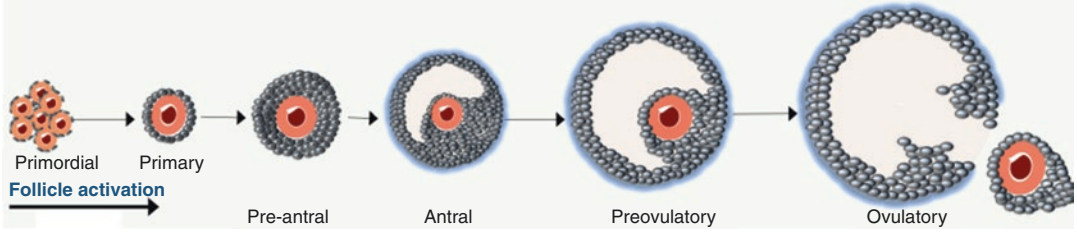

Fig. 2 Stages of follicle development: Primordial follicles represent a pool of non-growing follicles, which are continually initiated to grow throughout reproductive life. Once follicles are activated to grow (primary stage), granulosa cells proliferate to form multilaminar structures (preantral) and then form a fluid-filled cavity (antral), which undergoes expansion to reach preovulatory stages and the oocyte-cumulus complex being released at ovulation in response to LH signalling

enlargement phase of the antral follicle to the preovulatory or Graafian stage, associated with granulosa cell proliferation and antral fluid accumulation within the basement membrane; and (5) rupture of the Graafian follicle in response to the mid-cycle luteinising hormone (LH) surge to release the oocyte-cumulus complex at ovulation (Fig. 2) [6, 7, 9]. The complexity of this process makes attempts at re-capitulating it in vitro an enormous technical challenge [6, 7].

Work on developing systems to support the growth of primordial follicles entirely in vitro has been ongoing for almost 30 years [6, 7, 9], and complete in vitro growth (IVG) with subsequent maturation (IVGM) followed by in vitro fertilisation (IVF) has resulted in mice being produced [10, 11]. Early work on this culture system resulted in the birth of one mouse (Eggbert), which subsequently developed many abnormalities as an adult leading to concerns regarding the safety of these culture techniques [10]. Following alterations in the culture medium and improvements to the technique, several mouse embryos and offspring with no detectable abnormalities were successfully obtained [11]. These studies provide proof of concept that complete oocyte development can be achieved in vitro, and this has driven the development of culture systems that could be applied to other species, particularly human; however, the protracted growth period and large size of human follicles are some of the factors making interspecies translation of these techniques difficult to achieve [6, 7, 12, 13].

The development of a successful IVG system for mouse oocytes/follicles has highlighted the necessity for a multistep system to support IVG human follicles. Each stage of follicle development from activation of primordial follicle growth to maturation has changing requirements, and so different conditions need to be provided at

appropriate times in vitro. Regulation of growth and development of primordial follicles occurs via complex interactions of paracrine factors [14] modulated by oocyte-somatic cell interactions [15] and microRNAs [16], all of which are influenced by biomechanical forces [17]. Whilst defining systems to support such a complex multilayered process in vitro is clearly technically challenging, good progress is being made in developing in vitro systems that support complete human oocyte development from primordial to maturation [18, 19].

Human Oocyte Development In Vitro

Several published studies have reported on culture systems designed to support the growth of defined stages of human follicle/oocyte development with most starting with growing stages rather than primordial follicles [19, 20]. The growth and development of human oocytes within a multistep culture system from the primordial stage to fully grown oocytes that can resume meiosis and reach metaphase II following IVM has been reported (Fig. 3) [18]. This system focuses on the key stages of development and the sequence of conditions necessary to support human oocyte development in vitro without the need to maintain large intact follicles [6, 7, 18, 21]. Whilst the reported system has not yet been fully optimised, it supports the strategy of using a multistep approach and focussing on oocyte development to achieve complete in vitro development of human oocytes [18].

The follicle unit functions both as an endocrine structure and as a vehicle to support oocyte growth and development, but by focusing on oocyte development and the maintenance of appropriately differentiated somatic cells, the need to maintain large follicular structures in vitro can be avoided. The culture system needs to accommodate the changing requirements to support development of oocytes and their surrounding somatic cells, hence the need for a multistep system. The multistep system that supports development of human oocytes from primordial stages to maturity [18] can be broken down into three major steps, each of which supports specific stages of follicle/oocyte development: (1) culturing small pieces of ovarian cortex to support activation of primordial follicles, (2) isolation and culture of growing preantral follicles to achieve oocyte growth and development to antral stages and (3) aspiration and maturation of oocyte-cumulus complexes (Fig. 3).

Activating Non-Growing (Primordial) Follicles

Primordial follicles that are isolated from ovarian tissue and placed in culture remain quiescent and do not activate growth; therefore, to achieve activation, primordial follicles need to be maintained

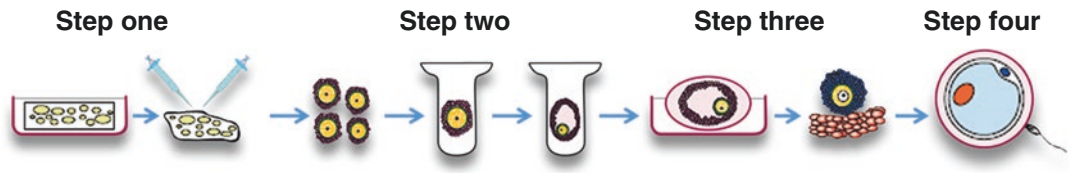

Fig. 3 A multistep culture system to support in vitro growth (IVG) of oocytes from human primordial follicles to maturation [18]. Step one: flattened strips of ovarian tissue are cultured free floating in medium containing human serum albumin, ascorbic acid and basal levels of FSH. Once follicles have reached multilaminar, stages they can be isolated mechanically using needles. Step two: isolated follicles are cultured individually from preantral to antral stages. Step three: the final stages of oocyte growth and development are achieved by removing the oocyte-cumulus complex from the antral follicle and culturing the oocyte and its surrounding somatic cells. Step four: oocyte-cumulus complexes placed within medium for in vitro maturation (IVM). Oocytes are then analysed for the presence of a metaphase II spindle and a polar body. The ultimate goal is to determine whether these complexes can be fertilised and can form embryos, but this remains to be demonstrated

within small pieces of ovarian cortex containing stromal cells [18, 22–27]. Systems that support the initiation/activation of human primordial follicles have been developed [18, 22–30]. Each culture system results in varying rates of activation, which is correlated with the preparation and architecture of the starting material. This observation highlights the importance of mechanical signalling to cell pathways, regulating primordial follicle activation [17].

Ovarian tissue that has been mechanically loosened supports activation of human primordial follicles, and these can develop to multilaminar (secondary) stages within 6 days [18, 26] (Figs. 3 and 4). It has become clear that the critical step in setting up this culture system to support follicle activation is the preparation of tissue. Step one involves the removal of any growing follicles and most of the underlying stromal tissue so that the tissue consists of predominantly ovarian cortex containing primordial and primary follicles, and this is referred to as a micro-cortex (Fig. 4). Culturing these fragments of micro-cortex in serum-free medium results in a significant shift of follicles from primordial to growing stages within relatively short culture periods of 6–10 days [26] (Fig. 4). The density of stromal cells and tissue architecture are emerging as critical factors contributing to the regulation of activation of growth in vitro. Preparation of human ovarian tissue as solid cubes of cortical tissue results in slow initiation of growth [23] when compared with cortex prepared and cultured as flattened "sheets," where much of the underlying stroma is removed [18, 26]. The fragmentation of ovarian tissue that occurs during the preparation of tissue into fragments of micro-cortex affects the Hippo signalling pathway [14, 31, 32], which controls organ size through regulating cell proliferation and cell death [33]. Fragmentation and biomechanical force affects these processes, and the effect of the Hippo signalling pathway can explain the differences observed between culture systems where dense cubes of ovarian cortex are prepared, resulting in low levels of follicle activation [23, 24, 27], whilst significant activation occurs in fragments of micro-cortex which has underlying stroma removed and tissue interactions disrupted [18, 26].

In addition to the Hippo signalling pathway, a key cell signalling pathway involved in regulating primordial follicle activation is the phosphatidylinositol-3′-kinase (PI3K-AKT) pathway [31, 34–37]. It is now well-recognised that the PI3K-AKT signalling pathway plays a critical role in regulating follicle activation and that a component of this pathway, the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), acts as a negative regulator and suppresses initiation of follicle development [36, 38]. Disruption of this component of the PI3K-AKT pathway using knockout models [38] and pharmacological inhibitors [35, 39] promotes activation of follicle growth in mice and in xenotransplanted [35] or cultured human [31, 37] and bovine ovarian tissue [40]. Follicle activation is

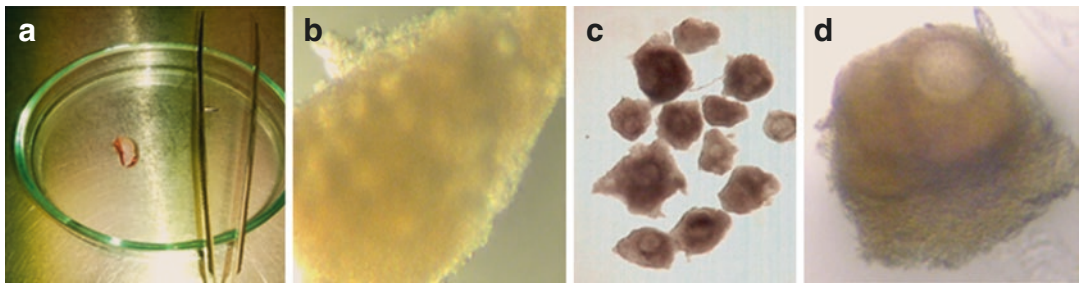

Fig. 4 Follicle growth in micro-cortex: Photomicrographs of (**a**) a typical piece of ovarian tissue before being prepared into micro-cortex for step one of culture. (**b**) Micro-cortex after 7 days in culture showing evidence of activation of many follicles. (**c**) Growing follicles that have been mechanically isolated from micro-cortex after 7 days. (**d**) Isolated follicle with surrounding theca cells selected for individual culture in step two of multistep culture [18]

also affected by other components of this pathway, namely, mammalian target of rapamycin complex 1 (mTORC1), a serine/threonine kinase that regulates cell growth and proliferation in response to growth factors and nutrients and has a positive effect on follicle activation [41] .

The action of PTEN can be inhibited pharmacologically using compounds such as bisperoxovanadium (bpV(HOpic)) [42], and the addition of this to cultured micro-cortex results in increased activation of primordial follicles and resulted in higher numbers of secondary follicles after 24 h of culture [35, 37], and treatment with rapamycin (an inhibitor of mTORC) resulted in decreased activation of primordial follicles [43]. Several studies have demonstrated that manipulation of the PI3K pathway results in altered activation of primordial follicles [14, 31, 32, 35, 37, 40], and this has been applied clinically to overcome infertility associated with primary ovarian insufficiency (POI) [32, 44] with a live birth being reported [32]. Whilst this is an extremely encouraging development, a note of caution needs to be added as the use of similar inhibitors in vitro increases activation of primordial follicles, but oocyte quality at later stages of development is compromised [31, 37]. This negative effect of PTEN inhibition on isolated secondary follicles appears to alter the oocyte's capacity for efficient DNA repair as it has been reported that inhibition of PTEN in cultured bovine ovarian cortex results in high levels of DNA damage and reduced capacity of DNA repair mechanisms [40]. Normally, oocytes have a good capacity for efficient DNA repair [45], and it is not known how final oocyte quality is affected if this is altered [36].

Once follicles have been initiated to grow within ovarian micro-cortex (Figs. 3 and 4), they can develop to the preantral/secondary stage, but when they reach this stage, the cortical environment that they are embedded within becomes inhibitory to further growth, resulting in a loss of follicle integrity and oocyte survival [24, 26]. This inhibitory effect can be overcome by removing growing follicles from the micro-cortex and placing them in individual culture wells to limit the effect of follicle interactions [26, 46].

Growing Preantral Follicles in Vitro

Follicles that have reached multilaminar stages can be mechanically isolated from the ovarian cortex with or without using enzymes. Enzymes such as collagenase and DNase can be used to isolate growing follicles from stromal tissue, but this can be deleterious to follicle morphology and survival [47]. The use of highly purified enzyme preparations such as Liberase may mitigate against the damage observed following treatment with collagenase [48, 49], but enzymes need to be used cautiously as they can compromise surrounding theca layers which are required to retain follicular structure [26, 50]. Recent work on isolated bovine follicles has demonstrated that the presence of intact theca layers supports oocyte growth in vitro in the absence of growth factors [50]. By mechanically isolating follicles, the damaging effects of enzymes can be avoided as the basal lamina and theca layers are maintained, thus preserving follicle integrity, but it is a time-consuming process, which results in a relatively low yield of follicles being isolated [18, 26, 28].

Culture systems that support further growth of multilaminar follicles that have either been isolated from ovarian tissue as growing follicles ([51–55]) or developed in vitro from primordial stages [18, 26, 28] have been reported. Growing isolated human follicles in vitro presents challenges for maintaining their structure as they can grow up to several millimetres. To address this problem, several groups have applied tissue engineering principles and utilised biomatrices such as alginate to encapsulate human preantral follicles, thus supporting their structure and promoting their growth in vitro [52, 54, 55]. This encapsulation provides an extracellular matrix-like material that can facilitate molecular exchange between the follicle and the surrounding culture medium, whilst its rigidity prevents dissociation of the follicle unit, but it is flexible enough to accommodate expansion [56]. In addition to alginate, there has been development of a range of scaffolds to support human follicle growth in vitro. These include de-cellularised ovarian tissue [53, 57] and 3D microporous scaf-

folds [58, 59]. The use of scaffolds has great potential, and the production of engineered scaffolds to support follicle development provides a reproducible and accessible option [59].

The use of a supporting matrix or scaffold may be advantageous, but it is not required to promote the development of isolated follicles and is not used during the multistep culture system that has been developed for human follicles (Fig. 3) [18]. Individual multilaminar follicles are simply placed within v-shaped microwell plates with serum-free medium containing a low dose of FSH, Activin-A and ascorbic acid [18, 26, 28]. Growth and differentiation of preantral follicles takes place within this system, and their three-dimensional architecture is maintained in vitro [26, 28, 37], and cow follicles [46] with antral formation occur within 10 days (Figs. 4 and 5).

The capacity of human oocytes/follicles to develop in vitro following isolation from the cortex is quite remarkable. Human preantral follicles, isolated from ovarian cortex and cultured within an alginate matrix with the addition of FSH, undergo complete oocyte growth within 30 days [55], with some becoming capable of meiotic maturation and reaching metaphase II [19]. Primordial follicles activated to grow within cultured fragments of cortex and mechanically isolated as preantral follicles (Fig. 4c, d) become steroidogenic and undergo differentiation, forming antral cavities within 10 days in vitro [26] (Fig. 5a). Maintaining growth of multilaminar follicles in vitro is dependent on sustaining viable oocyte-somatic cell interactions, and it has been demonstrated that the addition of FSH and Activin-A to the culture media at this stage supports these interactions and results in improved health of follicles and antral formation both in a bovine [60] and human follicles [18].

Growing human preantral follicles to in vivo preovulatory sizes is technically challenging. The most successful culture system that supports complete development of mouse oocytes from primordial stages does not attempt to grow intact antral follicles to preovulatory stages in vitro but focuses on oocyte-granulosa cell complexes (OGCs) to promote oocyte development [11]. The rationale and basis for developing step three of the human multistep culture system is informed by the mouse system. During step two of the multistep system, isolated follicles that were cultured individually form antral cavities within 6–8 days, and at this stage, oocyte-granulosa cell complexes can be retrieved by applying gentle pressure to the follicle (Fig. 5c) [18]. Complexes with complete cumulus and adherent mural granulosa cells are then selected for further growth on membranes in step three of the multistep system (Fig. 5). The aim of this stage is to promote oocyte growth given that oocyte size is an indicator of meiotic potential [61], and following this step, oocytes of at least 100 microns can be obtained and selected for further maturation [18].

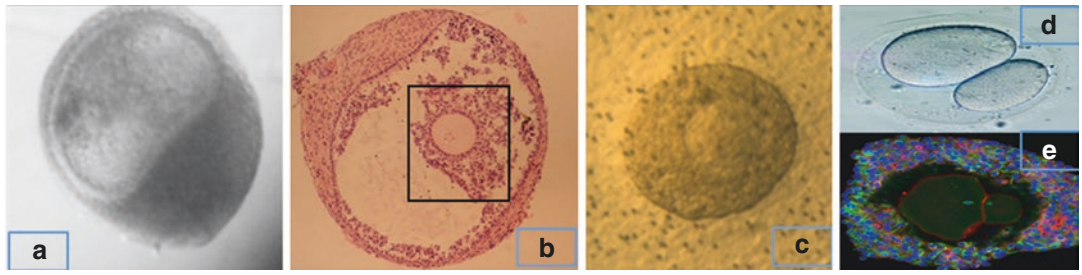

Fig. 5 Photomicrographs of in vitro grown human ovarian follicles: (**a**) in vitro grown antral follicle after step two of culture [18]. (**b**) Histological section of in vitro grown antral follicle, highlighting the oocyte cumulus cell complex that can be removed after a total of 16–20 days in culture. (**c**) An in vitro grown oocyte-cumulus cell complex isolated and cultured on a membrane for further growth (step three) and then placed in maturation medium for 24 hours. (**d**) IVG oocyte after maturation and formation of a polar body, indicative of progression to metaphase II; this is confirmed in (**e**) where the metaphase II spindle of the oocyte is immunostained

Optimising Culture Conditions

The various culture systems that have been developed show differences in oocyte growth rate, and these are most striking between systems where intact follicles are cultured [19, 55] compared to those where complexes are removed for further growth after an antral cavity has formed [18, 26]. Detailed analysis of culture systems that result in different growth rates is required to determine optimal conditions for IVG. Regulation of follicle/oocyte development in vivo is complex and multilayered, balancing influences that promote growth with those that lead to degeneration. The optimal requirements to support IVG are still not fully understood, which makes it difficult to make significant advances in improving culture conditions [9, 62]. The basic culture medium being used varies between groups, but McCoy's 5A is routinely used in the human multistep system [18, 26]. Initially, the culture systems used to support activation and growth included medium containing serum, but this has been better characterised and is now serum-free with the addition of human serum albumin (HSA) and a combination of insulin and transferrin and selenium (ITS) and ascorbic acid [18, 26]. Several growth factors and hormones have been tested in a range of species, but results are conflicting, and there is no clear understanding on the concentrations that should be used or the timing of adding these factors [62].

The key to the success of all follicle culture systems is the need to maintain oocyte-somatic cell interactions, which are essential to support and sustain oocyte development. Many of the factors regulating cell-cell communication are members of the TGF beta superfamily; these include the oocyte factors, growth differentiation factor-9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) [15], which in humans are expressed in the oocyte and cumulus granulosa cells where they promote the establishment of cell-cell interactions and influence cell proliferation [63]. Improvements in oocyte maturation and subsequent fertilisation rates in human [64] have been associated with increased mRNA levels of both GDF-9 or BMP-15 in cumulus cells, and adding either factor to cultured human ovarian cortex has resulted in increased activation of primordial follicles [65]. Continued growth of multilaminar follicles in culture is dependent on maintaining oocyte-somatic cell interactions [66], and the addition of Activin-A in combination with a low-dose FSH maintains oocyte-somatic cell interactions, leading to improved health of multilaminar stages and antral formation in a bovine model [60], and has been successfully applied to the human multistep system [18].

Meiotic Maturation

Oocyte-cumulus complexes that are harvested at the end of the IVG process need to undergo in vitro maturation (IVM) to support resumption of meiosis to the point of metaphase II. Methods of IVM of immature human oocytes have been developed for over 50 years [67], but the first live birth following IVM was not reported until 1991 [68]. IVM is now performed clinically in a limited number of centres, resulting in varying levels of success with these rates heavily influenced by oocyte source [69]. The rate of successful maturation of immature oocytes is less than that of oocytes harvested from stimulated ovaries [69], and whilst this reflects the quality of oocytes harvested for IVM, it also highlights the need for improvements in IVM protocols [70]. Promising developments are being made in refining and optimising IVM protocols by using a biphasic system where oocytes are pretreated with c-type natriuretic peptide followed by conventional IVM (CAPA-IVM), and recent results indicate significant improvements in maturation rate and oocyte quality [71]. IVM as an independent procedure is dealt with in greater detail in Dr. deVos's chapter in this volume.

Some IVG oocytes derived from the multistep culture system undergo meiotic maturation following an IVM protocol with approximately 30% of oocytes that survive the entire culture period forming metaphase II spindles (Fig. 5d, e) [18]. However, polar bodies formed by the IVGM oocytes are significantly larger than normal (Fig. 5d, e) [18]. It is not known what leads to

these large polar bodies, but the proximity of the spindle to the oocyte cortex can influence polar body size [72], and loss of spindle contact with the oocyte cortex can lead to extrusion of large polar bodies [73]. The end point of any IVG system is to produce developmentally competent and epigenetically normal oocytes; therefore, future research needs to focus on optimising each of the stages and gaining further understanding of the epigenetic status of IVG oocytes and of any embryos formed [74].

Culturing Different Tissue Sources

Evidence is emerging to suggest that no single culture strategy will support tissue obtained from all patient groups. Before culture systems could ever be applied clinically, it would be essential to demonstrate how tissue from a variety of patient sources could develop in vitro. These sources include tissue from prepubertal girls, patients following chemotherapy and other conditions such as Turner's syndrome. A significant application of IVG would be to prepubertal girls who have few options for fertility preservation [75]. However, it has been demonstrated that significant differences exist in the follicle population with age and stage of pubertal maturation [28]. Follicles derived from prepubertal girls have a different growth trajectory in vitro compared to those derived from adults; therefore, culture systems developed for adult tissue may not be suitable for prepubertal girls [28]; therefore, adaptations and refinement of the culture systems for specific age groups will be required. Ovarian tissue taken from patients with Hodgkin's lymphoma (HL) after treatment with adriamycin, bleomycin, vinblastine and dacarbazine (ABVD) has been cultured, and differences in the rate of follicle development have been observed between ABVD-treated tissue and tissue from age-matched healthy controls [76]. Young women with Turner's syndrome are increasingly accessing fertility preservation and having ovarian tissue cryopreserved [77]. Ovarian tissue obtained from these patients varies greatly in terms of follicle number and quality, each of which presents challenges for successful IVG [77]. It is clear that there will be no "one-size-fits-all" culture system and that optimisation for each patient group will need to be achieved by altering the conditions for each of these groups.

Conclusions

The primary objective in developing culture systems that support IVG of oocytes from ovarian biopsies is to produce developmentally competent oocytes that can be fertilised. The current status and utility of the systems discussed in this chapter is predominantly as models of human gametogenesis that can be used to gain better understanding of the factors regulating stages of human oocyte development, a process of which we are still lacking knowledge. Additionally, these systems have a pivotal role to play in improving fertility preservation programmes as they can be and should be used to assess the quality of cryopreserved ovarian tissue prior to transplantation.

The ability to develop human oocytes from immature stages to maturation and fertilisation in vitro could revolutionise fertility preservation practice by providing an alternative to autologous transplantation and has the potential to become the next-generation IVF. Currently, there is no fully optimised culture system for human oocytes, but there is now proof of concept that complete in vitro growth of human oocytes from primordial to maturation is possible [18]. The culture systems detailed in this chapter provide us with greater insight to the process of human oogenesis and improve understanding of human oocyte development. Whilst the methodology is technically demanding and there are many variables that affect outcomes, many positive developments have been achieved, but there is still a great deal of research and testing to be carried out to demonstrate the safety of these techniques before reaching clinical application [21].

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Automation in Oocyte and Ovarian Tissue Vitrification

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Brief History of Vitrification

Preservation of cells, tissue samples, and whole organs by cooling has been an area of active research for long time [1]. Slow freezing and vitrification are the two methods that have been proposed to accomplish the task; however, more recently, a third option called freeze drying has been suggested as well, albeit still investigational. Freezing is the phase transition of liquid becoming ice by lowering the temperature to below its freezing point, and vitrification is a phenomenon at which a liquid solidifies rapidly, without the formation of ice crystals; thus, the process is referred to as a glass transition and the result is an amorphous solid. Historically, Gay-Lussac in 1836 saw that water enclosed in small tubes can be supercooled to −12 °C, and this finding seeded already the idea of vitrification [cited in 2]. In 1938, a century later, Luyet and Hodapp published the first report on successful cryopreservation of spermatozoa done by vitrification [3].

Following the first success of freezing mouse embryos [4], Rall and Fahy were the first to report success vitrification using a relatively large volume sample in 1985 [5]. They were applying a mixture of DMSO, acetamide, and polyethylene glycol in a relatively large volume inside a 0.25-mL straw, which was then plunged into liquid nitrogen (LN]. Oocytes, however, were discovered to be much more difficult to cryopreserve than embryos due to their increased chilling sensitivity. This difference in their preservation ability was mainly attributed to differences in membrane lipid compositions, with less polyunsaturated fatty acids (PUFA) in the oocytes as compared to embryos [6]. Changes in oocyte membrane fatty acid composition by either nutrition or fusion with liposomes affected biophysical parameters and were shown to decrease the chilling sensitivity of bovine oocytes [6, 7]. In the late 1980s and early 1990s, the "minimum drop size (MDS)" method was developed by Arav et al. [8, 9]. This method (MDS) applied the minimum volume size capable of maintaining oocytes or embryos without damage due to desiccation. The volume used for vitrification with the MDS method was in the range of 0.07 μL (70 nL), and the concentration of the vitrification solution was about 50% lower than that of the vitrification solution used with large-volume vitrification [9]. The MDS method has been adopted worldwide

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and has led to the success of oocyte vitrification [10, 11, 12, 13]. It allowed for faster cooling rates, decreased chilling injury, and the use of lower concentrations of cryoprotectants (CPs) due to volume reduction.

Principles of Vitrification

As stated earlier, vitrification is the biophysics process by which a sample solidifies without the formation of ice crystals, thus resulting in a glassy amorphous state. It requires high viscosity, high cooling rates, and a small sample volume [1] expressed in the equation {probability of vitrification = CR or WR × μ × 1/V}. This means that the probability of vitrification increases with the increasing cooling/warming rate (CR or WR) and viscosity (μ) and with the decreasing volume (V). These conditions allow the cryopreservation of cells and tissue samples with no ice formation prior to their transformation into a glassy form, therefore drastically reducing freezing damage.

Traditional freezing, on the other hand, requires nucleating factors that will induce ice crystal growth, either spontaneously, as temperature is lowered, or deliberately, by doing what is referred to as seeding [1]. Nucleating factors occur spontaneously in a solution when the temperature is lowered; however, the lower the volume of the sample, the lower is the chance for such nucleating factors to occur. When temperature is lowered and the viscosity is increased, the freezing point of a solution is decreased; however, at the same time, the glass transition temperature (Tg) of a solution is increased. Based on this difference, various means to outrun and avoid freezing, such as increasing the viscosity (i.e., concentration of CPs) and cooling rates while decreasing the sample's volume [9, 11], can be applied. The first approach to vitrification focused on increasing viscosity by using high CP concentrations [5]. However, these concentrations were damaging to the oocytes, causing osmotic stress or chemical toxicity [14]. Consequently, the next approach was to reduce the CP concentration by increasing the cooling rates and reducing the volume [9, 15]. Today, many successful protocols for cryopreservation utilize the method of vitrification (as opposed to slow freezing) by plunging oocytes and embryos into LN and using what is referred to as open systems [1, 10, 13]. The open system method poses a potential risk of contamination and cross-contamination [16, 17] due to direct contact of the oocytes or embryos with the LN or LN slush.

Oocyte Vitrification

Oocytes are very different from sperm or embryos with respect to cryopreservation. The volume of the mammalian oocyte is in the range of three to four orders of magnitude larger than that of the spermatozoa, thus substantially decreasing the surface-to-volume ratio. However, this is not the reason why mature oocytes are sensitive to low temperatures and to slow freezing. Mature oocytes are very sensitive and susceptible to slow freezing damages. This is due to their chilling sensitivity which occurs at low temperatures, impacting different cellular components: the zona pellucida (ZP), plasma membrane, meiotic spindles, cytoskeleton, etc.

The plasma membrane of oocytes at the MII stage has a low permeability coefficient, thus making the movement of CPs and water slower [1, 18]. In addition, the freeze-thaw process causes premature cortical granule exocytosis, leading to zona pellucida hardening and making sperm penetration and fertilization impossible [19]. This is the main reason for resorting to intracytoplasmic sperm injection (ICSI) for the insemination of thawed/rewarmed oocytes. Oocytes also have high cytoplasmic lipid content, which in turn increases chilling sensitivity [20]. They have less sub-membranous actin microtubules [20, 1], making their membrane less robust. Cryopreservation can also cause cytoskeleton disorganization and chromosome and DNA abnormalities [21]. The meiotic spindle, which has been formed by the MII stage, is very sensitive to chilling and may be compromised as well [1]. It does, however, tend to recover to some extent after thawing or warming and in vitro culture (IVC). Oocytes are also more susceptible to damaging effects of reactive oxygen species (ROS] [22]. Many of these parameters change after fertilization, thus making embryos

less prone to chilling sensitivity and easier to cryopreserve [23, 24].

Vitrification requires the presence of high concentrations of CPs. It is therefore important to minimize the damage caused to cells by the osmotic stress or chemical toxicity. Still, there is not an ideal cryoprotectant that meets such requirements and that can be used across different species and for the various developmental embryonic stages. Ideally, vitrification studies should therefore be preceded by osmotic and cytotoxic studies specific for the cells or tissue to cryopreserve. The presence of cryoprotectant in the vitrification solution decreases the probability of intracellular crystallization, which is considered to cause most damage when very rapid cooling takes place, but on the other hand, the high concentration of the cryoprotectant required is toxic and causes osmotic injury to the oocytes even without cooling.

Different methods have been used to reduce this "solution effect": (1) short time of exposure to cryoprotectants [25, 26], (2) use of low toxicity cryoprotectants [10, 14] or mixtures of them, (3) addition of nonpermeating cryoprotectants [14], (4) reduction of the cryoprotectant concentration [10], and (5) exposure at low temperatures [9]. Of these methods, the use of nonpermeating cryoprotectants has been shown to be very useful either because the shrinkage of the oocyte and consequently the amount of intracellular water at risk of ice crystallization during rapid cooling and warming is lower [27] or because of the reduced amount of the cryoprotectant that penetrates the cell, thus reducing the possible toxic effects [14]. In addition, the carbohydrates used as nonpermeating CPs have a stabilizing effect on membranes [28]. In the study reported by us (14), trehalose was less harmful than sucrose. Determination of the Boyle-Van't Hoff relationship for both sucrose and trehalose produced the same regression line, so it is possible that this beneficial effect could be a consequence of its interaction with the membrane polar lipid groups [28]. Only 10 min of exposure are required for equilibration in propylene glycol and DMSO solutions or mixtures of them [14]. The results of the vitrification provide evidence that propylene glycol can be used successfully. However, concentrated solutions of permeating cryoprotectant are required for successful cryopreservation of oocytes when rapid cooling and warming rates are used. In earlier reports on immature pig oocytes, we showed that when lower concentrations of CPs are used, despite apparent vitrification, membrane destruction was unavoidable [29]. In 1990, Kasai was the first to describe the use of ethylene glycol for mouse embryo vitrification [30]. Today, the most frequent solutions are based on a mixture of DMSO and ethylene glycol [10].

Small volume (MDS method) is the solution for most problems that occur during vitrification.

There are three major problems associated with vitrification: [1] crystallization (during cooling], [2] devitrification (crystallization during storage or during warming), and [3] fractures of the glassy solution which can cause devitrification due to the release of energy by the fracture. Surprisingly, at 1 μl, fractures appeared only when the concentration of the vitrification solution (VS) is high (100% VS = 38% ethylene glycol, 0.5 M trehalose, and 4% bovine serum albumin in TCM medium), but not at lower volumes [26]. This means that the probability of fractures increases with the increasing glass transition temperature (Tg) or the viscosity of VS. At the low concentration of VS (50% VS), fractures were observed only at very high cooling rate. We suggest here a simple explanation of this phenomenon, based on the following equations:

$$\text{Probability of fracturing} = \mathbf{CR\ and\ WR} \times \mu \times \mathbf{V}$$

$$\text{Since probability of vitrification is} = \mathbf{CR\ and\ WR} \times \mu \times \mathbf{1/V}$$

1. Increasing the cooling rate (CR) will increase the probability of vitrification; however, it will also increase the probability of fractures.
2. Increasing the viscosity ($\boldsymbol{\mu}$) will increase also the probability of vitrification because Tg will increase [28], therefore increasing the probability of fractions.
3. The only parameter that will increase the probability of vitrification and at the same time decrease the probability of fractures is reducing the volume (V) to the value of the "minimum drop size."

The reason for the increasing probability of fractures in high concentrations of VS is thought to be related to the Tg temperature. We know that fractures can form only at temperatures below that at which the liquid turns into glass (Tg) and above the LN temperature (−196 °C). We also know that a solution with a higher CP concentration will have a higher Tg. Therefore, if the temperature gradient increases, as in the case of higher Tg, then the probability of fracturing will also increase. Finally, the results of vitrification of bovine oocytes at the MII or GV stage, with a concentration of 75% VS, have been reported [14]. We achieved 72% and 38% cleavage and blastocyst rate formation, respectively, for the vitrified MII oocytes and 27% and 14% cleavage and blastocyst rate formation, respectively, for oocytes vitrified at the GV stage. We conclude that the new vitrification procedure, which features small volumes, direct contact with supercooled LN, and low concentrations of VS, reduces chilling injury and provides a high probability of vitrification in the absence of glass fractures.

It was shown by Seki and Mazur [31] that warming rate is more important than cooling rate. Mice oocytes maintained high survival rates when warmed rapidly after being frozen at slow cooling rates. They also noticed that at slow warming rates, the survival of oocytes decreased as the cooling rate increased. It was also shown by Hopkins et al. [32] that for most of the CPs, the critical warming rate is by far higher than the critical cooling rate. The explanation for this can be the fact that the number of ice nucleation increases as cooling rates increase. Ice nucleation is dominant at lower temperatures, and therefore higher cooling rates will form more ice nuclei during rapid cooling [33]. At relative slow warming rates, recrystallization will increase due to these ice nuclei, and more damage will occur. Therefore, the importance of high cooling rate is mainly for reducing chilling injury, and as a result, warming rate needs to be higher. We have shown recently [34] that to maximize the warming rate, one should maintain the distance between liquid nitrogen and the warming solution as little as possible, i.e., between 50 mm and 250 mm.

Parameters Needed for Automatic Vitrification

The most important feature in automation is the possibility of standardizing the whole procedure of both vitrification and rewarming process by using the same device. A key element for oocyte survival is a high cooling and warming rate, which is achieved by using small volume and a small-size carrier for the cells. Both these important requirements are present in the Sarah automatic vitrification device, which will be described next.

The Sarah prototype device for automatic vitrification [35, 36] allows the time of exposure and the incubation conditions (temperatures, CO2, and humidity) for either oocytes or tissue to be very accurate. This standardizes the exposure time and condition for each of the vitrification and warming steps, resulting in a consistent reproducibility of the process and the outcome results. But above all, the vitrification process (rapid cooling) and warming are carried out using small volume of the vitrification solution and small cryo-carriers. The equilibration steps with the CP solution should be gradual to avoid osmotic stress and chemical toxicity. The rapid cooling and warming are particularly important for oocytes and ovarian tissue, as we have shown for oocytes that if cooling to 0 °C takes more than 0.5 seconds, a chilling injury will inevitably occur [35, 36]. Therefore, adopting most of the open system cryo-carriers (Cryotop, Cryolock, OPS), creating cooling and warming rates of over 20,000 °C/min, results in the optimal cooling rates of less than 0.5 second to reach 0 °C.

As described earlier, the volume of the drop should be minimized (MDS) for reducing not only ice nucleation but also fracture probability [36]. When considering the need of storage space, it should also be important that the device will be small and easy to operate. In addition, it would be ideal that all the procedures—exposure to equilibration and vitrification solutions, cooling into LN, as well as the warming and dilution—be automated and performed in a short time. Finally, the automatic procedure should be safe and with zero error, but if an

error occurs, the device should alert the operator, who in turn should continue the vitrification process manually, avoiding the loss of the biological samples.

Automation Systems (E. Vit and Sarah]

To advance the use of oocytes, embryos, and tissue vitrification, we developed a system in which embryo vitrification, warming, and dilution can be performed within a straw [35]. This system has been tested with ovine in vitro produced embryos (IVP) that were vitrified using the "E. Vit" device (easy vitrification), composed by a 0.25-mL straw with a 50-µm pore polycarbonate grid at one end [36].

The automatic device used to vitrify oocytes and embryos (Sarah) consists of a vertical robotic handle where a special straw holder, which can load up to six straws, is attached. This robotic arm moves up and down in a vertical plane, at predetermined time intervals, and by so doing carries the biological samples contained in the straws between different solutions (vitrification and equilibration) arranged into nine cups placed in a temperature-controlled metal carousel plate (see Fig. 1). The final station on the carousel plate is the one containing LN, where the straws are ultimately plunged, and the entire cycle of vitrification is considered completed in about 17 min. The straws utilized in these preliminary experiments were 0.25-mL straws (CBS, L'Aigle, France). Prior to attaching the straws to the holder, the oocytes or embryos are first manually loaded into the straws, and then the straws are closed at one extremity by special capsules (50-µm pores) (Fertile Safe Ltd., Nes-Ziona, Israel) (see Fig. 2]. After the holder is placed on the robotic handle and the proper vitrification protocol has been selected from the touch screen, it is sufficient to press the "ON" button for the vitrification cycle to begin. Once the entire steps are completed, the holder plunges the straws into a special insulated vessel containing LN or sterile liquid air [38]. The special straw holder is then disconnected from the handle, and the straws can

Fig. 1 The Sarah device

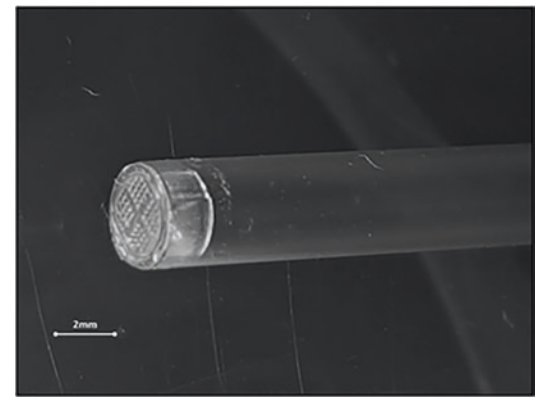

Fig. 2 Detail of capsule closing one end of the straw

be either stored as such or inserted into a 0.3-mL heat-sealed straw (if closed systems are preferred) and then placed in LN tanks for long-term storage.

The results of mice and bovine oocytes [35] and ovine blastocysts [37] showed that oocyte survival, blastocyst re-expansion, embryo survival, and hatching rate were all the same as the fresh control. In addition, testicular tissue vitrification using E. Vit straws [39] and mice and

ovine ovarian tissue (in preparation) showed a very high survival following vitrification using E. Vit.

In summary, the most important features of an automated vitrification system should be the ability to produce high survival and viability of both oocytes and ovarian tissue by (a) generating high cooling and warming rates with the adoption of the MDS volume, (b) exposing the cells to the increasing and decreasing concentrations of CP solutions, and (c) using many steps to avoid osmotic damages [36, 37]. Furthermore, an ideal device should enable both time and temperature control and reduce the vitrification working time required per each patient. Disposable containers such as straws should be easy to label and easy to store in standard LN tanks without making changes in storage space or requiring specialized canisters. These features are all present in the Sarah device reported here [35]. Currently, there is no methodology able to fully automate the vitrification process. Few publications have described solutions for automated vitrification; however, the only commercially available semiautomated vitrification machine is the one called Gavi, from Genea [40]. The Sarah device is a simplified methodology that uses commercially available straws and is almost completely operator-independent since it includes also the immersion into LN, thus providing flexibility to embryologists since the biological samples are vitrified by the device and kept in LN until placed in a long-term storage. The Sarah device can operate up to six straws, and since each straw can load five oocytes (or embryos), a total of 30 oocytes/embryos can be vitrified simultaneously per patient, thus shortening the time required to complete the entire task. This is an important feature, particularly for busy units. But the most important characteristic of the Sarah or E. Vit devices is the use of a very small capsule connected at the extremity of the straw, which allows the use of the minimal volume size of the samples. As already stressed earlier but worth repeating, the minimal drop size is the most critical feature for a successful vitrification, allowing the high cooling and warming rates of over 20,000 °C/min. Our results with fertilized oocytes (2PN embryos) are comparable with the results obtained with other vitrification systems such as open pulled straws and Cryotop [35] or when vitrification was done with long or short equilibrations [37]. In summary, a key element for oocyte survival is a high cooling and warming rate, which is achieved by using small volume and a small-size carrier for the cells. Both these important requirements are present in the Sarah automatic vitrification device. This device has simplified the vitrification process due to two main advantages: [1] the embryos/oocytes are within the same straw during the entire vitrification cycle, from the initial exposure in the ES medium until the final step being the plunging into LN or LN slush; and [2] the movements between the different solutions are done in an automated and precisely timed manner. This means that the time and the temperatures that the oocytes or embryos are exposed to each solution are predetermined and very accurate. These two advantages eliminate the need for searching the embryos/oocytes, allowing the simultaneous transfer of multiple oocytes or embryos between the various solutions at once. Importantly, the exposure time to each of the vitrification steps is standardized, not operator-dependent, resulting in a consistent reproducibility of the process and of the results. It is anticipated that this new device, by maintaining all the important features needed for successful vitrification such as rapid cooling and warming, small volumes, small carriers, and relatively low concentrations of CPs and controlled temperature and time for each step, would be a game changer for simplifying and standardizing vitrification.

In conclusion, E. Vit is a 0.25 or 0.3 straw and 1-mm capsule that can vitrify oocytes, embryos, and tissue slices. It can be operated in a safe open system or with a semiclosed system, able to reach cooling and warming rates of >20,000 °C/min. The Sarah automatic device can vitrify up to 30 oocytes or up to 6 blastocysts (1 blastocyst/straw) simultaneously in about 17 min [35].

Take-Home Messages

Automatic vitrification of oocytes, embryos, and ovarian/testicular tissue is necessary for the standardization of the vitrification procedure. An automatic procedure will cover all the steps from gradual exposure of the equilibration and vitrification solution, the cooling into LN, the warming, and the dilution from vitrification solution. A safe, not expensive, and rapid procedure is under development and soon will be used clinically.

Appendix: Protocol for Vitrification and Warming of Ovarian Tissue

Step-by-step procedure for ovarian tissue vitrification in 0.3-ml straws for E. Vit:

1. Isolate cortical strips 1 × 2 mm and 20 mm long.
2. Prepare 25, 50, 75%, and 100% equilibration solution (100% ES = 7.5% EG+ 7.5% DMSO+ 20%SSS in holding medium).
3. Prepare vitrification solution (VS = 20% EG + 20% DMSO+ 0.5 M Trehalose +20%SSS in holding medium).
4. Connect syringe to the E. Vit 0.3 ml (CBS sperm straw) as shown in Fig. 1.
5. Load the tissue by aspiration with holding medium (HM) until it reaches the capsule.
6. Evacuate the HM and load the 25% ES until it reaches the capsule for 5 min.
7. Evacuate the 25%ES and load the 50% ES until it reaches the capsule for 5 min.
8. Evacuate the 50%ES and load the 75% ES until it reaches the capsule for 5 min.
9. Evacuate the 75%ES and load the 100% ES until it reaches the capsule for 5 min.
10. Load the 100% VS until it reaches the capsule for 5 min.
11. Evacuate the VS from straw and remove syringe and plunge the straw in LN slush (using the VitMaster).
12. Place the straw in cryovial and store in LN container [39].

Ovarian Tissue Warming

1. Prepare warming solution (WS) 1 M (2 vials), 0.5, 0.25 0.125 M of trehalose solution (+20% SSS) in holding medium.
2. Remove straw from LN and plunge in 1 M WS (37 °C) for 10 s, and then aspirate 1 M WS at RT for 5 min.
3. Connect the syringe to the straw.
4. Evacuate 1 M WS and replace with 0.5 M WS for 5 min.
5. Evacuate 0.5 M WS and replace with 0.25 M WS for 5 min.
6. Evacuate 0.25 M WS and replace with 0.125 M WS for 5 min.
7. Wash and use the strips for transplantation.

Of note, similar protocols have been used to vitrify also testicular tissue. Post-warming results showed 66%, 59%, and 31% survival after immediate, 2 h, and 24 h culture, respectively [39].

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Gamete Production from Stem Cells

Krista Maye Symosko, Gerald Schatten, and Charles Allen Easley IV

Introduction

Currently, global estimates report 15% of reproductive-aged couples are infertile [1]. Among the infertile couples, 28% are affected by different pathologies that lead to the absence of functional gametes [2]. Not only do these couples have the inability to have their own biological children, but their infertility diagnoses often result in both emotional challenges [3–6] and financial hardships [7–9]. Although genetic factors are associated with infertility, recently, research has shown that men and women may become infertile due to environmental exposures and medical interventions [10]. Therefore, as infertility rates continue to rise, understanding root causes and designing proper therapies to restore fertility are paramount.

Humans are exposed to many of the 70,000 commercially and occupationally available chemicals [11] each day, posing significant risks to male and female fertility. Over the last several years, numerous scientific studies reported several types of environmental exposures are linked to decreased fertility levels in men and women, including flame retardants [12], heavy metals [13–16], and cigarette smoking [17–29]. As evidence continues to emerge on the impacts of environmental exposures on fertility, more mechanistic research is needed to design proper therapies.

Several medical interventions, such as chemotherapy, radiation therapy, and immunosuppressant treatments, are known to affect fertility negatively [30–35]. For example, children and adults undergoing high-dose chemotherapy or whole-body or targeted radiation to the gonads may become permanently infertile [36–37]. While adults have the opportunity to preserve their fertility before undergoing their cancer treatments, prepubescent boys and girls are unable to bank either sperm or oocytes [35, 38] and, thus, may be unable to have a child with their partner in the future. As more children survive their cancer diagnoses [31, 35, 39–46], therapies targeting fertility restoration are needed.

Advances in assisted reproductive technologies (ART) such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have assisted infertile couples in achieving successful pregnancies. In cases of male factor infertility,

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adult patients who can provide a semen sample have the option to cryopreserve their gametes to be used in future IVF and ICSI procedures to fertilize their partner's oocyte [47]. While more invasive, testicular biopsies, testicular sperm extraction, or percutaneous sperm aspiration paired with ICSI have all been utilized to achieve pregnancies, even in patients diagnosed with azoospermia [48–50]. Conversely, male patients who either cannot provide a semen sample or are prepubescent have the option to cryopreserve testicular tissue [35, 46]. Though this procedure requires foresight, it is minimally invasive and may be the only option to preserve fertility in some patients.

Compared to men, the infertility treatment options for women are more challenging. Typically, young reproductive-aged women may undergo embryo or oocyte cryopreservation, both of which can be used for future IVF procedures. Though experimental, ovarian tissue cryopreservation for future transplantation is a potential method for prepubertal and adult females [51]. However, some studies indicated the possibility of reintroducing malignant cells following ovarian tissue grafting of patients with leukemia [52], thus prompting further investigation as a fertility preservation method for cancer patients [51]. Overall, current fertility treatment options are invasive, costly, and solely rely on both partners producing functional gametes [53–54]. If one partner is unable to do so, the only option available is to use donor gametes [55]. Therefore, alternative approaches for fertility restoration are needed to allow patients who cannot produce gametes to have their own genetic offspring.

Stem cell-based therapeutics are being explored to produce in vitro-derived gametes for fertility restoration in both men and women. Pluripotent stem cells (PSCs) differentiated in vitro into transplantable spermatogonial stem cell-like cells (SSCLCs) or functional gametes are two options being examined to restore fertility in men, while in vitro-derived oocytes from PSCs are being studied to restore female fertility.

In this chapter, students will learn:

- Current scientific advancements of stem cell-based therapeutics to restore both female and male fertility.
- Future scientific directions of generating gametes in a dish.

Introduction to Pluripotent Stem Cells

Over the last several decades, research has shown the potential to use stem cell-based therapeutics in medicine. Mainly, fertility specialists are interested in utilizing patient-specific PSCs to restore fertility in individuals who are either at risk or diagnosed with infertility. PSCs can be differentiated into cells associated with all three germ layers, including ectoderm, mesoderm, and endoderm [56]. Recently, scientific advances have shown PSCs' ability to differentiate into somatic cells and gametic lineages, showing their "promiscuity" to differentiate into various cell types [57]. Thus, PSCs provide a unique opportunity to potentially generate functional gametes in vitro. Following a brief introduction to three types of PSCs, including embryonic stem cells (ESCs), somatic cell nuclear transfer-embryonic stem cells (SCNT-ESCs), and induced pluripotent stem cells (iPSCs), this chapter will focus on utilizing patient-specific stem cells to restore both female and male fertility.

Embryonic Stem Cells

In the early 1980s, mouse ESCs were isolated from the inner cell mass (ICM) of mammalian blastocysts. With a normal diploid karyotype, these ESCs could proliferate indefinitely and differentiate into all three germ layers when injected into an organism to form teratomas [58–59]. Eventually, scientists at the Wisconsin National Primate Research Center derived the first non-human primate (NHP)-ESC line from rhesus macaque and marmoset embryos [60–61].

Subsequently, the first pedigreed NHP-ESC [62] and baboon NHP-ESC [63] lines were developed, all of which had similar properties to the mouse ESCs derived earlier. Overall, the significant development of NHP-ESCs derived in the early 1990s led to the derivation of the first human ESC line designated H1, followed by four others (H7, H9, H13, and H14) [64]. Today, ESCs are valuable tools for proof-of-concept benchtop experiments for understanding signaling pathways and mechanisms associated with proper gamete formation. In addition, human ESCs have been utilized in clinical trials for spinal cord injuries, Parkinson's disease, and macular degeneration [65–66]. Though ESCs have therapeutic potential in regenerative medicine, they have limited clinical use in reproductive medicine since ESCs are not patient-specific. Therefore, in order to develop stem cell-based therapeutics to restore fertility, patient-specific stem cells, including both SCNT-ESCs and iPSCs, represent better cell sources for fertility restoration.

Somatic Cell Nuclear Transfer-Embryonic Stem Cells

In 1962, Gurdon demonstrated cellular reprogramming through SCNT by transferring the nucleus of a *Xenopus laevis* somatic cell to an enucleated egg to generate an embryo genetically identical to the somatic cell donor [67–68]. Through manipulation, the nucleus of the somatic cell could be reprogrammed to an embryonic, pluripotent state. Followed by limited progress until the late twentieth century, the most famous reproductive cloning experiment utilizing an SCNT-derived embryo was the birth of Dolly the sheep [69]. Eventually, Dolly's birth would lead to the development of a powerful platform to genetically modify patient somatic cells for therapeutic cloning.

In the late 2000s, adult rhesus macaque skin fibroblasts were used to derive SCNT-ESCs [70]. Shortly afterward, human SCNT-ESCs were generated by transferring a somatic cell nucleus into an enucleated oocyte [71]. Upon further optimization [72–76], SCNT-derived PSCs have been used for therapeutic purposes, including cell transplantation and disease modeling [76] (Fig. 1). However, the ethical concerns surrounding the use of human embryos for research and the relatively low efficiency in generating SCNT-ESCs [77] have hindered their usage in clinical settings. Therefore, iPSCs may provide a more ethically favorable method for modeling diseases in vitro [66].

Induced Pluripotent Stem Cells

During the mid-2000s, Yamanaka and colleagues [78] generated iPSCs by reprogramming adult mouse cells into ESC-like cells by expressing Octamer-binding transcription factor 4 (OCT 4), SRY-Box 2 (SOX 2), Kruppel-like factor 4 (KLF 4), and *c-MYC*. Throughout the next 2 years, iPSC lines were rapidly derived from adult rhesus fibroblasts [79], adult human fibroblasts [80–81], and other adult human somatic cells [82]. Shortly thereafter, numerous disease-specific human iPSCs were generated [83]. Not only do iPSCs provide an unlimited source of patient-specific stem cells, but their generation also provides an opportunity to study, predict, and develop personalized therapeutics for diseases without the usage of human embryos [84]. Additionally, these newly derived stem cells have the potential to overcome the risk of immune rejection and, thus, minimize the usage of immunosuppressant drugs in transplantation trials [66].

Though promising, there were limitations to iPSCs that originally hindered their usage in the clinic. Initially, iPSCs were derived utilizing retroviruses or lentiviruses [80, 82], which integrate into the host cell's genome [85]. To overcome this challenge, iPSCs are reprogrammed using safer and more efficient methods [66, 85]. Today, iPSCs are used in clinical trials as possible therapeutics for age-related macular degeneration and graft-versus-host disease [65–66].

Whether SCNT-ESCs or iPSCs are better suited for clinical translation is currently debated [75–76, 86–87] (Table 1). SCNT-ESCs may pos-

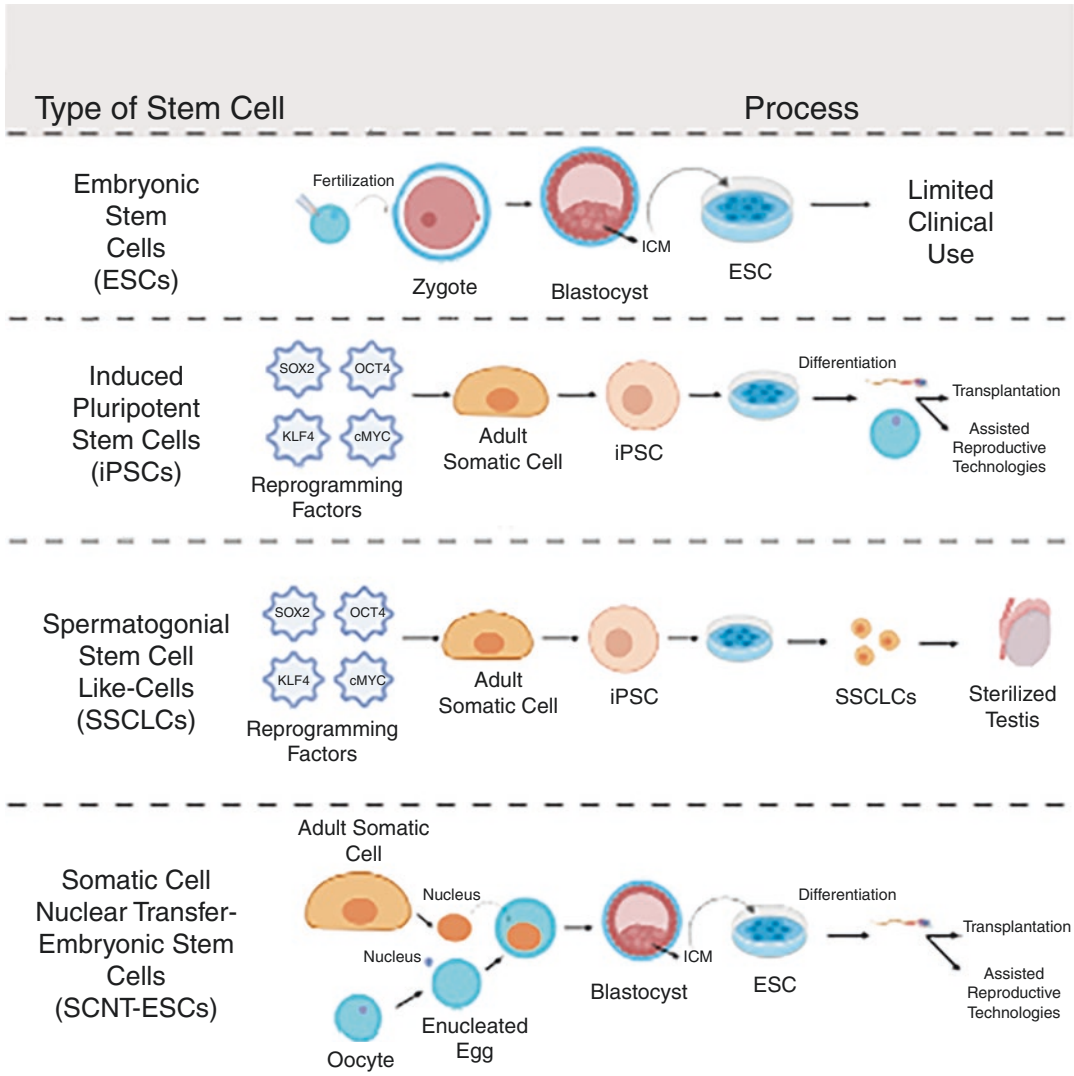

Fig. 1 Currently, stem cell-based therapeutics are being developed to restore fertility in men and women. Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of in vitro fertilized derived blastocysts, but they have limited clinical use for restoring fertility. By utilizing reprogramming factors OCT 4, SOX 2, *c-MYC*, and KLF 4, adult somatic cells can generate induced pluripotent stem cells (iPSCs). These iPSCs can be differentiated into sperm or eggs to be used during assisted reproductive technologies (ART) or possibly be transplanted into sterilized ovaries to restore fertility. For men, in vitro-derived iPSCs can be differentiated into spermatogonial stem cell-like cells (SSCLCs) for transplantation into a sterilized testis to restore spermatogenesis for natural conception. Somatic cell nuclear transfer embryonic stem cells (SCNT-ESCs) are derived by transferring the somatic cell nucleus into the enucleated oocyte to generate a blastocyst. From the blastocyst stage, the ICM can be derived to form SCNT-ESCs. These cells can then be differentiated in vitro to form sperm, which could be used during ART or transplanted into a sterilized testis. Created with Biorender.com

sess a greater ability to differentiate into more functional germ cells than iPSCs due to the erasure of epigenetic marks during oocyte-mediated reprogramming [75]. However, the ethical concerns surrounding SCNT-ESCs make iPSCs more favorable for clinical translation. Overall, both SCNT-ESCs and iPSCs represent patient-specific cell types that can potentially be used to restore fertility in sterile patients via in vitro gametogenesis.

Table 1 Summary of advantages and disadvantages of utilizing induced pluripotent stem cells and somatic cell nuclear transfer-embryonic stem cells to restore male and female fertility

Type of stem cell	Advantage	Disadvantage
Induced pluripotent stem cell (iPSC)	– Easy to culture in vitro – Differentiate to all cell types within an organism – Capable of self-renewal – Have been used to model several human diseases – Autologous to the adult donor – Ability to overcome immune rejection	– Utilized retroviruses and lentiviruses which carried the risk of mutations – Possible reactivation of transgenes – Tumorgenicity – Retain epigenetics of the original cell type – Chromosomal aneuploidies
Somatic cell nuclear transfer-embryonic stem cell (SCNT-ESC)	– Easy to culture in vitro – Differentiate to all cell types within an organism – Capable of self-renewal – Genetically identical to somatic cell – Ability to overcome immune rejection	– Ethical concerns of the sources and derivation from oocytes

Pluripotent Stem Cells' Treatment Options for Infertility

For the past two decades, several laboratories reported the ability to generate primordial germ cells (PGCs), the precursor cells that contribute to both female and male gametogenesis, from mouse, NHP, and human PSCs [88–107]. In 2011, Hayashi et al. [91] demonstrated that primordial germ cell like-cells (PGCLCs) generated from mouse PSCs could be transplanted into sterilized mouse testes. These transplanted cells colonized the gonad and underwent spermatogenesis to produce functional haploid sperm cells. The following year, Hayashi et al. (2012) [108] performed similar work with PGCLCs from female mouse PSCs. Upon transplantation into sterilized mouse ovaries, these cells developed into oocytes, which were then used to produce healthy offspring following in vitro maturation and fertilization. The importance of this work highlighted the capabilities of PSCs to differentiate into functional germ cells. The remaining sections in this chapter focus on how researchers are utilizing PSCs to generate both in vitro-derived female and male gametes for future infertility treatment (Fig. 1).

Stem Cell-Based Therapeutics for Infertile Women

In 2003, mouse ESCs were differentiated into oogonia that had the capability to enter meiosis and form follicle-like structures. However, the functionality of these cells was not examined [109]. As mentioned above, Hayashi and colleagues (2012) [108] made a significant advancement by differentiating both female mouse ESCs and iPSCs to form PGCLCs. When these cells were aggregated with female gonadal somatic cells and transplanted under the ovarian bursa in an immunocompromised female mouse, immature germinal vesicle-stage oocytes developed [108]. These oocytes underwent in vitro maturation and fertilization, giving rise to fertile offspring. Soon after, Hikabe et al. (2016) [110] reconstituted complete oogenesis in vitro. During their study, metaphase II oocytes were generated from mouse ESCs and iPSCs in vitro, and following IVF, normal offspring were produced. Furthermore, ESCs were rederived from the blastocysts from the in vitro-derived oocytes [110].

During the same year, Morohaku and colleagues (2016) [111] also reconstituted the entire process of mouse oogenesis in vitro to generate metaphase II oocytes. By utilizing in vitro cultured female mouse gonads, secondary follicles were isolated and matured to metaphase II oocytes. After the oocytes underwent IVF and reached the two-cell stage, these embryos were transferred into pseudo-pregnant mice, and healthy pups were born [111]. A year later, Jung

and colleagues (2017) [112] induced human ESCs to enter folliculogenesis in vitro to form oocyte-like cells and granulosa cells. Though the production of functional, in vitro metaphase II oocytes from human PSCs has not been accomplished, current scientific advancements continue to provide steps toward achieving the goal of producing functional human oocytes in vitro.

By utilizing different approaches to stem cell-based therapeutics to restore female fertility, scientists are seeking to develop platforms that could support the maturation of in vitro-derived oocytes to allow natural pregnancies to occur. In 2010, Krotz and colleagues [113] created an "artificial" human ovarian environment utilizing a three-dimensional (3D) in vitro maturation culture system. When combined with follicular cell subtypes, including theca, granulosa, and oocytes, the 3D culture system supported human oocyte maturation into metaphase II oocytes [113]. Subsequently, the development of two platforms led by Teresa Woodruff and colleagues could 1 day support the maturation of oocytes. In 2015, Laronda and colleagues [114] examined the use of artificial ovaries to restore fertility. These artificial ovaries indicated that human ovarian scaffolds could support viable ovarian cells and retain their endocrine function. In 2017, Xiao and colleagues [115] demonstrated the ability to model the human 28-day menstrual cycle in vitro. Called EVATAR, this microfluidic platform supported individual ovarian follicle growth, maturation, and ovulation. Each of these 3D culture systems has the potential to be paired with patient-specific in vitro-derived human PGCLCs to drive oogenesis and the production of fertilizable oocytes. However, before these culture systems could be translated to the clinic, more long-term studies with appropriate animal models are needed to determine if healthy offspring can be generated.

Stem Cell-Based Therapeutics for Infertile Men

Currently, the most advanced stem cell-based fertility preservation technique for men rendered sterile by medical interventions is SSC transplantation [41, 43, 116–123]. During the last 20 years, several groups have successfully demonstrated SSC transplantations in numerous mammalian species [43, 118–120, 124–131]. In 2012, Orwig and colleagues reestablished spermatogenesis in sterilized NHP's following SSC transplantation [116], providing evidence that this method of fertility restoration is ideal for reintroducing a patient's own germline stem cells. However, there is still a risk of reintroducing cancer cells in patients with testicular cancers or certain forms of leukemia after treatment [132–134], although this risk has been dramatically reduced due to improvements in separating SSCs from cancer cells [135]. In order for SSC transplantation to be used in the clinic, the testicular biopsy must be obtained before undergoing cancer treatment [46], and the SSCs must be retrieved from the testicular biopsies.

Additional research shows that the recipient's age may impact donor germ cell engraftment [46]. In a study completed by Brinster and colleagues [125], the reconstitution of spermatogenesis was significantly higher in the preadolescent-aged mice than the adults following SSC transplantation. Furthermore, the somatic environment must remain intact to support SSC recolonization [136]. Damage to the somatic environment may prevent SSC expansion and differentiation following transplantation [53–54]. Thus, in cases where SSC transplantation is impossible and for those patients who are unable to cryopreserve their gametes or SSCs for transplantation, there are no current treatment options [54]. Hence, deriving functional gametes from PSCs may be a more favorable option for restoring fertility in these patients.

In 2011, Hayashi and colleagues [91] demonstrated that PGCLCs could be generated from mouse PSCs and then transplanted into sterilized mouse testes to restore spermatogenesis and produce functional haploid cells that were used with ICSI to produce offspring. Recently, evidence has shown human PSCs could be differentiated into *VASA* and Deleted-in-Azoospermia-like (*DAZL*)-expressing PGCLCs [89, 96–97, 99–100, 102]. Though transplantation of both endogenous and in vitro-derived PGCLCs has shown promise in recolonizing sterilized mouse testes,

there has been limited success in other mammals, including primates [137]. While this work was important and groundbreaking, there have been difficulties with the clinical translation of this work due to the inability of PGCs to recolonize the testis in higher mammalian species.

Recently, it was demonstrated that human ESCs and iPSCs could be differentiated in vitro into SSCLCs [138–139]. Unlike PGCs, this germ cell stage has been shown to recolonize the gonad and restore fertility in several animal models [116, 140–141]. While current research focuses on deriving SSCLCs from NHPs and examining whether SSCLCs derived from patient-specific NHP PSCs can be autologously transplanted into the testis to restore fertility, future experiments are needed to address the similarities between endogenous and in vitro-derived SSCLCs. Thus, further research using NHPs could lead to the first stem cell-based therapy to treat male infertility.

In 2012, Easley and colleagues [138] demonstrated that advanced spermatogenic cells could be generated in vitro from PSCs (Fig. 1). In the study, both human male ESCs and iPSCs were directly differentiated into advanced spermatogenic lineages, including SSCLCs, differentiating spermatogonia-like cells, primary and secondary spermatocyte-like cells, and haploid round spermatid-like cells [138]. In 2018, Zhao and colleagues [139] independently validated and improved upon this work by differentiating patient-specific human iPSCs from patients diagnosed with non-obstructive azoospermia (NOA) with Sertoli cell-only syndrome into SSCLCs and spermatid-like cells, suggesting that gametes could be derived from skin cells from infertile men. Although the functionality of these in vitro-derived gametes could not be assessed due to ethical and legal concerns, these experiments showed the feasibility of differentiating human iPSCs into advanced spermatogenic stages, including round spermatids [138–139]. In the future, patient-specific iPSCs could be differentiated into SSCLCs and transplanted into the patient's testis for recolonization and fertility restoration [54] (Fig. 1).

Summary of the Derivation of Female and Male Gametes from Pluripotent Stem Cells

During the last 60 years, several scientific advancements have laid the foundation for today's achievements (Fig. 2). From the pio-

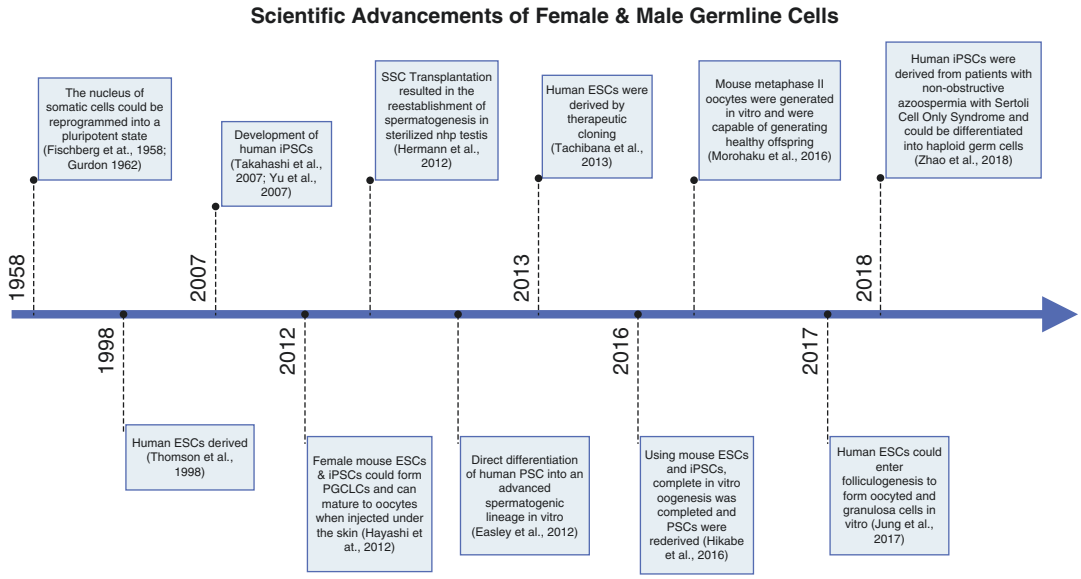

Fig. 2 This figure represents a historical view over the last 60 years of deriving female and male gametes in vitro. Embryonic stem cells (ESCs); induced pluripotent stem cells (iPSCs); primordial germ cell-like cells (PGCLCs); spermatogonial stem cell (SSC); pluripotent stem cell (PSC). Created with Biorender.com

neering work of Gurdon [68], Thomson [64], Yamanaka [80], and Hayashi [108], human PSCs have been shown to differentiate into PGCLCs [93, 100–103, 105–106] and eventually into both ovarian-like cells [112] and haploid spermatid-like cells [138–139]. Although fully functional and mature human gametes have not yet been generated in vitro, these results indicate that it may be feasible to differentiate patient-specific PSCs into gametes usable in a clinical setting. In conclusion, the production of gametes from stem cells may 1 day make it possible for infertile couples to have their own biological children.

Future Directions

Before male and female gametes derived in vitro can be translated to the clinic, further evaluations are needed at the NHP level. First, researchers must ensure the blastocysts formed using in vitro-derived male or female gametes can develop in utero correctly, producing normal and healthy offspring. Additionally, studies will need to address postnatal development to ensure normal growth and aging are occurring [54, 142]. Although the clinical applications for using patient-specific in vitro-derived gametes are still years away, the recent scientific advancements make it possible for patients rendered sterile by environmental exposures or medical interventions to have their own biological children.

Overall, the culmination of stem cell-derived gamete research during the last 25 years has moved scientists one step closer to deriving both male and female gametes in vitro to restore fertility in infertile patients [66, 142–143]. Today, scientists are working toward deriving in vitro male and female gametes from patient-specific PSCs to provide a stem cell-based treatment to restore fertility. Ultimately, this therapeutic approach could provide the opportunity for both infertile men and women to have healthy offspring since current treatment options fail to address their infertility.

Take-Home Messages

- In vitro-derived mouse ESCs and iPSCs have generated metaphase II oocytes that were capable of producing offspring.
- Human in vitro-derived ESCs and iPSCs have been shown to differentiate into advanced spermatogenic lineages, but complete spermatogenesis resulting in functional sperm has not been generated in a dish.

Key Readings

Easley et al. 2012 [138].
Easley et al. 2013 [53].
Easley et al. 2014 [54].
Easley et al. 2014b [142].
Eguizabel et al. 2019 [66].
Evans and Kaufman 1981 [58].
Fischberg et al. 1958 [67].
Gurdon 1962 [68].
Hayashi et al. 2011 [91].
Hermann et al. 2012 [116].
Jung et al. 2017 [112].
Kurek et al. 2020 [143].
Martin 1981 [59].
Takahashi and Yamanaka 2006 [78].
Thomson et al. 1995, 1998 [60, 64].
Valli et al. 2014 [134].
Wolf et al. 2017 [75].
Zakrzewski et al. 2019 [56].
Zhao et al. 2018 [139].

Glossary

Azoospermia The absence of sperm in the ejaculate.

Gonocytes Precursor cells of spermatogonia that differentiate in the testis from primordial germ cells.

Infertility A couple's inability to conceive after 1 year of unprotected intercourse.

Non-obstructive Azoospermia A medical diagnosis describing the absence of sperm in the ejaculate due to the complete failure of spermatogenesis.

Pluripotent Stem Cells Cells that can differentiate into all three germ layers (ectoderm, mesoderm, and endoderm), but not extraembryonic structures.

Primordial Germ Cells An undifferentiated stem cell type that will differentiate into both male and female gametes.

Sertoli Cell-Only Syndrome A cause of male infertility where Sertoli cells are the only cell type present with the seminiferous tubules.

Therapeutic Cloning The transfer of a somatic cell nucleus into an enucleated oocyte to generate stem cell lines with the same genome as the donor.

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Techniques of Ovarian Transplantation

Jessica Subirá

Abbreviations

AMH	Anti-Müllerian hormone
OCC	Ovarian cortex cryopreservation
OCT	Ovarian cortex transplantation

Introduction and General Principles

Since the birth of the first baby after autotransplantation of frozen-thawed ovarian tissue in 2004 [1], several groups have reported worldwide their experience with this technique. Despite the surgical heterogeneity present among these groups, several fundamental principles apply to all of them: (1) the election of the best vascularized site (i.e., the ovary if present), (2) the reduction of the time of ischemia to a minimum, and (3) the suture of the fragments with stitches or by placing surgical films or glues [2].

It must be reminded that more than 50% of all the follicles present in the graft will be lost during the process of ischemia due to hypoxia and subsequent neoangiogenesis that occurs during the first days after the transplantation [3–5]. This phenomenon explains the dramatic reduction on the follicle pool, and it is the reason why the election of a well-vascularized site that permits the formation of new vessels is paramount for the survival of the remaining follicles. But there is another mechanism explaining this tremendous loss of ovarian reserve in the graft. During these days, there is also a massive activation of primordial follicles that appears to be confined to the first 3 days post-OCT according to recent evidence [6, 7]. These two mechanisms should be the target of new molecules or surgical modifications to act on the initial stages of graft dynamics to hopefully maintain a larger number of follicles and thus prolong the life of the tissue. We will look into these recently developed options further along this chapter.

The slow-freezing technique was firstly introduced with the majority of groups having used it from the first cases. In fact, almost all the pregnancies achieved come from slow-frozen tissue. However, throughout these years, some groups have used vitrification instead with reports of at least an equivalent outcome and a simpler tissue processing procedure [8–10]. More groups are joining the use of vitrification, and perhaps it is only a matter of time that it replaces slow-freezing as it happened with embryos and oocytes some years ago. Concerns are raised by the use of higher concentration of cryoprotectants and their potential toxic effect on the tissue, but last reports are reassuring [11].

In this chapter, we will review the current techniques described by different groups for the transplantation of the ovarian tissue. We will also

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compare the results yielded by each group in terms of recovery of endocrine function and pregnancies (discussed in detail on the previous chapter of this book). Finally, we will review the most recent advances in the research of adjuncts to reduce follicle loss during transplantation and introduction of technique modifications.

Surgical Approach: Orthotopic Vs Heterotopic

Since the first auto-transplantation of frozen-thawed ovarian tissue performed by Oktay group in 1999 [12], several fertility preservation teams started presenting their experience with OCT, first as case reports and lately as a wider number of procedures.

The orthotopic approach involves the transplantation of the cortical pieces inside the pelvic cavity, namely, the ovary itself, the broad ligament, or a subperitoneal pouch [13]. It provides a number of advantages including possibility of natural conception as long as the graft remains active and better environment for follicular growth. However, it requires an abdominal surgery (usually minimally invasive via laparoscopy) and carries the theoretical risk of reintroduction of malignant cells (only significant in some neoplasms according to current evidence: this issue will be separately discussed in the following chapter). It is currently not known if one site is superior to other in terms of efficacy. The FertiPROTEKT network is currently conducting an open international multi-center study (NCT02780791) to determine the best site to perform an orthotopic OCT [14].

The heterotopic site has also been used in some OCT. The main sites used include the forearm, the subcutaneous abdominal wall, the subperitoneum, and the rectus muscle. The simplicity of the procedure and the accessibility of the tissue for monitoring of follicular growth or even malignancy are clear advantages. Moreover, it would be an alternative to the orthotopic site in cases of severe pelvic adhesions and would facilitate repeated transplantations [15, 16]. However, it neglects the possibility of natural conception. Other major limitations with regard to fertility restoration are the report of only two live births from the same procedure in which fragments were transplanted inside the abdominal cavity [17]. Only recently a new live birth has been reported after transplantation on an heterotopic site [18]. This makes the heterotopic site only optimal when the OCT is performed with the sole intention of hormonal recovery or when the patient does not have a suitable orthotopic site. It appears that pelvic-specific conditions of temperature, oxygen, and pressure within the peritoneal cavity are needed for optimal follicle growth and their absence would result in poorer outcomes [19]. The great majority of live births obtained after OCT come from orthotopic sites, and therefore, unless proven otherwise with well-designed studies, it must remain the via of choice when looking for pregnancy (Table 1).

Table 1 Comparison of orthotopic versus heterotopic ovarian cortex transplantations

	Orthotopic	Heterotopic
Site	Pelvic cavity (ovary, broad ligament, subperitoneal pouch)	Forearm, rectus muscle, subcutaneous abdominal wall
Patient suitability	For both recovery of hormonal function and fertility	Only intended for recovery of hormonal function (one live birth reported only)
Technique	More complex; requires abdominal surgery and different techniques according to different groups	Simple, subcutaneous placement of thawed fragments
Relative surgical contraindications	Frozen pelvis, severe adhesions	None
Monitoring of malignancy	More difficult, especially if transplanted adjacent to native ovary	Easier due to location and accessibility, easy removal of graft if needed

Orthotopic: Different Methods Described by the Groups

There are almost as many techniques described as groups performing transplantations (Table 2). As stated above, several surgical principles must be met and are consistently reported by all of them: well-vascularized site, adequate placement of the fragment with the cortex facing the abdominal cavity, and microsurgery to suture the fragments (J [2].). Another constant is the prioritization of the ovary as a site if there is at least one present in good condition, namely, with adequate size to allow the placement of the fragments and not being severely atrophic.

Donnez's group was the first to achieve a live birth after OCT [1]. The first step is the decortication of a large area on the native ovary to expose the medulla with its vessels. Then the cortex pieces are placed with the medulla facing the ovary and the cortex on top. Graft sizes vary from 0.5 to 2 cm^2 and must be 1–2 mm thick after removal of any remaining medulla. They can be sutured with either 7/0 or 8/0 polypropylene stitches or fixed with a surgical film such as Interceed®. If both ovaries are present, the procedure can be done on the contralateral ovary if sufficient tissue is available. The creation of a peritoneal window on the anterior leaf of the broad ligament is the only option for patients with bilateral oophorectomy. This procedure was originally described in two steps separated 7 days [1] to allow the new vessels to proliferate in the newly created peritoneal window. It can also be done in a single-step procedure and is extremely simple. Firstly, a peritoneal window is created, and secondly, the fragments are placed in and fixed with Interceed®.

The amount of tissue to use determines the number and location of the sites. If enough tissue is available, it can be grafted in several sites bilaterally (i.e., the ovary, the broad ligament). The decision as to how many fragments should be thawed must be taken along with the patient and must take into consideration several aspects: the age of the patient, the amount of tissue available, and whether or not the patient would reconsider a second transplantation (if the first fails or the tissue gets exhausted). In general, it is advisable to leave some tissue for a future re-transplantation if needed.

Other groups such as Silber's use a minilaparotomy and expose a larger area of medulla on the native ovary. In turn, the fragment placed is also larger (3–4 cm^2) and sutured with 9/0 Nylon stitches [20–23].

Table 2 Summary of surgical techniques for orthotopic OCT reported by different groups

Group	Approach	Size fragments	Location	Suture/technique
Donnez	Laparoscopy	0.5 × 2 cm^2 (1–2 mm thick)	Ovary, broad ligament window, subperitoneal pouch	Decortication of ovary or creation of pouch within the broad ligament or peritoneum. Microsuture 6/0 or 7/0 polypropylene stitches or simply fixed with Interceed®
Silber	Minilaparotomy	3–4 cm^2	Ovary	Sutured to the medulla of the ovary with 9/0 nylon stitches
Meirow	Minilaparotomy	0.5 x 1.5 cm^2	Ovary	Creation of tunnels underneath the tunica albuginea, fragments slipped, opening closed with 4/0 Vicryl stitches
Andersen	Combined laparoscopy/ minilaparotomy	0.5 x 0.5 cm^2	Ovary	Creation of two subcortical tunnels on each ovary, fragments slipped through, laparoscopy to assist mobilization of ovary
Pellicer	Minilaparotomy	1 x 1 cm^2	Ovary, subcortical pouches, broad ligament, subperitoneal pouches	Fragments are either sutured using 5/0 nylon stitches or placed inside the pouches

Meirow's group creates several ovarian tunnels just underneath the tunica albuginea, and the fragments are gently slipped through them. The incisions are closed with 4/0 Vicryl stitches [24].

Andersen's technique involves a combined laparoscopy/minilaparotomy procedure. The laparoscopy aids to the mobilization of the ovary which is then accessed through the laparotomy incision. Two subcortical tunnels are created on each of them, and the fragments are placed aligned inside of them with cortex in the right position [25].

Pellicer's group has used a combination of the techniques described originally by Donnez. The thawed ovarian tissue fragments are transplanted using three different techniques: (1) in subcortical pouches; (2) using microsurgical stiches; or (3) in subperitoneal pouches ([26]) (Table 2).

Results: Is the Technique Related to Success?

Success rates of OCC and subsequent transplantation have been extensively reviewed in the preceding chapter. As it has been described, success rates are consistently similar across all the groups reporting on OCT cases. Hormonal recovery rate exceeds 90% in all series, and live birth rate is in the region of 20–30% [27, 28]. As there is significant heterogeneity among surgical techniques between groups, one should wonder whether this could have any impact on success rates. In light of the evidence, it appears that is not the case. Surely all techniques used by the different authors respect the general principles stated above (vascularization, placement of fragments, and microsuture) which makes in turn success rates stable across them.

Modifications of the Original Technique: In Vitro Activation and Use of Robotics

In vitro activation of follicles (IVA) was first described by Kawamura [9, 10] as a method aimed to activate dormant primordial follicles present in women with premature ovarian insufficiency (POI). It comprises a two-step procedure both mechanical and chemical. Firstly, the ovarian tissue is fragmented into smaller pieces (1–2 mm^3) to produce a disruption on the inhibitory HIPPO pathway. Secondly, the tissue is incubated with Akt stimulators before retransplanting the fragments to the native ovary. The former produces growth of follicles from the secondary to the antral stage, whereas the latter activates dormant primordial follicles to initiate folliculogenesis. This approach has successfully restored fertility in POI patients with a number of pregnancies reported, and it represents a promising ovarian rejuvenation procedure [9, 10].

The addition and/or replacement of the usual OCT technique by IVA may provide some benefits: faster follicular activation of the primordial pool, quicker recovery of endocrine function, and achievement of pregnancy in the short term which may represent an advantage in some oncological patients who will need to resume treatment after giving birth (i.e., hormonal therapy in breast cancer). However, this rapid activation may also lead to quick exhaustion of the tissue and a shorter lifespan of the graft not allowing the achievement of more than one pregnancy nor the maintenance of hormonal function with all its benefits for several years [29].

Further studies will need to compare both techniques to elucidate whether IVA adds any benefit to conventional tissue preparation to OCT and/or to select patients accordingly.

Another new prospect in the field of OCT is the use of robotics to improve the surgical technique. This has been recently presented by Oktay in a video article [30] along with the use of an extracellular matrix of human de-epithelized skin (Alloderm) acting as a scaffold for the fragments to be transplanted. The use of robotics can add precision and reduce time from thawing to grafting. The use of this new scaffold has resulted in some live births, and the authors report improved follicle growth and response to stimulation [31].

Use of Adjuncts to Improve Graft Viability

Follicle survival is a key limiting factor to achieve success after OCT as we discussed above. Several groups are currently working on the development of strategies to improve revascularization times and in turn favor the survival of more primordial follicles. Growth factors such as VEGF (vascular endothelial growth factor) have an important role in neoangiogenesis. The use of hydrogels containing it can improve follicle survival by accelerating the formation of new vessels in animal models [32]. AMH has also been involved in the premature activation of follicles and a subsequent burn-out of the graft. The transplantation of the tissue incubated with AMH has resulted in a reduced loss of follicles after OCT [33]. Finally, the promising use of stem-derived cells like mesenchymal stem cells or adipose tissue-derived stem cells in a co-transplantation model may enhance angiogenesis and improve the success of the OCT [34–36].

Another area of interest lays with the assessment of the tissue before OCT. Presence of malignancy is consistently ruled out through various methods with several limitations, and it will be properly addressed in another chapter of this book. New methods are trying to guide the clinical decision on the amount of tissue that should be used. Takae et al. [37, 38] have been using imaging techniques such as optical coherence tomography or infrared ray examination to count the number of primordial follicles present and give an estimation of duration of graft function to help to decide the number of fragments to be used. This technique could even be used for non-invasive detection of metastases within the same tissue that will be transplanted solving one of the issues of ruling out malignant contamination before transplantation [39].

In conclusion, OCT is a reproducible technique with the aim to recover hormonal function and fertility. It requires a well-vascularized area to support the graft, and despite this, a great proportion of the follicles will be lost due to ischemia and premature activation. Sites inside the pelvis (orthotopic) are the optimal ones for fertility purposes, whereas the heterotopic sites are according to current evidence only suitable for recovery of endocrine function. Despite the surgical heterogeneity, success rates are maintained stable across all groups due to respect to key surgical principles. Although most groups have worked with slow-freezing, it is very likely that vitrification will finally replace the former because of its simplicity and equivalent results. Several groups are working on different research areas to improve follicle survival and OCT performance. Growth factors, hormones, matrix scaffolds, and robotics surgery may await the future of this young yet stablished fertility preservation procedure.

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Part II

Male Fertility Preservation

Impact of Cancer Treatment on Testicular Function

Rod T. Mitchell, Sheila Lane, and Mark Brougham

Introduction

Male fertility is dependent on the continuous production of sperm, which begins at puberty and continues throughout adult life. Treatment for cancer during childhood can affect future fertility, whilst treatment in adulthood may result in azoospermia that is either transient or in some cases permanent. The specific treatment regimen an individual receives is the primary determinant of subsequent reproductive function; however, additional factors may contribute. This includes, but is not limited to, the age of the individual and stage of reproductive development, the underlying malignancy and any pre-existing testicular pathology, e.g. previous or current cryptorchidism.

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Testicular function is under central regulation by the hypothalamo-pituitary-gonadal (HPG) axis. Damage to the hypothalamus and/or pituitary can affect gonadotrophin secretion, which results in impaired testicular function. To some extent, this may be treated by replacing or substituting the deficient hormones; however, for direct testicular damage resulting in loss of germ cells, effective treatment strategies are lacking. Where primary testicular damage is anticipated, sperm cryopreservation prior to cancer therapy is the only established clinical strategy for fertility preservation. For prepubertal boys, in whom sperm production is not established, no such clinical option is possible. Understanding the effects of cancer treatment on male reproductive function is crucial for counselling patients on fertility risk and can also inform future development of cancer treatment regimens aimed at reducing potential for gonadotoxicity.

In this chapter, we will describe the key events involved in testicular development and function. We will use case histories to describe the impact of traditional cancer treatments on testicular function at different stages of development, with emphasis on the impact of treatment in childhood during which sperm cryopreservation is not possible. We will also discuss novel treatments in the context of their potential for impacts on male reproductive function.

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Testicular Development and the Hypothalamo-Pituitary-Gonadal Axis

In order to understand the potential damage that cancer treatments can cause to testicular function, it is important to understand how the testis develops including the morphological and functional changes that occur at different periods from foetal life through to adulthood. Testicular function in humans is regulated by the hormones of the HPG axis. This involves the central production of gonadotrophins, luteinising hormone (LH) and follicle-stimulating hormone (FSH), from the pituitary in response to hypothalamic gonadotrophin-releasing hormone (GnRH) produced by the hypothalamus. Luteinising hormone stimulates the Leydig cells of the testis to produce testosterone, whilst follicle-stimulating hormone stimulates the Sertoli cells of the testis to support germ cell development. Together, gonadotrophin stimulation of the testis supports spermatogenesis in adulthood (Fig. 1). The activity of the HPG axis changes throughout life with a period of relative quiescence during childhood, which may impact on sensitivity to cancer treatment during different life stages.

Testicular Development and Function during Foetal Life

The testis forms from a bipotential gonad in foetal life [1]. This is primarily determined by the

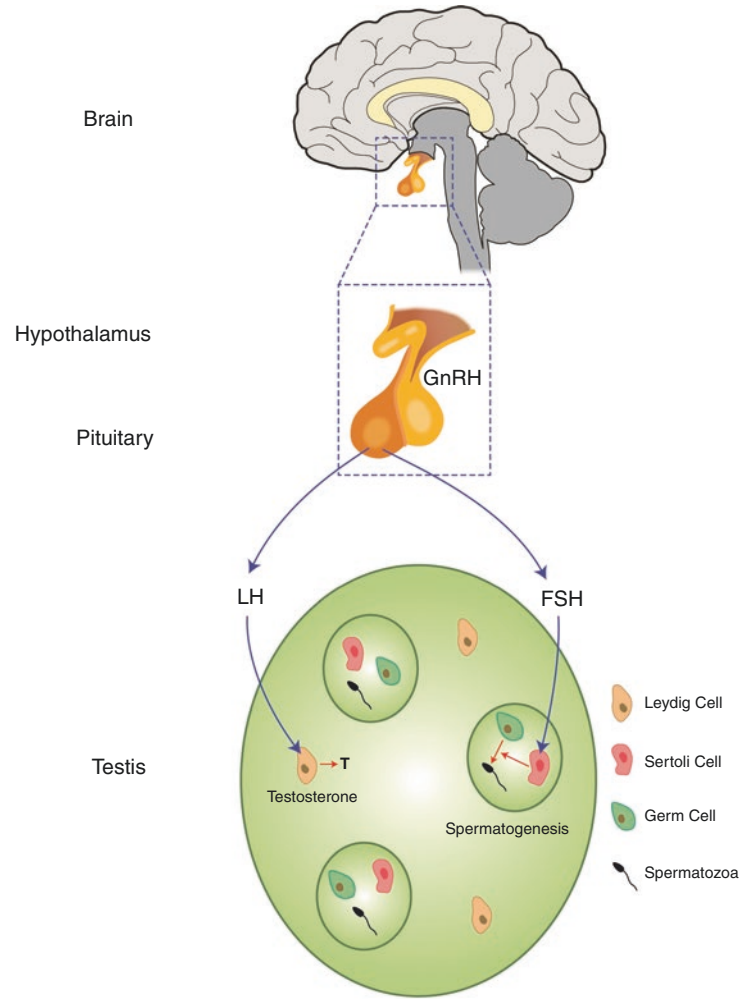

Fig. 1 The hypothalamo-pituitary-gonadal (HPG) axis in humans. Gonadotrophins (luteinising hormone, LH; and follicle-stimulating hormone, FSH) are released from the anterior pituitary in response to hypothalamic gonadotrophin-releasing hormone (GnRH). LH stimulates the Leydig cells of the testis to produce testosterone (T), whilst FSH stimulates the Sertoli cells to support germ cell development and spermatogenesis in adulthood. The HPG axis is quiescent during childhood. Negative feedback from testosterone and Sertoli cell-derived Inhibin B regulates gonadotrophin release from the pituitary

presence of a Y chromosome and a number of key 'male-determining' genes, with several additional genetic influences [1]. Specification of the Sertoli cell population drives the formation of the seminiferous cords to enclose the germ cells [2]. The formation of the cords separates the seminiferous epithelium from an interstitial compartment in which testosterone-producing cells are located. Testosterone production commences around 8–10 weeks' gestation and is initially under the control of placenta-derived human chorionic gonadotrophin (hCG). From mid-gestation, testosterone production is under the control of gonadotrophin stimulation. Therefore, in foetal life, the HPG axis is active with peak levels of gonadotrophins and testosterone at 11–14 weeks, with a decline from 17 to 20 weeks until just prior to birth when the levels decline to low/undetectable levels in the peri-natal period ([3, 4].

In addition to specification of the somatic cell populations of the testis, germ cells undergo a critical phase of development from gonocyte to (pre)spermatogonia. This process occurs asynchronously within the germ cell population over the remainder of foetal life and into the early postnatal period [5]. This key stage of differentiation of germ cells is critical for establishing the pool of spermatogonia that will be present during childhood and subsequently support future fertility in adulthood [5]. Whilst chemotherapy exposure in utero is relatively rare and use may be associated with foetal loss, it is possible that some 'milder' cytotoxic agents may have less severe global effects on the developing foetus that could impact gonadal development or function, particularly if chemotherapy is given during the first or early second trimester which is a key period for testicular development [6].

Infancy and the 'Mini-Puberty'

Following the reduction in gonadotrophins around birth, there is a re-activation of the HPG axis during the first week of postnatal life, with a peak of gonadotrophins and testosterone at 2 months [3]. Subsequently, gonadotrophin levels begin to fall until around 3 months of age at which point they are very low or undetectable. During mini-puberty, testicular volume increases from 0.6mls at birth to ~1.2mls at 6 months of age [7]. Mini-puberty also coincides with the period during which the remaining gonocytes complete their transition to (pre) spermatogonia, suggesting a role for testosterone in completing this transition [5]. The activity of the HPG axis and the development of gonocyte to spermatogonia are important considerations for exposure to cancer treatment during the first few months of life.

Testicular Development during Childhood

The end of the mini-puberty heralds a long period during which the HPG axis is relatively quiescent and levels of gonadotrophins and testosterone remain low or undetectable [3]. This 'childhood' period in males lasts until the onset of puberty, which is considered normal in boys from the age of 9 years [8]. This relative quiescence has previously been postulated to render the testis less susceptible to the damaging effects of gonadotoxic treatment; however, it is clear that prepubertal boys are at risk of impaired testicular function following treatment for cancer. Despite the quiescence of the HPG axis during this period, there is activity in the testis, which includes progressive maturation of the Sertoli cell population and slow turnover of the germ cells ([9, 10], with a small increase in testicular volume to approximately 1.5mls by 10 years of age in prepubertal boys [11]. This activity contributes to the sensitivity of the prepubertal testis to the damaging effects of cancer treatment. This includes the germ cells which consist of a population of spermatogonial stem cells (SSCs) supported by the partially differentiated Sertoli cells [12] (Fig. 2). Whilst spermatocytes and occasionally spermatids have been shown to be present from as early as 4 years of age, these are rare, and mature spermatozoa are not produced [7]. Clinical onset of puberty is preceded by nocturnal activation of the HPG axis with pulsatile release of gonadotrophins and testosterone. This peri-pubertal 're-awakening'

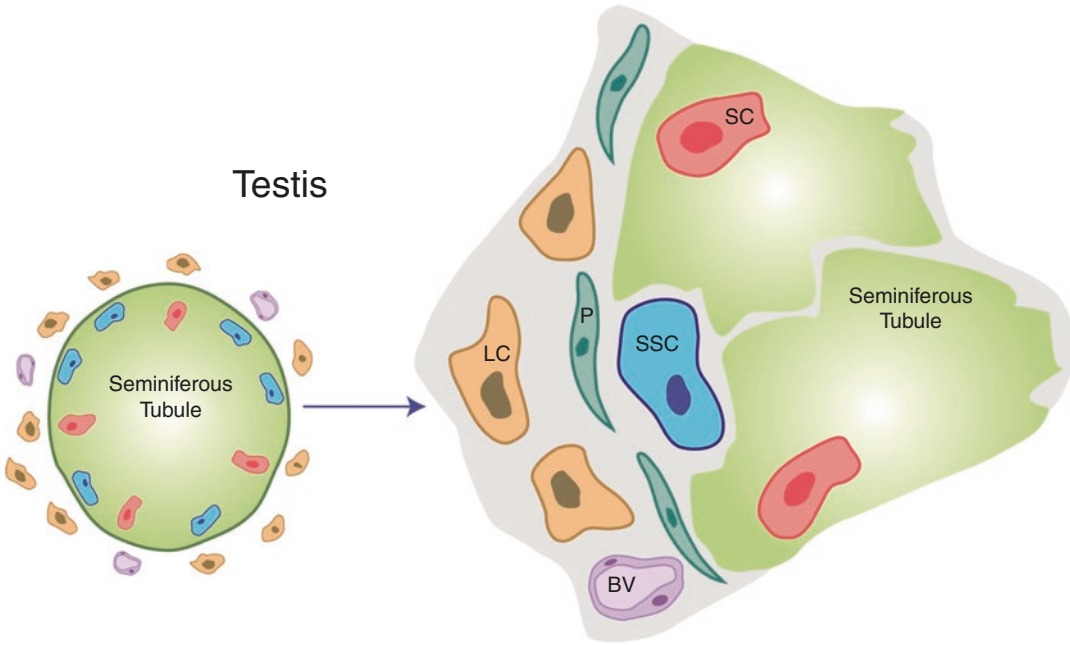

Fig. 2 The childhood testis. The seminiferous tubules of the prepubertal testis contain undifferentiated germ cells and Sertoli cells (SC). The spermatogonial stem cell (SSC) population must survive for future fertility to be possible. Leydig cells (LC) lie outside the seminiferous tubules in the interstitium. The Leydig cells do not produce testosterone during childhood. *P*, peritubular myoid cells; *BV*, blood vessel

period of development may also represent a period of differential sensitivity to cancer treatments.

Puberty and the re-Activation of the HPG Axis

Puberty in boys commences between 9 and 14 years with re-activation of the HPG axis resulting in FSH stimulation of Sertoli cells and LH stimulation of testosterone from Leydig cells. This results in testicular enlargement (≥4mls indicates the start of puberty) leading to the onset of spermatogenesis. Sertoli cells attain terminal differentiation including cessation of proliferation, and final Sertoli cell number determines capacity for sperm production [13]. The likelihood of presence of sperm in the testis has been estimated based on the testicular volume. Based on a study involving 1160 boys, sperm were present in the urine of <20% of boys with testicular volumes ≤8mls [14]. However, focal areas of spermatogenesis can be identified in some boys with testicular volumes as low as 6mls [15].

Adulthood: A Period of Continuous Sperm Production

The HPG axis remains active in adulthood supporting the continuous production of sperm from a pool of spermatogonial stem cells (SSCs). SSCs either can self-renew to maintain the population or can differentiate toward sperm [16]. The SSCs represent a population of undifferentiated spermatogonia. However, the identity and characteristics of these cells in human are poorly characterised. Undifferentiated human spermatogonia are currently divided into two populations, known as A_{dark} and A_{pale}, based primarily on their nuclear morphology. The A_{dark} population have been considered a reserve SSC population that can respond to undergo expansion following loss as a result of cytotoxic therapies, whilst the A_{pale} population are postulated to be self-renewing

progenitors that can proliferate during each spermatogenic cycle [17]. However, recent studies in rodents have demonstrated the heterogeneity of spermatogonial populations and stochastic developmental potential [18], and similar complexity is likely for the human making the identification of a 'true SSC' challenging [19]. Spermatogenesis is a complex process that requires spermatogonia to undergo meiosis to form haploid cells followed by spermiogenesis during which the cells complete major morphological and functional changes to produce spermatozoa. Cancer treatment during adulthood may impact on the differentiating germ cells resulting in transient azoospermia, whilst damage to the SSC population will likely result in permanent impairment of fertility (Fig. 3).

Spermatogonial Stem Cell Niche

SSCs are dependent on support from the surrounding somatic cell populations, and together this forms the 'SSC niche'. This unique local environment maintains the SSC population and supports their differentiation into sperm [16]. Sertoli cells are critical to this process and are located in direct contact with the SSCs. Germ-Sertoli cell contact is maintained as germ cells differentiate to sperm and move toward the lumen of the seminiferous tubule. Sertoli cells provide structural support to germ cells and contribute to the formation of the blood-testis barrier (BTB), through the development of tight junctions, providing immunological protection of meiotic cells on the luminal surface of the seminiferous epithelium. Sertoli cells produce growth factors, e.g. glial-derived neurotrophic factor (GDNF), that have been shown to contribute to the balance between self-renewal and differentiation of SSCs in mice [20]. In order to support sufficient SSCs for normal sperm production in adulthood, Sertoli cells must proliferate during the neonatal and peri-pubertal periods ([13, 21]. In addition, Sertoli cells undergo functional maturation, including the acquisition of androgen responsiveness and formation of the BTB [12]. Peritubular myoid cells, a population of smooth muscle cells,

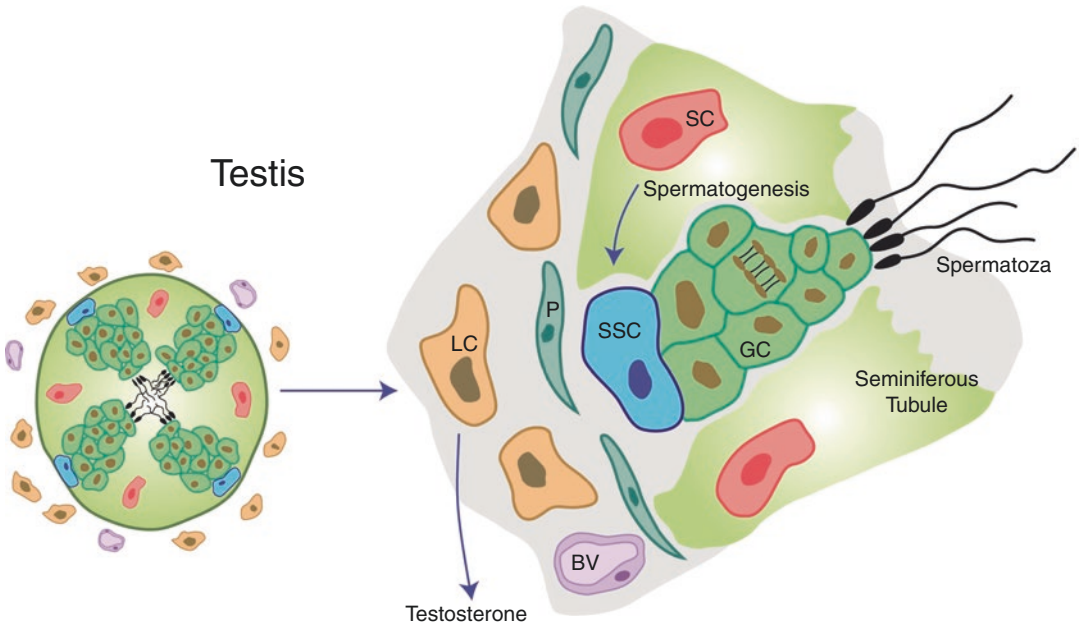

Fig. 3 The adult testis. Seminiferous tubules contain germ cells (GC) undergoing spermatogenesis from a self-renewing population of spermatogonial stem cells (SSC). Spermatogenesis is supported by Sertoli cells (SC). Leydig cells in the interstitial compartment produce testosterone to induce and maintain secondary sexual characteristics and to support spermatogenesis. P, peritubular myoid cell; BV, blood vessel

line the basement membrane of the seminiferous tubule providing structural support in addition to production of growth factors (e.g. GDNF) that can support SSC maintenance. The interstitial compartment also contributes to the SSC niche. For example, testosterone produced by the interstitial Leydig cells is required for qualitatively normal spermatogenesis in adult males. An additional role for the vasculature has also been proposed based on work in rodents which suggests the SSC niches are in direct apposition to the blood vessels in the interstitial compartment [22]. In addition to the local environment, the SSC niche is dependent on stimulation by pituitary-derived gonadotrophins. This includes follicle-stimulating hormone (FSH) acting on Sertoli cells and luteinising hormone (LH) on Leydig cells.

Whilst the importance of the SSC niche is being increasingly recognised, there is much still to learn about its regulation and role in support of spermatogenesis. However, current understanding highlights the importance of multiple factors extrinsic to the SSCs themselves. This has implications for understanding the effects of cancer treatment on fertility which may result not only from direct damage to the SSCs but from damage to somatic cells and other components of the SSC niche.

Impact of Cancer Treatment on Male Reproductive Function

Cancer treatment during prepuberty can impact future reproductive function in males. This may result from hormonal dysfunction due to central damage to the HPG axis or as a result of direct damage to the testis (Fig. 4).

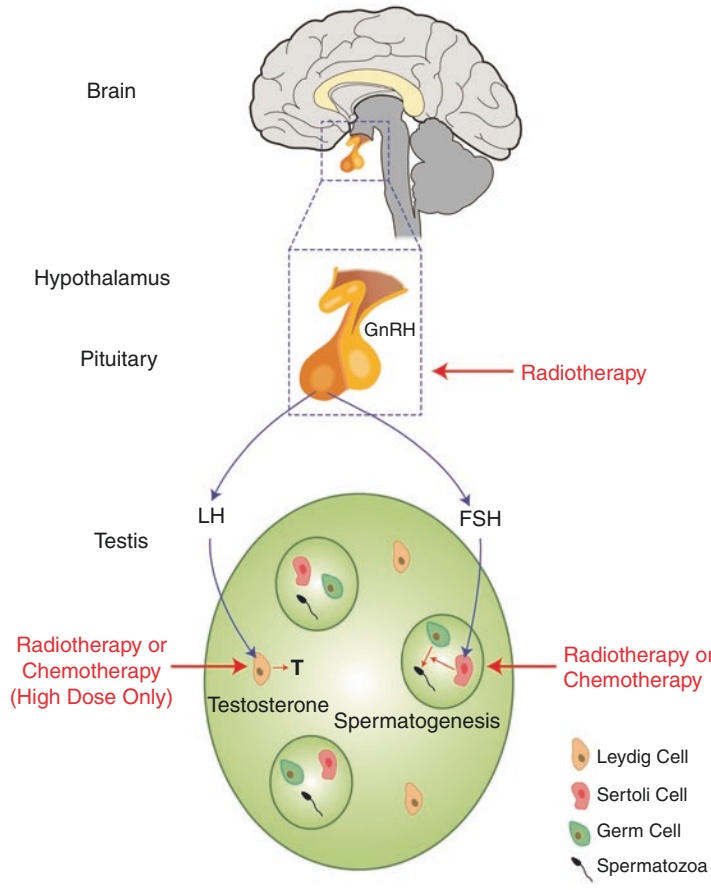

Fig. 4 Effect of cancer treatment on the hypothalamo-pituitary-gonadal axis. Radiotherapy exposure to the brain may result in damage to the hypothalamus and pituitary leading to gonadotrophin deficiency. Exposure of the gonads to chemotherapy and/or radiotherapy may result primary damage to the testis. Germ cell loss may occur at all ages leading to impairment of spermatogenesis in adulthood

Central Damage to the HPG Axis

Radiotherapy to the hypothalamus or pituitary can affect secretion of gonadotrophins leading to hypogonadotropic hypogonadism which will manifest with delayed puberty and infertility. Testosterone replacement may be given to induce puberty in individuals with hypogonadotropic hypogonadism. Providing there is no significant testicular damage, gonadotrophins may be given in adulthood to induce spermatogenesis for natural fertility [23].

Direct Damage to the Testis

Radiotherapy or chemotherapy can also cause direct damage to the testis, with germ cells the primary target. This germ cell loss may result in oligo- or azoospermia in adulthood, which will affect fertility. Loss of SSCs at all ages will lead to permanent azoospermia, whereas loss of differentiating germ cells in adulthood will result in transient azoospermia, providing the SSCs survive the treatment [24]. Testosterone production from Leydig cells may be affected at higher doses of chemotherapy and/or radiotherapy relative to those required to impair spermatogenesis. Puberty can be induced using testosterone in those with primary Leydig cell dysfunction. However, in cases where treatment has resulted in Leydig cell failure, azoospermia is expected, and currently there are no treatments available to restore natural fertility in these individuals.

The risk of infertility can be estimated based on the specific regimen that the patient receives and the contribution of the individual agents. The risk of toxicity may be estimated based on the cumulative cyclophosphamide equivalent dose (CED) for all agents used to treat a specific cancer [25]. A summary of the relative gonadotoxicity of regimens used in the most common childhood cancers is provided in Table 1.

However, it must be recognised that long-term follow-up data on fertility is limited in the paediatric population and there may be additional factors that will modify the risk such as the underlying diagnosis, history of cryptorchidism and previous treatment. As a result, assessment of fertility potential and counselling for fertility preservation should be conducted on an individual patient basis as demonstrated in the following clinical cases.

Table 1 Impact of common childhood cancer treatment regimens of male fertility

	Diagnosis	Gonadotoxic treatment	Estimated gonadotoxicity
Leukaemia	Acute lymphoblastic leukaemia (first line)	Cyclophosphamide or ifosfamide	Low–medium
	Acute lymphoblastic leukaemia (relapse)	Cyclophosphamide Testicular irradiation	Medium–high Very high
	Acute myeloid leukaemia	None	Low
Lymphoma	Non-Hodgkin's lymphoma (low risk)	Cyclophosphamide	Low
	Non-Hodgkin's lymphoma (standard and high risk)	Cyclophosphamide	High
	T-cell lymphoma	Cyclophosphamide	Medium
	B-cell lymphoma	Cyclophosphamide	Medium–high
	Hodgkin's lymphoma	Cyclophosphamide ± dacarbazine	Low–medium
		Cyclophosphamide + dacarbazine + procarbazine	Very high

(continued)

Table 1 (continued)

	Diagnosis	Gonadotoxic treatment	Estimated gonadotoxicity
Brain tumours	Ependymoma	Cyclophosphamide + cisplatin	High–very high
	Medulloblastoma	Cyclophosphamide + cisplatin ± lomustine	Very high
	Pineoblastoma	Cyclophosphamide	Very high
	Atypical teratoid/rhabdoid tumour	Cyclophosphamide + ifosfamide	High
		Cyclophosphamide + ifosfamide + thiotepa	Very high
	Glioma (high grade)	Temozolomide	High
	Intracranial germ cell tumour	Ifosfamide	Medium
		Ifosfamide Cisplatin	High–very high
GCT	Extracranial GCT	None	Low
		Cisplatin	High
	Extracranial GCT (relapsed/refractory)	Ifosfamide + cisplatin + vinblastine	Very high
Bone and soft tissue	Ewing's sarcoma	Ifosfamide ± cyclophosphamide Busulfan Melphalan	Very high
	Osteogenic sarcoma	Cisplatin ± ifosfamide	High–very high
	Soft tissue sarcoma (low risk)	None	Low
	Soft tissue sarcoma (standard/high risk)	Ifosfamide	High–very high
	MMT	Ifosfamide ± cyclophosphamide	Very high
	Synovial sarcoma	Ifosfamide	High–very high
	'Adult-type' soft tissue sarcoma	Ifosfamide	Very high
	Neuroblastoma (low and intermediate risk)	Cyclophosphamide ± cisplatin	Medium–high
	Neuroblastoma (high risk)	Cyclophosphamide Cisplatin Busulfan Melphalan	Very high
Wilms tumour	Wilms tumour (low risk)	None	Low
	Wilms tumour (high risk/metastatic)	Cyclophosphamide + pelvic radiotherapy	High–very high
	Wilms tumour (relapsed)*	Cyclophosphamide ± melphalan	High–very high
Others	Hepatoblastoma	None	Low
		Cisplatin ± carboplatin	High–very high
	Retinoblastoma	None	Low
	Langerhans cell histiocytosis	None	Low
		Fludarabine + melphalan	High
BMT	Allogenic bone marrow transplant	Cyclophosphamide Busulfan/melphalan/ treosulfan**/TBI	High–very high
	Allogenic bone marrow transplant	Fludarabine	Low

Relative gonadotoxicity of common childhood cancer treatment regimens
Note that gonadotoxicity is estimated and absolute risk is dependent on exact regimen, dose and frequency used
Table adapted from CCLG Oncofertility Consensus Document (https://www.cclg.org.uk)
TBI total body irradiation, *GCT* germ cell tumours
*some relapsed Wilms not treated with gonadotoxic therapies (LOW)
**gonadotoxicity of treosulfan is uncertain

Clinical Cases with Practical Tips

Exposure to Cancer Treatment during Early Childhood

Testicular Germ Cell Tumour

A 1-year-old boy presented with a 1-week history of an enlarged left testicle. There were no history of pain and no overlying skin or colour changes. He was otherwise entirely well. On examination, he had a firm, enlarged left testis, with a volume of approximately 6 ml, compared to his right testis, which was normal in both consistency and volume (~1 ml). Examination was otherwise unremarkable and he had no dysmorphic features.

Initial imaging with ultrasound demonstrated an isoechoic mass within the left testis with markedly increased vascularity. Tumour markers were sent, and whilst his serum human chorionic gonadotrophin (hCG) was normal, his alpha-fetoprotein (AFP) was markedly elevated at 494ku/l. This was highly suggestive of a testicular yolk sac tumour. Further staging investigations demonstrated no evidence of disease elsewhere, and as such, he proceeded to left inguinal orchidectomy. This was performed uneventfully, and pathology confirmed this was a localised, pure, yolk sac tumour.

In view of this, there was no indication for further treatment at this stage and no indication for consideration of fertility preservation techniques, as we would expect his contralateral testis to function normally. He did, however, require regular follow-up, and reassuringly his serum AFP normalised following surgery.

However, 8 months after surgery, his serum AFP began to increase, and this was confirmed on serial measurements. Further restaging imaging demonstrated enlarged para-aortic lymph nodes consistent with stage 3 disease. As such, he required chemotherapy, with four cycles of carboplatin (2400 mg/m^2 total dose), etoposide and bleomycin (JEB). Germ cell tumours tend to be very chemo-sensitive and therefore his long-term prognosis is very good.

Discussion and Review of the Literature

At initial diagnosis, no cytotoxic treatment is required for localised testicular GCT, and therefore potential for fertility would be expected to be preserved providing the remaining testis was normal. However, the subsequent requirement for gonadotoxic chemotherapy means that future fertility may be impaired. There are no established methods for fertility preservation in prepubertal boys; however, testicular cryopreservation can be considered as an experimental approach for particular patients at high risk of future infertility [26]. This should only be performed within a robust research programme. For tumours requiring orchidectomy, cryopreservation of testicular tissue from the contralateral testis is not recommended in case any damage results in the single remaining 'normal' testis [24].

Platinum-based chemotherapy has been the principal agent used in the treatment of germ cell tumours for many years, with very good effect. In the UK, carboplatin is favoured over cisplatin, particularly in children under 11 years of age. Although carboplatin is particularly myelosuppressive, evidence suggests it is associated with less ototoxicity and nephrotoxicity in the long term, which is of particular importance to the paediatric age group [27]. Carboplatin is also felt to be less gonadotoxic than cisplatin, with the latter agent implicated as a risk factor for future impaired fertility, although a recent publication using murine model suggested carboplatin may be equally gonadotoxic as cisplatin in prepuberty [28], although human studies would be required to confirm the clinical relevance of these findings. Future fertility is an important consideration in patients with gonadal germ cell tumours, in whom orchidectomy or oophorectomy is required in addition to exposure to gonadotoxic therapies.

Exposure to Cancer Treatment during Childhood

Acute Lymphoblastic Leukaemia

A 5-year-old boy presented to A&E with a history of intermittent fever, lethargy, pallor and poor appetite. On examination, he looked pale and unwell and had multiple bruises on both bony and soft tissue areas with a palpable liver and spleen. His blood count showed pancytope-

nia and presence of blasts on the blood film. A bone marrow aspirate confirmed the diagnosis of pre-B acute lymphoblastic leukaemia (ALL).

The family were informed of the diagnosis and advised that their son would be eligible for 'standard risk' treatment as per the UKALL 2011 clinical trial. The effect of treatment on future fertility was discussed but not felt to be sufficiently high to warrant fertility preservation. At the end of the first month of treatment, the patient had a reassessment bone marrow. He was known to have 'good risk' tumour cytogenetics. However, the day 29 minimal residual disease (MRD) result showed inadequate clearance of the leukaemia. Treatment was intensified as per the clinical trial protocol. The effect of treatment intensification on future fertility was discussed, but again was felt not to warrant fertility preservation treatment.

Leukaemia treatment for boys treated on the UKALL 2011 protocol lasts for 3 years. At the end of treatment, the patient was well with no detectable leukaemia in the bone marrow or cerebral spinal cord (CSF). Four months after the end of treatment, the patient, now aged 9 years and still prepubertal, developed leg pains and petechiae. A blood film showed low platelets, and a bone marrow aspirate confirmed relapse of the leukaemia. The CSF was clear of disease. This pattern of relapse is considered to be 'high-risk' disease. The treatment plan for high-risk relapse disease includes re-induction chemotherapy +/− monoclonal antibody and a stem cell transplant with total body irradiation (TBI). This treatment carries a very high risk of infertility. Fertility preservation options were again discussed. The family was referred to a centre able to offer testicular tissue cryopreservation, and, after counselling, plans were made to collect testicular tissue after re-induction treatment and prior to transplant.

Discussion and Review of the Literature

Acute leukaemia is the commonest malignancy of childhood. In the UK, 1 in 2000 children develops the disorder, with around 650 new cases being diagnosed annually in the 0–24-year age group [29]. Eighty per cent of children diagnosed with leukaemia have acute lymphoblastic leukaemia [30].

Leukaemia arises from genetic mutations in blood progenitor cells in the bone marrow. These mutations lead to developmental arrest of the progenitor cells at a particular point in their differentiation and also confer on the cells the uncontrollable capacity for self-renewal [31]. The bone marrow becomes overwhelmed by immature cells known as blast cells which prevent normal cell production; the reticulo-endothelial system and other extra-medullary sites including the testes also become infiltrated with leukaemic cells that similarly affect organ function.

Overt leukaemia in the testis with a swollen testis at presentation is reported in about 2% of children [32]. However, subclinical infiltration of leukaemic cells into the testes occurs more frequently. In one published series, testicular biopsies taken at diagnosis in children with ALL contained leukaemic cells in up to 25% cases [33]. In adult testicular autopsy samples from patients who have died of leukaemia, but had no clinical evidence of testicular involvement prior to death, leukaemic infiltrates in the testis were found in 40–60% of patients [34].

Relapse of leukaemia with testicular involvement is a well-recognised pattern of disease [35]. The incidence of testicular relapse has declined with modern risk-stratified chemotherapy [36], but this should not obscure the evidence of subclinical infiltration of the testis at diagnosis and relapse, and this risk must always be considered when fertility preservation treatment is discussed, especially if tissue rather than sperm storage is considered.

Leukaemic infiltrates within the testis may disrupt the SSC niche leading to reduction in sperm quality and quantity. Production of mature sperm takes between 60 and 90 days, and therefore sperm found in semen within 3 months of treatment will have been exposed to chemotherapy. These sperm may be at significantly increased risk of aneuploidy [37], and DNA damage has even been identified in sperm up to 2 years post-treatment [38]. Therefore, if semen cryopreservation is being considered, it should be performed before treatment commences.

First-line treatment for 'standard risk' ALL has an event-free survival (EFS) of over 90%

[29], and whilst there may be some disease- and treatment-related effects on the testis [39], the vast majority of patients are expected to retain their fertility. However, the effect of cancer treatment on fertility should be discussed with all patients and their families before the start of treatment even when the risk of planned treatment is considered relatively low.

Fertility preservation treatment for prepubertal boys involves surgical removal of testicular tissue for storage of spermatogonial stem cells (SCC) [26], a treatment still regarded as experimental. The risks associated with fertility preservation in prepubertal boys diagnosed with acute leukaemia, namely, surgical risk and the presence of leukaemic infiltrates within the tissue limiting its future use, outweigh potential benefits as the risk of infertility with first-line treatment is low. If the index case had been a post-pubertal teenager, the risk/benefit of fertility preservation would have been in favour of storage of mature sperm because storage of sperm in an ejaculated semen sample is non-invasive and does not delay start of treatment, whilst treatment for leukaemia in teenagers can require more intensive treatment than that required in younger children. Fertility preservation in each case must be based on a risk versus benefit basis. As noted previously, the quality of the sperm and the ability to produce sperm in a semen sample may be affected by subclinical leukaemic infiltrates in the testis. If a post-pubertal boy cannot produce a semen sample containing sperm, the possibility of collecting sperm from the seminiferous tubules by testicular extraction of sperm (TESE) should be considered [40].

Further discussion about fertility preservation should take place at the time of relapse as the risk of infertility from subsequent treatment may be significantly increased. Conditioning treatment for a stem cell transplant for relapsed leukaemia involves high-dose alkylating agent chemotherapy and TBI with doses of radiation in excess of 10Gy. In male patients, testicular radiation doses as low as 0·1–1·2 Gy can impair spermatogenesis, with doses of more than 4 Gy often causing permanent azoospermia [41].

Following relapse, as at presentation, the risk of circulating blasts and testicular infiltration is high. Storage of testicular tissue for preservation of stem cells is the only fertility preservation option for prepubertal boys or if sperm is absent from semen samples. The timing of tissue collection in relapsed patients is an important consideration. In girls, where ovarian tissue is stored for future fertility and where there is more experience of use of the stored tissue, the recommended practice is to store tissue once the patient has achieved bone marrow remission prior to stem cell transplant [42]. A similar approach with collection of testicular tissue would seem appropriate where possible.

The storage of testicular tissue for cryopreservation of spermatogonial stem cells is still in the experimental stage of development, and to date, there have been no human babies born from stored tissue. Animal experiments confirm the viability of spermatogonial stem cells after cryopreservation, and in a number of species including non-human primate, there have been live births from use of stored testicular tissue [43]. Future use of testicular tissues stored from patients with leukaemia will require assessment of leukaemic infiltration and may restrict use of tissue to in vitro methods for sperm maturation and intracytoplasmic sperm injection (ICSI). These issues must be discussed in full with the patient/family prior to storage of any tissue.

The use of novel therapies in cases of relapse or in patients presenting with very-high-risk cytogenetics is becoming a more established part of treatment protocols. Currently, there is little long-term data to help predict the effect that these new treatments will have on the development of the testis and future fertility.

Exposure to Cancer Treatment during Childhood

Medulloblastoma

A 7-year-old boy presented with a 1-month history of headaches, which were initially occurring mainly after school but were associated with blurred vision and intermittent diplopia. However,

they worsened over time and became associated with vomiting. He subsequently developed a squint and was noted to have poor co-ordination. On clinical review, he was noted to have bilateral papilloedema, prompting urgent referral and imaging. This demonstrated a large posterior fossa mass with associated hydrocephalus. No metastatic disease was seen elsewhere in the brain or spine.

He underwent urgent neurosurgery, and complete tumour resection was achieved. Pathology demonstrated the tumour to be a WHO Grade IV, classic medulloblastoma. Lumbar CSF was negative, and he was therefore Chang Stage M0. In view of this, he required cranio-spinal radiotherapy and chemotherapy thereafter.

Radiotherapy was delivered as proton radiotherapy, and he received 23.4Gy in 13 fractions to his cranio-spinal axis and a tumour boost in 17 fractions to a total dose of 54Gy. Following recovery from radiotherapy, he received chemotherapy, with cisplatin 280 mg/m^2, lomustine 300 mg/m^2, cyclophosphamide 8 g/m^2 and vincristine. He completed treatment, and there was no evidence of recurrent or residual disease on follow-up imaging.

In view of the likely gonadotoxicity associated with his planned chemotherapy, fertility preservation was discussed with the family. As he was prepubertal, there were no established methods of fertility preservation, but following discussion, he underwent prepubertal testicular harvest before starting any cytotoxic treatment. This was performed uneventfully, and the surgical procedure was combined with his staging lumbar puncture and insertion of central line.

Discussion and Review of the Literature

It is important to recognise that in addition to the direct testicular insult from such chemotherapy, there will also be effects secondary to radiotherapy. He received proton radiotherapy, which is more focal than photons due to the nature of the proton beam and lack of 'exit' dose. This is in line with current recommendations to reduce radiation exposure outside the central nervous system, and as such, his testes did not receive any radiation, which is important as gonads are exquisitely sensitive to such damage. Radiation doses as low as 0.1–1.2Gy can have detectable effects on spermatogenesis in adult men, with doses over 4Gy causing a more permanent detrimental effect [44]. However, the entire central nervous system has been exposed to radiation, and this includes the hypothalamic-pituitary axis.

Cranial irradiation causes hypothalamic-pituitary dysfunction [45], with the resultant hormone deficiency dependent on the total dose of radiation received and the fractionation schedule [46]. In addition, age at the time of radiotherapy is important, with younger children more sensitive to radiation-induced damage [47].

Of the pituitary hormones, growth hormone (GH) tends to be the most radiosensitive, with doses as low as 18Gy causing GH deficiency. Subsequently, with increasing dose and time from treatment, deficiencies are seen in gonadotrophin, corticotrophin and thyrotropin secretion. As such, pituitary deficiencies are likely to be multiple and manifest rapidly and completely in younger children, in those receiving higher radiation doses and in those with tumours in close proximity to the hypothalamic-pituitary area. In contrast, those in whom the hypothalamic-pituitary axis receives a lower radiation dose may only develop single hormone deficits that evolve more slowly over time.

Patients receiving radiation doses to the hypothalamic-pituitary axis of 35–45Gy have demonstrated subsequent deficiencies in follicle-stimulating hormone (FSH) and luteinising hormone (LH) [46]. As discussed, the prevalence of gonadotrophin deficiency increases with time following radiotherapy. The clinical sequelae of gonadotrophin deficiency exhibit a broad spectrum of severity, from subclinical abnormalities detectable only by gonadotrophin-releasing hormone (GnRH) testing to a significant reduction in circulating sex hormone levels and delayed puberty. In the latter, exogenous testosterone may be used to induce puberty at an age-appropriate time, although this will not support subsequent fertility. Spermatogenesis may be induced in adulthood using hormone replacement regimens that include gonadotrophins (hCG ± FSH). Success of this approach will largely depend on

the degree of primary testicular damage caused by the chemotherapy exposure. Recent studies have investigated the utility of using gonadotrophins to induce puberty as an alternative to testosterone [3]. This may have the added benefit of promoting spermatogenesis although the regimens require multiple weekly injections, compared with 1–3 monthly for testosterone, and there is a lack of long-term follow-up data [3].

For young boys who receive a combination of cranial irradiation and gonadotoxic chemotherapy, long-term endocrinology follow-up is essential to monitor for central gonadotrophin deficiency, primary testicular failure or a combination of the two. This includes clinical examination of testicular volumes using a Prader orchidometer and biochemical assessment of LH, FSH and testosterone.

Exposure to Cancer Treatment during Peri-Puberty

Ewing's Sarcoma

A 12-year-old boy presented with a 3-month history of pain in his left leg. Initially the pain was thought to have been caused by a soft tissue injury sustained during a football match. The pain improved with rest but then over the next few weeks worsened to the point of waking him at night and preventing weight bearing on the affected leg. An X-ray of the leg showed a destructive lytic lesion in the metadiaphysis and diaphysis of the femur, with a prominent soft tissue mass extending from the bone. These X-ray features were in keeping with a diagnosis of Ewing's sarcoma of the proximal femur. The diagnosis was confirmed with subsequent biopsy. The patient underwent full staging investigations which showed no evidence of metastatic disease.

The patient and family were counselled that multimodal treatment was required and this would include high-dose cyclophosphamide and ifosfamide chemotherapy. This international treatment protocol has a cure rate around 70% for non-metastatic ES [29] but is known to cause significant long-term morbidities including a high risk of infertility (Table 1). Fertility preservation treatment options were discussed as part of the initial cancer treatment consultation. The patient was in early puberty with testicular volumes 6–8 ml. He was physically and psychologically too young to be able to store sperm from ejaculate. The patient and family were keen to explore other methods of fertility preservation. A referral was made to a fertility centre able to offer micro-TESE (testicular extraction of sperm) and storage of either sperm or testicular tissue. These procedures were discussed with the patient, and TESE was arranged at the same time as the insertion of a central venous catheter line required for chemotherapy. No sperm were retrieved using micro-TESE, but testicular tissue collection and cryopreservation was performed for storage of SSCs.

The patient has been followed up for 5 years post-treatment with no evidence of recurrence of the tumour. Reproductive function has been monitored as part of follow-up care, and this has demonstrated arrest of puberty. He had a low testosterone with elevated LH indicating Leydig cell failure. Testosterone replacement was commenced for completion of puberty and for long-term health. The testes remain small (~6 mls), and he has an FSH above the normal range, indicating Sertoli cell failure and germ cell loss likely to predict infertility.

Discussion and Review of the Literature

Ewing's sarcoma (ES), malignant peripheral neuroectodermal tumour (PNET), Askin tumour and atypical Ewing's sarcoma are part of a family of tumours known collectively as Ewing's sarcoma family of tumours (ESFT). ESFT can arise in any bone of the body or in soft tissues. ES is the second most common malignant bone tumour in adolescents and young adults, with high propensity to metastasise due to its aggressive behaviour. Extra-skeletal Ewing's sarcoma has been reported in many locations including the testis and scrotum [48].

From a biological point of view, Ewing's sarcoma family tumours (ESFT) are tumours of neural crest derivation that differentiate along a neuroendocrine lineage and are described as 'small round blue cell tumours'. All ESFT

tumours are characterised by a balanced chromosomal translocation between the EWS gene (22q12) and genes which are considered as members of the E26 transformation-specific (ETS) family of transcription factors (FLI1 (11q24) and ERG (21q22)) ([49, 50].

Over the last 20 years, multi-model intensive treatment protocols have led to a significant improvement in the survival rate for Ewing's sarcoma but at the cost of significant long-term morbidity. Ewing's sarcoma treatment involves chemotherapy with high-dose alkylating agents cyclophosphamide and ifosfamide, surgery and in some cases radiotherapy. Kenney et al. [51] reported male infertility rates of 66% following ES treatment which fits with data from the US Childhood Cancer Survivor Study (CCSS) [52], showing that male ES survivors are significantly less likely to report siring a child than unaffected male siblings (RR = 0.38, 95% CI = 0.24 to 0.59, $P < 0.001$).

Chemotherapy with alkylating agents, with or without radiation to sites below the diaphragm, has been associated with a fertility deficit in approximately 60% of men [53]. The duration and permanence of induced azoospermia depend on the dose of the cytotoxic agent and the additive effects of different agents. The effect of treatment on fertility and fertility preservation treatment options should be discussed with all patients at diagnosis, but this is particularly important where the risk of post-treatment infertility is significant.

Fertility treatment options available to a patient will depend upon the age and maturity of the child/young adult, their state of health at the time of diagnosis and access to available facilities able to offer treatment. In each case, the risk of infertility must be weighed against the risk of the fertility preservation treatment.

In adolescent males who have already entered puberty, the most established approach to preserving fertility is the cryopreservation of ejaculated mature sperm. Cryopreserved mature sperm can be used later in life for intrauterine insemination or in vitro fertilisation, with or without ICSI if azoospermia occurs post-treatment. In most post-pubertal males, the process of providing sperm for cryopreservation is effective, inexpensive and non-invasive. Mature sperm collected prior to cancer treatment will be free from any risk of micro-metastatic contamination. Collection of sperm must take place prior to the start of chemotherapy as sperm found in ejaculate after cancer treatment has started will have been exposed to the mutagenic effects of the chemotherapy which may have detrimental effects on the sperm DNA.

Silber et al. [54] demonstrated that sperm do not survive epididymal transit and do not reach the ejaculate if the human testis contains fewer than 3–4 million sperm. In boys with post-treatment azoospermia, surviving Sertoli cells and islands of spermatogenesis may exist, but the level of sperm production be too low to find sperm present in ejaculate. Similarly, in peripubertal boys who cannot produce an ejaculate pre-cancer treatment, sperm may be present within the testis but at a low concentration. Whilst there are no exact markers to define the time when sperm will be present in the testis, an early morning testosterone level and testicular volume may be useful in predicting presence of sperm within the testis. It may be possible, in such cases, to extract mature sperm from testicular tissue using a technique known as micro-TESE (microscopic testicular extraction of sperm). Micro-TESE under general anaesthetic involves opening the testis and inspection of the seminiferous tubules under the dissecting microscope. Areas of stem cell activity can often be identified and sperm within the tubules extracted and cryopreserved ([40, 55].

Ewing's sarcoma tumours are high-grade aggressive tumours characterised by a high metastatic dissemination potential [56–59]. Studies have shown that around 30% of patients had detectable evidence of metastasis at diagnosis [60] and a much higher percentage have circulating ES cells although no detectable micrometastases [61]. EWS metastases spread haematogenously to lungs, bones, bone marrow and in some cases germinal tissue [62]. It is essential that risk of testicular tissue containing metastatic contamination is discussed with patients and families prior to collection of tissue

and that robust tests are developed to enable detection of minimal residual disease (MRD) in germinal tissue prior to use. A recent publication by Chaput et al. describes use of EWS-FLI1 mRNA detection by qPCR as an accurate tool to assess EWS MRD in ovarian and testicular tissues cryopreserved by slow or vitrification freezing methods [63].

In conclusion, ES treatment poses a significant risk of long-term infertility in males. The detrimental psychological impact of post-treatment infertility is well documented, and fertility preservation treatment should be discussed with all patients prior to the start of cancer treatment. In each case, the risk of infertility must be balanced against the risks and benefits of storage of sperm or testicular tissue.

Exposure to Cancer Treatment during Adulthood

Clinical Case 6: Adult—Testicular Cancer

A 22-year-old male presented with a painless mass in the left testicle. Ultrasound scan of the testicle revealed a lesion within the testicular parenchyma. Orchiectomy was performed with regional lymph node *biopsy* and a CT scan of the pelvis. A pathological diagnosis of a pure seminoma testicular germ cell tumour (TGCT) was confirmed. Chemotherapy was commenced with three cycles of BEP (bleomycin, etoposide and cisplatin). The patient received counselling prior to commencing chemotherapy, and given the fertility risk of the proposed treatment, semen cryopreservation was undertaken to store sperm for future use for IVF/ICSI in the event that the patient was rendered infertile by their treatment. The patient recovered well from treatment, and no recurrence was identified on subsequent radiological monitoring. Follow-up of reproductive function demonstrated an increased FSH, indicating Sertoli cell failure and a low/normal testosterone with mildly elevated LH indicating compensated Leydig cell failure. Semen analysis 1-year post-diagnosis revealed azoospermia.

Discussion and Review of the Literature

Testicular cancer represents a risk to fertility not only as a consequence of the treatment required but also as a result of the primary diagnosis. Pre-treatment oligospermia is described in up to 50% of cases of TGCT [64, 65]. Indeed, the presence of testicular cancer, its precursor lesion or associated disorders are frequently identified in men undergoing assessment in infertility clinics. The prevalence of TGCT in patients with abnormal semen analysis has been shown to be 20 times higher than the general population [66].

TGCTs are often described as part of the testicular dysgenesis syndrome (TDS) disorders which consist of cryptorchidism, hypospadias, TGCT and infertility. TDS disorders are considered to have a developmental origin, resulting from events that occur in foetal life, with contributions from both genetics and environment [67]. TGCT arise from the transformation of pre-malignant germ cells termed germ cell neoplasia in situ (GCNIS). These pre-neoplastic cells result from the failure of differentiation of gonocytes to spermatogonia which usually occurs from foetal life to infancy [67]. GCNIS cells present in the testis become malignant TGCT in young adulthood [67]. The fact that these cancers can be considered a developmental disorder provides an explanation for abnormalities in the remainder of the testis. As a result, abnormal function and even the presence of GCNIS cells may also be present in the contralateral testis in a patient with TGCT. In a study of 218 men with testicular cancer, 24% were shown to have permanent impairment of spermatogenesis in the contralateral testis [68]. The increased incidence for TGCT to occur in the remaining testicle indicates the presence of GCNIS in contralateral testis at initial presentation and further supports the hypothesis of a developmental origin for these tumours. In some cases, biopsy of the contralateral testis may be indicated, and if GCNIS is detected, radiotherapy (or chemotherapy) may be required which may result in azoospermia [69].

In addition to the impact of TDS, the presence of a TGCT itself may negatively impact spermatogenesis and fertility. TGCT has been associ-

ated with an increased presence of anti-sperm antibodies (ASA) in 72% of patients [70]; however, a more recent study reported a much lower (5.8%) incidence of ASA in 190 patients with testicular cancer. Changes in temperature, local hormone production, blood flow or alterations in the blood-testis barrier may all contribute to alteration of the SSC niche with subsequent impacts on spermatogenesis.

The mainstay of chemotherapy treatment for TGCT involves regimens that include platinum (cisplatin or carboplatin) agents, often in combination with etoposide ± bleomycin. The platinum agents are considered to be the major contributor to gonadotoxicity in these regimens; however, inclusion of ifosfamide would be expected to increase gonadotoxicity [71]. Several studies have reported evidence of germinal epithelial damage, indicated by raised FSH levels and/or reduced sperm counts, in men receiving chemotherapy for TGCT [72–75]. However, distinguishing the testicular effects of the underlying pre-treatment condition from those of the treatments can be challenging. In an attempt to separate these factors, studies have compared pre-treatment reproductive function with post-treatment outcomes. In a study investigating 170 men with TGCT, 40 (24%) were reported to be azoospermic prior to treatment, and 54 (32%) were azoospermic a median of 30 months after platinum-based chemotherapy [76]. Furthermore, of the men who were normospermic prior to chemotherapy, only 64% remained normospermic after treatment [76]. The probability of recovery of a normal sperm count was found to be higher in the men who had received carboplatin instead of cisplatin and also in those that had received five cycles or more of chemotherapy [76]. Paternity rate has also been reported in a cohort of patients receiving chemotherapy for TGCT [72]. Paternity (natural conception or assisted reproductive technologies) was 10% at 5 years in the high-dose (>850 mg) cisplatin group, compared to 35% in those receiving low-dose (≤850 mg) cisplatin. In the high-dose group, there was evidence for long-term recovery of spermatogenesis with paternity rates increasing to 85% at 15 years post-treatment [72].

Semen cryopreservation is considered the most suitable option for fertility preservation in men with TGCC. However, this may not always be possible as a result of the patient condition. In patients who are unwell or suffering from impotence, this may not be an option. Another group of patients in which semen cryopreservation may not be possible are adolescents and young adults who may not be able to produce a sample due to psychological or physical maturity factors.

Novel Treatments for Childhood Cancer

In recent years, new technology has enabled a much greater understanding of the biology and pathological processes that underlie many adult and paediatric cancers. This new understanding has led to the development of new treatments targeted at cell surface antigens, upregulation of the adaptive immune system as well as use of agents that target key signalling pathways involved in tumour development and growth. Whilst these new agents potentially allow more ‘precision’ and a personalised approach to cancer treatment, their mechanism of action are often not confined to the target cancer cells alone.

Clinical long-term follow-up alongside basic scientific research is essential for a better understanding of the safety and efficiency of these novel therapies and the effect they have on testicular development. In addition, the need for pre-treatment fertility preservation must be continually assessed as more is learned about the effects of these agents on fertility.

Tyrosine kinase inhibitors (TKIs), such as imatinib, dasatinib and sorafenib, have been used with impressive outcomes in certain leukaemias [77], and crizotinib in anaplastic large cell tumours [78]. These agents have been shown to have off-target effects on a variety of endocrine systems leading to growth deceleration, alterations in bone mineralisation and abnormal thyroid metabolism, particularly when used in prepubertal patients. The mechanism for these off-target effects remains unclear, but it is possible that it is due to disruption of growth hormone signalling, inadequate signal transduction through insulin-like growth factor 1 (IGF-1) receptor (a tyrosine kinase receptor) or inhibition of platelet-derived growth factor receptor signalling and consequent

effects on macrophage colony-stimulating factor receptor (c-fms) [79]. The effect of these new treatments on the developing testis and future fertility is at the present time unclear. However, clinical data obtained from patients receiving these treatments, combined with advances in experimental approaches to understand the effects and mechanisms for testicular damage [80], should help to predict how these new treatments will affect the prepubertal testis and determine the need for fertility preservation treatment in children and young adults.

Take-Home Messages

Male reproductive function can be impaired following cancer treatment administered at any age. The risk of impairment of fertility is primarily dependent on the treatment that the individual will receive, but this risk will also be influenced by additional factors including the underlying diagnosis, presence of an underlying reproductive disorder and previous treatment. Central damage to the hypothalamo-pituitary unit results in impaired secretion of gonadotrophins, which may be treated with hormone replacement to induce or maintain secondary sexual characteristics and fertility potential. However, direct damage to the testis may result in loss of SSCs leading to azoospermia in adulthood. For adult males, semen cryopreservation prior to treatment may be possible; however, for prepubertal boys, the options for fertility preservation are limited to cryopreservation of testicular tissue harbouring SSCs which remains experimental. The risk of infertility in an individual can be estimated based on the planned treatment, although limited long-term follow-up data particularly in children make absolute predictions very challenging. In addition, cancer treatments are constantly evolving, and novel therapies are frequently emerging. The impact of new agents and regimens on future fertility in males should be a focus of ongoing research.

Definitions

Azoospermia

– Lack of sperm in seminal fluid.

Cryptorchidism

– A condition in which one or both of the testes fail to descend from the abdomen into the scrotum.

Gonocyte

– Undifferentiated germ cell present in the foetal and early postnatal human testis which differentiates into a spermatogonium.

Hypogonadotropic hypogonadism

– A condition involving low sex steroid levels (hypogonadism) associated with low levels of gonadotrophins, follicle-stimulating hormone (FSH) and luteinising hormone (LH).

Leydig cell

– A somatic cell type present in the interstitium of the testis that is able to produce testosterone.

Sertoli cell

– A somatic cell type present in the seminiferous epithelium that supports germ cell development and spermatogenesis.

Spermatogenesis

– The process by which haploid spermatozoa develop from spermatogonia in the seminiferous tubules of the testis.

Spermatogonium

– A pre-meiotic germ cell present in the prepubertal testis.

Spermatozoa

– A haploid post-meiotic germ cell capable of fertilising an oocyte.

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ART in Male Cancer Patients Including Sperm Donation

Luciano Negri, Pasquale Patrizio, and Paolo Emanuele Levi-Setti

Introduction

Chemo/radiotherapeutic treatments can adversely affect spermatogenesis by depleting spermatogonial stem cells. The extent of the gonadotoxic damage may range from complete loss of the spermatogonial stem cells, causing what is histologically known as Sertoli cell-only syndrome, to various degrees of hypospermatogenesis [1]. Since it is impossible to predict whether cancer Chemo/radiotherapeutic protocols may cause permanent azoospermia, sperm banking, possibly before starting cancer treatments, should be recommended to every male cancer patient interested in preserving future chances of fatherhood with their biological children [2].

When cancer treatments cause irreversible azoospermia, cryopreserved sperm can be used in conjunction with assisted reproduction techniques (ART). Male cancer survivors seem more likely to avail of assisted reproduction than the general population. Gunnes et al. [3] reported a relative risk of resorting to ART close to 3.32 (95% CI: 2.68–4.11), while Stensheim et al. [4] have observed that 6% of the first post-cancer pregnancies were initiated by ART (male controls 2%, $p < 0.001$). The choice of which ART procedure depends on the quality of the banked sperm after thawing [2, 5]. ART procedures with donor sperm like intrauterine insemination (IUI-D) or IVF with donor sperm are generally offered when patients failed to cryopreserve sperm before cancer treatment and no sperm are present in testicular biopsies. If testicular sperm extraction (TESE, micro-TESE) is successful, sperm can be used for intracytoplasmic sperm injection (ICSI) [6, 7].

An important observation is the low utilization rate of cryopreserved sperm; in fact, only about 8% of patients who cryopreserved sperm use their frozen samples for ART techniques [8].

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Plausible reasons for the low utilization rate are the low likelihood to marry and have children than the general population or for recovery of spermatogenesis or for lack of interest in having children [9–11]. Among 11,451 male adolescent and adult cancer survivors, 23% initiated at least 1 pregnancy after cancer compared to 32% among the males in the age-matched comparison group ($p < 0.001$) [4]. Since only a small proportion of patients discard their banked sperm (16%, 95% CI 15%–17%) [8], it is also possible that most cancer patients will plan to use their frozen sperm in the future. Therefore, it cannot be ruled out that the low worldwide usage rates may be underestimated, compared to the actual rate of final use. Another plausible explanation for the low use of cryopreserved sperm is the restoration of fertility after cancer treatments [12, 13]. For example, patients receiving high doses of alkylating agents for Hodgkin's lymphoma are very likely (~90%) to become irreversibly azoospermic, while for patients receiving ABVD cycles, the risk of permanent azoospermia is very low (0–4%) [14, 15].

In literature, the majority of ART treatments for cancer survivors relate either to the use of cryopreserved sperm for intrauterine insemination (IUI) or for conventional in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) and, to a lesser extent, to the use of surgically recovered testicular sperm (TESE or micro-TESE). Few data are available on the use of fresh semen after oncological treatments or donor sperm.

What Kind of Art?

The primary objective is to offer the couple an effective, low-complex, and low-cost ART procedures. Intrauterine insemination (IUI) is simple and affordable but requires sperm of good quality. Conventional IVF is more complex and expensive but can be used with borderline suboptimal sperm parameters or in the presence of female infertility and provides higher success rates than IUI. ICSI is more expensive than IVF, particularly if associated with TESE, but it is the most efficient option when sperm parameters (number, percent motility, and morphology) are very poor.

To decide what type of ART should be utilized, it is important to first assess the quality and quantity of the available sperm. The total motile sperm count (TMSC), which is obtained by multiplying semen sample volume by the sperm concentration and the percentage of type *A* and *B* sperm motility, divided by 100 is a good formula to decide what option to choose. *A* and *B* motility [16] corresponds to forward motility of 2010 WHO criteria [17]. TMSC appears more effective than the WHO classification system (volume, sperm concentration, motility, and morphology) in predicting spontaneous ongoing pregnancy [18] and ICSI outcome [19]. The TMSC before cryopreservation is also able to predict with a fair accuracy the recovery rate of motile sperm after thawing [20]. Of note, basal TMSC may be influenced by the type of cancer, being usually lower in lymphoid leukemia, myeloid leukemia [21, 22], and testicular cancer [5, 21, 23, 24]. However, oncological patients even before starting any type of cancer treatment have already lower TMSCs than those of healthy donors [25, 26]. Semen samples can be processed by procedures like swim-up [27] or density gradient centrifugation [28]. These different laboratory procedures allow to select and concentrate most of the motile and viable spermatozoa for future ART treatments [21, 29, 30]. Since it is not possible to predict with absolute precision the sperm motility and viability after thawing, i.e., whether the sample is suitable for IUI, IVF, or ICSI, generally a vial can be thawed and analyzed few days after the initial banking. This "thawing test" will provide a definitive indication on the quality of the sperm sample after cryopreservation and will allow a correct counseling to the couple [30–32].

Cryopreservation/thawing causes loss of sperm viability and decrease in TMSC in all semen samples [33, 34]. In general, either a count of five million TMSC after thawing or ten million TMSC after processing pre-freeze sample are used as a cutoff for IUI [35–38]. In the oncological population, the extent of loss of viability and TMSC after thawing seems also related to the type of cancer [21, 39]. Using post-thawing

TMSC >5 x 10^6 as threshold, Hotaling et al. [21] have shown that 63% of patients with myeloid leukemia, 59% of patients with testicular cancer, and 53% of patients with lymphoid leukemia achieve these numbers.

Maximizing sperm recovery is an important objective for oncological patients [40]. This can be achieved by collecting more semen samples [31, 41] and banking the largest number of vials. Sexual abstinence does not seem to significantly affect sperm quality before and after thawing Agarwal et al. [42] described similar post-thaw TMSC values with 1–2-day abstinence compared to higher abstinence time. Since the target is to allow the couple a simple and low-cost procedure as IUI and since, in young women, most pregnancies achieved with IUI occur during the first three to four treatment cycles [43, 44], it would be advisable to cryopreserve more than four vials, each one suitable for one intrauterine insemination, reserving those vials in excess for use with IVF or ICSI procedures [45].

Female reproductive health and age strongly influence the outcome of ART procedures [46–48]. Low ovarian reserve [49], endometriosis, and tubal factor are indications for IVF-ICSI procedures. Although female age is universally considered a prognostic influencer on ART outcome [47], it is noteworthy that most of the studies on ART in cancer survivors do not report the age of female partners (see Tables 1 and 2). Recent trends to delay childbearing are leading to a progressive increase of women's age even in partners of cancer survivors [64, 65]. For instance, IUI outcomes in non-oncological patients range from 38.5% for women under 30 to 26.3% for 36–40-year-old women and 12.5% for those over 40 ($p < 0.000001$) [48]. In ICSI procedures using fresh, thawed, or testicular sperm of cancer survivors, the maternal age is inversely related to the probability of full-term pregnancy [62, 65]. When the female partner is ≥40 years, the cumulative delivery rate/couple is 15.7% vs. 58.3% seen in younger women ($p = 0.0037$). In the same population, the mean female age for couples achieving pregnancy is 33.9 years (range 25.4–41) vs. 37.3 years (range 28.1–45.9) for couples not achieving pregnancy ($p = 0.0014$) [65]. The issue of cost for the ART procedure must also to be considered [66]. Sperm cryopreservation fees and annual storage costs are aligned worldwide [67, 68]. Usually, it can be completed for less than $1000. Storage fees vary, depending on the storage facility, but are in the $275–500 range per

Table 1 IUI outcomes in cancer survivors. Not reported (NR), clomiphene citrate (CC), human menopausal gonadotrophin (hMG), follicle-stimulating hormone (FSH)

Author	Couples	Female age	Cycles	Ovarian stimulation	Clinical PR/cycle	Live birth rate/cycle
Audrins et al. [50]	12	–	53	NR	2 (3.8%)	1 (1.8%)
Fitoussi et al. [12]	12	–	80	NR	–	2 (3%)
Kelleher et al. [51]	–	–	35	NR	11 (31%)	–
Lass et al. [52]	6	–	12	NR	3 (25%)	3 (25%)
Ragni et al. [53]	–	–	40	NR	3 (8%)	–
Chung et al. [26]	3	–	7	NR	0	0
Agarwal et al. [54]	–	–	42	*CC-hMG*	3 (7%)	3 (7%)
Schmidt et al. [55]	–	29.6	55	NR	8 (14.5%)	6 (11%)
Meseguer et al. [56]	–	30.2 ± 3.1	5	NR	1 (20%)	–
van Casteren et al. [57]	–	–	7	Natural cycle	1 (14%)	–
Crha et al. [58]	4	–	9	NR	2 (22%)	2 (22%)
Freour et al. [39]	31	–	66	NR	8 (12%)	8 (12%)
Bizet et al. [24]	–	–	39	NR	5 (13%)	4 (10%)
Žáková et al. [59]	–	–	6	NR	3 (50%)	1 (16.6%)
Botchan et al. [5]	22	–	81	*CC-FSH*	9 (11%)	8 (10%)
Muller et al. [60]	31	30 ± 5	108	NR	15 (14%)	14 (13%)
Total			*645*		*74/565 (13.1%)*	*52/558 (9.3%)*

NR not reported, *CC* clomiphene citrate, *hMG* human menopausal gonadotrophin, *FSH* follicle-stimulating hormone

Table 2 Conventional IVF results with cryopreserved semen in cancer survivors

Author	Couples	Female age	Cycles	Clinical PR/cycle	Live birth rate/cycle
Khalifa et al. [61]	10	32.6 ± 0.9	12	4 (33%)	3 (25%)
Audrins et al. [50]	11	–	11	5 (45%)	5 (45%)
Fitoussi et al. [12]	3	–	8	1 (12.5%)	0 (0%)
Kelleher et al. [51]	–	–	28	6 (21.4%)	–
Lass et al. [52]	6	–	14	2 (14.2%)	2 (14.2%)
Ragni et al. [53]	–	–	6	0 (0%)	0 (0%)
Chung et al. [26]	2	–	7	1 (14.2%)	1 (14.2%)
Agarwal et al. [54]	–	–	26	6 (23%)	5 (19.2%)
van Casteren et al. [57]	–	30.5 ± 3.9	32	8 (25%)	–
Hourvitz et al. [62]	–	33.4	54	–	13 (24%)
Bizet et al. [24]	–	–	7	2 (28.5%)	2 (28.5%)
Botchan et al. [5]	8	–	12	0 (0%)	0 (0%)
Depalo et al. [63]	19	34.7 ± 5.3	20	5 (25%)	5 (25%)
Muller et al. [60]	–	31 ± 4	79	33 (41.7%)	27 (34.1%)
Total			*316*	*73/262 (27.8%)*	*63/256 (24.6%)*

year. For IUI cycles, recent studies report cost ranges between 865 and 2623 US$; for IVF the price ranges are between 15,918 and 19,234 US$, while for ICSI they are between 17,544 and 25,000 US$ [68, 69]. While most European countries and Australia cover the costs for variable number of IVF-ICSI cycles including costs for medications, in many other parts of the world, patients must pay for ART treatments. In the United States, only 15 states have insurance mandates to provide coverage for the cost of ART [66]. Consequently, even a single IVF-ICSI cycle can be prohibitive for a person's average annual disposable income [70]. In addition, considering that estimated cost per successful outcome can range from US$ 10,696 for IUI with clomiphene citrate to US$ 19,566 for IUI with gonadotropins and US$ 61,377 for IVF [71], it can be concluded that IVF is unaffordable for patients at or below median income levels. Cancer survivors who failed to bank sperm prior to chemo/radiotherapy and are azoospermic must incur in an additional expense ranging between 8000 and 10,000US$ for micro-TESE [68].

Intrauterine Insemination (IUI)

IUI is the first choice for cancer survivors having cryopreserved sperm with at least five million TMSC after processing and thawing. It is a simple technique, accessible for most patients [72] as long as there is proof of tubal patency. IUIs can be performed in natural cycles or with ovarian stimulation using clomiphene citrate or human menopausal gonadotrophin (hMG) and follicle-stimulating hormone (FSH) [73]. With the use of ovarian stimulation, monitoring of follicular growth is necessary to avoid the risk of multiple pregnancies [74]. IUI with clomiphene citrate have a low risk for multiple pregnancies, but the live birth rates are lower than gonadotropins [75].

Analyzing IUI outcome from 16 studies performed in cancer survivors, the clinical pregnancy rate per cycle is 13.1% (74 out of 565 cycles) and the live birth rate 9.3% (52 out of 558 cycles) (Table 1) [5, 12, 24, 26, 39, 50–60]. It is worth mentioning that all available studies are retrospective and most do not report relevant information, as the number of patients, the female age, the use of ovarian stimulation, and the quality of sperm used. In the 123 IUI cycles reporting hormonal stimulation [5, 54], 11 births were recorded (8.9%). Therefore, it can be suggested that IUI outcomes in cancer survivors do not deviate from those with cryopreserved donor sperm in natural cycles [361 ongoing pregnancies out of 4269 IUI (8.4%)] [76]. However, when these rates are compared to IUI cycles performed with ovarian stimulation in donor sperm [252 clinical pregnancies (22.3%) and 214 birth

(18.9%) out of 1131 IUI cycles in 351 women using 100 mg clomiphene citrate +75 IU of FSH or hMG], they are lower [37]. Very similar results were described by other investigators [73, 77–80]. Although the available data are limited, IUI outcome seems less successful when using banked sperm from oncological patients.

The cost per delivery in non-oncological population range from 10,696 US$ for IUI with clomiphene citrate to 19,566 US$ for IUI with gonadotropins [69, 71]. No data are available on the cost per child in cancer survivors, but assuming a live birth rate per cycle about half than donor sperm stimulated IUI, the cost is likely higher.

Intrauterine Insemination with Donor Sperm (IUI-D)

The use of intrauterine insemination with donor sperm (IUI-D) is an appropriate therapeutic option for some cancer survivors. It is recommended for azoospermic patients without cryopreserved sperm and with no sperm even after attempted TESE or micro-TESE or for couples who cannot afford the cost of micro-TESE and ICSI procedure. The IUI-D is also indicated in couples who have repeated IVF-ICSI failures with their cryopreserved semen [81]. IUI-D has a birth rate per cycle ranging from 10.8% to 18%. In comparison, the IVF birth rate with donor sperm is 21.9%. The risk of multiple pregnancies can be as high 13% in IUI and 33% in IVF [82].

In unselected population, IUI-D live birth rates per IUI cycle range from 8.4% using cryopreserved donor sperm in the natural cycle [76] to 18.9%–21.3% using hormonal stimulation with letrozole or clomiphene citrate, human menopausal gonadotropins, or FSH in various combination [37, 80]. Clinical pregnancy rate is also associated with the number of cycles and the patient age. The cumulative pregnancy rate increases progressively from 23.0% to 26.5% [37, 80] after 1 cycle to 42.7%–61%, respectively, after ≥5 cycles. Referring to patient age, the clinical pregnancy rate per cycle ranges from 26.7% in patients 25–29 years old, 20.6% in patients 30–34 years old, and 17.1% in patients 35–39 years old. In women ≥40 years old, live birth rate is close to zero. When the IUI-D is performed in couples with azoospermia, the cumulative results with six cycles are higher than other male clinical indications (low total progressive motile count, antisperm antibodies, etc.), in both natural and hormonal stimulation cycles [73].

Cost-effectiveness analysis of IUI-D in cancer survivors is difficult to estimate because of few reports and the wide variance in price per donated sperm sample [83]. Today, in Europe, the cost of a vial ranges between 409 and 639 euros plus shipping costs of 300–500 euros. Buying three vials from a single donor, the cost rises to 2400 euros, and, if a couple wants to reserve a single donor for more than one pregnancy and request additional genetic screening, the cost (for ten vials) may approximate 24,100 euros.

In Vitro Fertilization (IVF)

IVF is an option after IUI failure [45] or when the TMSC is less than five million after thawing. Another indication is the availability of a limited number of vials and, therefore, the need to perform an ART procedure with high success rate. The TMSC cutoff values used to decide for conventional IVF are generally experience-based [45]. Suggested lower limits range from 0.2 million [84], 0.5 million [85], and one million [86]. The risk of fertilization failure is high when teratozoospermia coexists (< 5% normal form using strict criteria) [87]. Some authors suggest ICSI procedure when TMSC is less than one million [88].

Compared to IUI, conventional IVF guarantees higher clinical pregnancy and live birth rates per cycle, 27.8% and 24.6%, respectively, in line with results in non-oncological population (Table 2) [5, 12, 24, 26, 50–54, 57, 60–63]. Therefore, the cancer effect does not seem to impact IVF outcome. However, most of the data on IVF outcomes (13 retrospective studies reporting 316 cycles) show limitations

and imprecisions, not providing key information as the number of patients and the female age at the time of controlled ovarian stimulation. Compared to IUI, conventional IVF is an expensive and complex procedure [68, 69]. This aspect may be irrelevant for a European or Australian cancer survivor, being the procedure covered by the National Health Service, but it becomes burdensome for patients in the rest of the world because, frequently, the cost of IVF is not covered (estimated cost per successful outcome $61,377) [71]. Effect of woman's age, >38 years, nearly triples the cost per delivery compared with younger women [89].

Intracytoplasmic Sperm Injection (ICSI)

ICSI is indicated for the most severe forms of male infertility [90], ensuring the highest rates of fertilization. Therefore, it is the ideal option for cancer survivors with low number of cryopreserved sperm [51, 62, 91, 92]. Sometimes, ICSI is proposed as an alternative to IUI or conventional IVF, leveraging the higher fertilization rates, so that in countries where ICSI is guaranteed by the National Health Service or by health insurances, it is a common practice to use frozen/thawed sperm as a criterion for ICSI [40]. In general, ICSI is the most expensive of the ART procedures, but there are no cost-benefit studies in the oncology population.

The use of ICSI for cancer survivors is associated with good pregnancy and live birth rates (1023 cycles, 38.2% clinical pregnancy rate per cycle; 32.9% live birth rate per cycle) (Table 3) [5, 24, 26, 39, 51–58, 60, 62, 64, 65, 93–95], and, when calculated per couple (320 couples, 548 cycles), the live birth rate per couple reaches 58.1%. However, these data must be interpreted with caution for the absence of the female age in half of the studies [65]. According to the available data, the miscarriage rate is 19% (342 clinical pregnancies, 65 miscarriages, and 277 live births out of 841 cycles). These figures do not differ from the rates found in ICSI for male factor in the non-oncological population [96, 97].

Table 3 ICSI results in cycles using cryopreserved semen (*6 fresh sperm)

Author	Couples	Female age	Cycles	Clinical PR/cycle	Live birth rate/cycle
Kelleher et al. [51]	–	–	28	12 (42.8%)	–
Lass et al. [52]	3	–	6	4 (66.6%)	3 (50%)
Ragni et al. [53]	36	–	42	11 (26.1%)	–
Chung et al. [26]	1	–	3	1 (33.3%)	1 (33.3%)
Agarwal et al. [54]	–	–	19	7 (36.8%)	4 (21%)
Schmidt et al. [55]	–	29.6	49	19 (38.7%)	15 (30.6%)
Revel et al. [93]	21	33 ± 6	62	26 (41.9%)	18 (29%)
Meseguer et al. [56]	30	30.9 ± 2.9	30	15 (50%)	12 (50%)
Zorn et al. [94]	20	31.3 ± 4.3	31	7 (22.5%)	4 (12.9%)
Ishikawa et al. [95]	4	–	6	3 (50%)	3 (50%)
Hourvitz et al. [62]	118	34.8 ± 3.9	169	96 (56.8%)	85 (50.2%)
van Casteren et al. [57]	–	34.0 ± 4.1	53	16 (30.2%)	15 (28.3%)
Crha et al. [58]	28	–	38 + 6*	13 (29.5%)	9 (20.4%)
Freour et al. [39]	51	–	100 + 12IVF	26 (23.2%)	–
Bizet et al. [24]	–	–	71	23 (32.4%)	18 (25.3%)
Botchan et al. [5]	38	–	91	34 (37.3%)	23 (25.2%)
Garcia et al. [64]	29	36.7 ± 6	50	20 (40%)	18 (36%)
Muller et al. [60]	–	31 ± 5	101	47 (46.5%)	40 (39.6%)
Levi-Setti et al. [65]	28	37.2 ± 4.8	56	11 (19.6%)	10 (17.8%)
Total			*1023*	*391/1023 (38.2%)*	*277/841 (32.9%)*

Table 4 ICSI results in cycles using testicular sperm

Author	Number of couples	Female age	Number of cycles	Clinical PR/cycle	Live birth rate/cycle
Chan et al. [6]	9	–	9	3 (33.3%)	2 (22.2%)
Damani et al. [102]	15	–	26	9 (34.6%)	8 (30.7%)
Meseguer et al. [103]	5	33.5	8	1 (12.5%)	1 (12.5%)
Zorn et al. [94]	13	29.1 ± 1.6	39	9 (23%)	4 (10.2%)
Hibi et al. [104]	3	–	7	2 (28.5%)	2 (28.5%)
Hsiao et al. [101]	27	31.6 ± 4.9	36	18 (50%)	15 (41.6%)
Shiraishi et al. [105]	26	34.1 ± 2.9	58	7 (12%)	5 (8.6%)
Shin et al. [106]	31	–	–	23	18
Dar et al. [107]	12	29.8 ± 5.1	17	11 (64.7%)	10 (58.8%)
Levi Setti et al. [7]	30 *	36.7 ± 3.9	60	18 (30%)	12 (20%)
Levi Setti et al. [7]	18 **	35.3 ± 5.0	28	15 (53.5%)	10 (35.7%)
Total	*189*		*288*	*93/288 (32.3%)*	*69/288 (23.9%)*

. *Non-obstructive azoospermia. **Retrograde ejaculation/failure of emission

ICSI Using Testicular Sperm

When sequelae of oncological treatments result in persistent azoospermia and no prior sperm was cryopreserved, patients can be offered testicular sperm extraction (TESE) [98] or micro-TESE [99] with ICSI [6]. The ICSI procedure with micro-TESE is by far the most expensive (US$17,544–US$25,000 for ICSI + US$8000 to $10,000 for micro-TESE) [68, 69]. The rate of sperm recovery with TESE or micro-TESE in cancer survivors is around 46%, perfectly in line with the results in the non-oncological population affected by non-obstructive azoospermia (NOA) [100]. Not all forms of cancer benefit from successful sperm recovery [101], being higher in testicular cancers and lower in non-Hodgkin's lymphomas and leukemias. A summary of ten studies on this topic (288 ICSI cycles) (Table 4) [6, 7, 94, 101–107] reported a clinical pregnancy rate per cycle of 32.3% and the live birth rate per cycle of 23.9%, a rate like non-oncological NOA patients (live birth rate per cycle: 24%) [100]. However, miscarriage rates are higher compared to the use of cryopreserved sperm (25.8% vs. 19%). Perhaps, the high rate of miscarriage may be due to damage to the sperm DNA caused by chemotherapy/radiotherapy [108], but in none of the analyzed studies, specific tests were performed on sperm used for ICSI, and no data on female-related causes of miscarriage, such as female age [47], BMI [109], or polycystic ovary syndrome [110], are clearly reported.

Take-Home Messages

Over the last three decades, the improvement of assisted reproduction procedures has provided a wider range of options for male cancer survivors. In addition to intrauterine insemination and donor sperm inseminations, conventional IVF or ICSI can be proposed for cases with extremely low number of available sperm or when sperm is extracted from testicular biopsies.

Sperm cryopreservation before starting antineoplastic treatments plays a pivotal role for protecting future fertility. The choice of ART depends on the quality of the cryopreserved sperm and on the number of cryostored vials. Sperm samples of good quality and number may suggest the use of less invasive and costly procedures.

When sperm quality is sub-optimal or the number of vials banked is low (i.e., 3–4), conventional IVF or ICSI is recommended.

If no cryopreserved sperm is available and the cancer survivor became azoospermic, testicular sperm can be surgically retrieved with a success rates close to 50%. Testicular sperm used for ICSI produce success rates slightly lower than those of ejaculated cryopreserved sperm.

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Sperm Cryopreservation

Biljana Popovic Todorovic, Greta Verheyen, Veerle Vloeberghs, and Herman Tournaye

Introduction

Human spermatozoa were one of the first reproductive cells to be successfully cryopreserved. The idea goes back to the late eighteenth century when in 1776, Lazzaro Spallanzani, an Italian priest and physiologist, reported that when sperm were cooled down by snow, they became immotile [1, 2]. A century later Montegazza suggested that "a man dying on the battlefield may beget a legal heir with his semen frozen and stored at home" [1, 3].

In the late 1930s and early 1940s, it was observed that sperm could survive freezing to temperatures below −160 °C, although the survival was low without a cryoprotectant. In 1949, Polge et al. first reported on glycerol's unique ability to protect sperm cells being cryopreserved on dry ice, heralding the beginning of a new era in reproductive medicine [4]. The discovery of cryoprotectant sped up research in this area, namely, in animal and veterinary medicine.

The turning point in human sperm cryopreservation was the report of the first human pregnancy with frozen sperm in 1953 [5]. A technique for cryopreservation of human spermatozoa was introduced which showed that the sperm, after being frozen and stored in dry ice (−78 °C), were capable of fertilization and the subsequent induced development of normal progeny [3]. Yet, moral and legal issues hindered the use of this new technique, and 10 years later, the 11th International Congress of Genetics in 1963 marked the onset of human sperm banking [6].

A method for freezing human semen in liquid nitrogen vapour and its storage at −196 °C was introduced in 1963 and was followed by reports of normal births with its use [7]. The basic principles of the technique refined since 1963 have proven suitable for the establishment of clinical cryobanks, which have resulted in the birth of healthy offspring in various parts of the world.

The successful use of artificial insemination with donor and husband/partner semen in the early 1970s resulted in an increased use of sperm cryobanking [7].

Cryostorage of human spermatozoa is now an integral component in assisted reproductive technology (ART) procedures and particularly important in the preservation of fertility for cancer patients prior to chemotherapy or radiation therapy.

The field of sperm cryobiology has rapidly evolved with better understanding of sperm and their interactions with different types of cryoprotectants as well as tools for measuring post-thaw

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sperm parameters. The methodology for freezing needs to maximize post-thaw survival and sperm DNA integrity as well as the potential to create a viable embryo after in vitro fertilization IVF/ICSI. Lastly, the type of storage vessel also needs consideration and perhaps optimization for the number of sperm available.

Indications for Sperm Cryopreservation

Homologous and Donor Insemination

Sperm freezing is an established tool in the management of couples with infertility, and the scope of its clinical applications is widening rapidly. Its use developed firstly for storing donor sperm prior to intrauterine insemination (IUI) and later for in vitro fertilization (IVF) [8]. In fact, due to quarantine criteria, donor semen must be cryopreserved prior to insemination. Men must undergo rigorous clinical assessment prior to sperm donation [9]. Serum blood tests are performed to exclude viral infections such as HIV, hepatitis B and C, syphilis, human T-lymphotropic virus (HTLV) and cytomegalovirus (CMV). The donors also need to be tested for *N. gonorrhoeae* and *C. trachomatis* NAT on urine or a swab obtained from the urethral meatus. More recent outbreaks of viruses such as Ebola [10] and Zika [11] dictate that sperm bank policies must evolve constantly to ensure sperm donors are screened appropriately for viral pathogens.

Autologous sperm freezing is commonly utilized to provide a back-up ejaculate for patients with severe oligozoospermia or cryptozoospermia, undergoing intracytoplasmic sperm injection (ICSI). Storage, pooling and concentration of several oligozoospermic samples from one partner may provide a number of (progressively) motile cells for ICSI treatment.

It is also convenient to freeze sperm due to absence of the male partner as well as in the circumstances that patients may/have experienced anxiety-related anejaculation or unexpected azoospermia on the day of the procedure [3].

Donor banked sperm can be used as a back-up after preparing patients at counselling at the outpatient clinic in case no suitable sperm can be produced by the patient.

Cryopreservation Associated with Surgical Procedures

Cryopreservation of testicular spermatozoa can be performed at the time of diagnostic testis biopsy or therapeutic testis biopsy on the day of ICSI in azoospermic men with severe spermatogenic failure but with focal spermatogenesis or in men with irreversible obstructive azoospermia. This procedure may obviate the need for repeat invasive procedures. It also allows ovarian stimulation of the female partner to be timed and avoids the expense and frustration of having an ICSI cycle end unsatisfactorily with no sperm on the day of egg retrieval [12].

Sperm can also be cryopreserved before surgical infertility treatment, such as varicocele ligation in men with severe oligozoospermia, to protect against possible postoperative azoospermia in the event of testicular artery injury or ligation. Sperm can also be cryopreserved at diagnostic transrectal ultrasound with seminal vesicle aspiration prior to planned transurethral resection of the ejaculatory ducts (TURED). It provides insurance in case azoospermia ensues postoperatively [3].

Although it is not performed as commonly as initially anticipated, sperm can also be cryopreserved preceding vasectomy. This technique provides the opportunity for possible successful assisted reproduction in the future if circumstances change regarding the desire for progeny [3].

Sperm retrieval and freezing may be required in patients for whom ejaculation is not possible. Spinal cord injury (SCI) causes erectile dysfunction, anejaculation and reduced semen quality [13]. Abdominopelvic surgery including retroperitoneal lymph node dissection (RPLND), aorto-iliac reconstruction and colorectal excision may also cause anejaculation [14, 15]. Medical causes of ejaculatory failure include multiple sclerosis with an ejaculatory dysfunction rate of

50–75% [16] and diabetic neuropathy where retrograde ejaculation may be present in a third of patients [17]. Retrograde ejaculation due to surgery or diabetes may respond to sympathomimetic medications in a third of cases [18]; otherwise, surgical sperm retrieval for ART is required. Sperm freezing may be also performed prior to cytotoxic therapies for non-malignant conditions such as glomerulonephritis [19] and inflammatory bowel disease [20]. However, there is a paucity of data evaluating clinical outcomes following sperm freezing for these conditions.

Fertility Preservation

Malignancy is the major indication for sperm banking for preserving fertility. Testicular cancer, Hodgkin's lymphoma and leukaemia confer higher risks of male infertility/sterility when compared with other tumour types [21].

Cancer therapies, including chemotherapy and radiation therapy, can impair male reproductive function by directly damaging the germinal epithelium, causing oligozoospermia or azoospermia, or by damaging the neural pathways that regulate erection and ejaculation [22]. In addition, cytotoxic chemotherapy may also lead to transmission of genetic damage to children conceived after treatment [22]. The effect of cancer treatment on the incidence and severity of testicular dysfunction depends on various factors, including type of therapeutic regimen and cumulative dose [23].

Rates of recovery of spermatogenesis are highly variable and dependent on cancer type, treatment modality and underlying testicular function, but even highly gonadotoxic treatment for testicular cancer can see recovery of spermatogenesis in 50% of patients after 2 years [24]. The Childhood Cancer Survivor Study demonstrated that 46% of survivors of childhood cancer reported infertility when compared with 17.5% of their siblings [25].

As it remains difficult to predict, on an individual basis, whether permanent sterility will ensue after cancer treatment, it is recommended to offer semen cryopreservation to any patient before embarking on cancer treatment [26, 27]. In addition, decrease in sperm parameters and increase in DNA damage have been observed before cancer treatment [28]. Moreover, a patient's knowledge that his fertility can be secured through sperm cryopreservation may help in the emotional battle against cancer [29].

Until the early 1980s, sperm freezing before cancer therapy was useless if density and motility were drastically impaired, as artificial insemination was the only fertility treatment option. Although pregnancy chances increased and sperm quality criteria decreased with the development of IVF in the early 1980s, it is only since the introduction of ICSI that patient eligibility for cryobanking substantially increased and that banking of one single semen sample may be sufficient.

Premortem and Postmortem Sperm Cryopreservation

The retention of fertilizing capacity of a partner or donor in his temporary or permanent absence is possible with the use of cryopreservation [7]. Sperm can be cryopreserved prior to entry into military service or anticipation of toxin exposure. Pregnancy with ICSI using sperm from a deceased partner has also been achieved [30]. The postmortem use of frozen sperm to attempt to achieve pregnancy has been available for patients with cancer since the commencement of sperm cryopreservation, but only recently has assisted reproduction technology provided a realistic chance of success for those in need. Although sperm retrieval from deceased or incompetent individuals may be achieved readily, it is incumbent on the practitioner to consider the legal and moral implications of these procedures before proceeding [31].

Sperm Banking for Transgender Reassignment

Meta-analysis of prevalence studies in transsexualism has shown that the prevalence of transgen-

der individuals is 4.6/100,000 in the populations studied [32]. Nearly three times as many individuals undergo male-to-female reassignment ("trans women") when compared with female-to-male reassignment ("trans men"). Trans women are treated with oestrogen therapy with or without bilateral orchiectomy, which would render them temporarily or permanently infertile. However, transgender individuals often desire and expect to conceive in the future with appropriate fertility treatment [33]. For this reason, the Endocrine Society recommends that fertility counselling should be offered to patients undergoing gender reassignment [34]. Furthermore, the ethics committee of the ASRM recommends offering the option of gamete freezing to all individuals undergoing gender reassignment [35]. However, further studies by health professionals are required to explore how effectively sperm banking is delivered and utilized for ART by patients. In conclusion, sperm banking is emerging as an important element of gender reassignment therapy; however, many clinicians have limited knowledge of this novel therapeutic pathway, and further work is needed to develop clinical pathways to treat individuals seeking treatment.

Semen Sample Production

The simplest method of producing a semen sample for sperm banking is masturbation; however, other techniques are available if this is not possible. Disposal penile vibratory stimulation devices are noninvasive and simple [36].

Electro-ejaculation may be required in cases associated with disruption of the ejaculatory reflex arc such as in spinal cord injuries [37], yet requires general anaesthesia whenever a complete spinal cord injury is not present.

Surgical sperm retrieval techniques such as percutaneous epidermal sperm aspiration [38] and testicular sperm extraction may be useful when a low sperm yield is anticipated, as in cases of azoospermia [12].

Table 1 summarizes methods for sperm collection as well as selection of sperm for cryopreservation and storage (with permission from [39]).

TESE testicular sperm extraction, *TESA* testicular sperm aspiration, *MESA* microsurgical epididymal sperm aspiration, *PESA* percutaneous epididymal sperm extraction, *MACS* magnetic activated cell sorting, *ICSI* intracytoplasmic sperm injection.

With permission from [39].

Sperm Storage Consent

The legal framework defining the safety and quality standards for tissues and cells is set out in Directive 2004/23/EC, also referred to as the European Union Tissue and Cells Directive, adopted in 2004 by the European Parliament and Council. It covers all steps in the process from donation to distribution, including procurement, testing, processing, preservation and storage.

All patients who are cryopreserving sperm need to sign an informed consent. The laws

Table 1 Individual methods for sperm collection and selection of sperm for cryopreservation and storage

Collection methods	Selection options	Storage options Biological carriers	Non-biological carriers
Masturbation	Pre-freeze swim-up	Empty zona pellucida	Straws
TESE	Density gradient	Volvox globulator spheres	ICSI pipette
Micro-TESE	MACS		Cryoloop
TESA	Zeta potential selection		Microdroplet
MESA			
PESA			
Open epididymal fine-needle aspiration			
Microsurgical testicular sperm extraction			

regarding the storage of storage of gametes are often country dependent, but in general, all informed consents contain information on the length of storage period which is limited to 10 years but may be extended (HFEA). In the case when one of the partners is prematurely infertile or is likely to become prematurely infertile, the storage can be prolonged up to the age of 55 years (HFEA). In addition, the consent form needs to have information of what happens to gametes in case the patient dies or loses the ability to decide for oneself (i.e. becomes mentally incapacitated).

The European Union Tissue and Cells Directive provides recommendations regarding information and consents for the gamete/sperm donors. The information must cover the purpose and nature of the procurement and its consequences and risks; analytical tests, if they are performed; recording and protection of donor data; medical confidentiality; therapeutic purpose and potential benefits; and information on the applicable safeguards intended to protect the donor. The donor must be informed that he has the right to receive the confirmed results of the analytical tests, clearly explained. Information must be given on the necessity for requiring the applicable mandatory consent, certification and authorization in order that the tissue and/or cell procurement can be carried out.

Cryopreservation Methods

The fact that the spermatozoa are of small size and are relatively abundant allowed for the development of effective freezing protocols since smaller structures are less susceptible to damaging ice crystal formation during cryopreservation.

Sperm cryopreservation protocols can be divided into three general types: (1) slow programmed freezing, where temperature is slowly decreased in a stepwise manner using a programmable cell freezer; (2) vapour freezing, where the sperm are exposed to liquid nitrogen vapour at different levels above the liquid nitrogen surface, followed by immersion into liquid nitrogen [40]; and (3) vitrification, where the sperm specimen is quickly plunged into liquid nitrogen [40].

The major steps in the process of cryopreservation and warming can be summarized as follows: (1) addition of cryoprotectant agents (CPAs) to sperm/testicular tissue before cooling; (2) cooling of the mixture towards a low temperature followed by plunging in liquid nitrogen (−196 °C) in which the material is stored; (3) warming the sperm/tissue; and (4) removal of the CPAs after thawing.

The slow freezing protocol was the first method used to successfully freeze spermatozoa and remains the most commonly used technique worldwide [41]. The initial protocols consisted of two to three steps and on average took 2–4 h to complete [42]. Firstly, spermatozoa accumulated by ejaculation or other techniques were kept at room temperature for 10 min. Secondly, samples are gradually frozen from 20 °C to 5 °C at a rate of 0.5–-1 °C/min and then cooled from 5 °C to −80 °C at rate of 1–10 °C/min. Finally, samples are plunged into liquid nitrogen [43]. Nowadays, programmable freezing machines contain different programmes (long and short). The choice of a programme by the clinic must be preceded by the validation process.

Fast vapour freezing is based on a direct contacting of samples to liquid nitrogen vapour for 10–15 min [44]. The temperature drop cannot be as precisely controlled, leading to variation in cooling temperatures and reproducibility from sample to sample.

Both the slow freeze and vapour freeze techniques yield a cryosurvival rate of 40% to 60%, with higher cryosurvival seen in normozoospermic samples as opposed to compromised samples from subfertile men. It is not clear whether any one method is superior. With the vapour freeze technique, the temperature drop cannot be as precisely controlled, leading to variation in cooling temperatures and reproducibility from sample to sample. However, in contrast to the slow programmed method, during vapour freezing, sperm are cooled at a faster rate with shorter duration of exposure to cryoprotective agents, which can reduce sperm damage.

The third method of sperm cryopreservation includes vitrification which is a process that solidifies liquid into an amorphous or glassy state [45]. With this technique, cells are cryopreserved at ultrarapid cooling rates of over 1000 °C per minute. The idea is to avoid a phase change and thus the potential for damage by ice crystal formation. Currently, vitrification is the most commonly used technique in the preservation of oocytes and embryos, which requires addition of high concentration of permeable CPAs [45]. However, vitrification for sperm cryopreservation has not been as widely applied.

Despite the fact that data suggest superiority of vitrification to the traditional slow freezing [46], the debate on the preferred method of sperm cryopreservation is still ongoing. Pregnancy outcome data are needed to further validate this technique.

Cryoprotectants

An important aspect of the cryopreservation process is the selection of appropriate cryoprotectants. During the freeze-thaw process, sperm are exposed to a number of non-physiologic environments and subjected to both osmotic and oxidative stress. This may negatively affect sperm function, ultimately impairing the sperm's fertilizing capacity and ability to generate a viable embryo. However, in order to prevent intra−/extracellular ice crystal formation, cryoprotective agents are mixed with the sperm specimen prior to freezing.

The cryoprotectant is not added as a pure substance, but as part of a buffered solution, called cryoprotectant medium (CPM), containing permeating and non-permeating cryoprotectants and other compounds.

Slow programmed freezing and fast vapour freezing, the so-called conventional freezing methods, involve the use of CPAs to minimize osmotic damage and to minimize intracellular and extracellular ice crystal formation [46, 47]. There are two categories of CPAs: one is permeable CPAs including dimethyl sulfoxide, glycerol, glycol, ethylene and methanol, and the other is non-permeable CPAs including albumins, dextrans and egg yolk citrate [48]. It is well known that permeable CPAs stabilize the plasma membrane and non-permeable CPAs minimize the intracellular ice formation. However, in most cases, non-permeable CPAs play a supporting role that augments the effectiveness of permeable CPAs [49].

To date, glycerol is one of the most successfully used permeable cryoprotectants. Glycerol easily traverses the plasma membrane and can slowly equilibrate within sperm cells to reduce intracellular fluid volume and also provide an osmotic buffer for intracellular solutes.

In order to improve cryosurvival, the cryoprotectant buffer is enriched by compounds referred to as extenders. Widely used buffers are the combination of glycine and citrate [50], the combination of zwitterionic buffers TES-TRIS (TEST) [51] or HEPES [52]. Egg yolk is a common extender because of its protective effect on freezing damage by increasing membrane fluidity. Prins and Weidel [53] compared eight buffer systems with and without glycerol as cryoprotectant and observed the highest post-thaw motility with the use of TESTCY (TES-TRIS citrate egg yolk buffer) containing glycerol. Recently, little interest is shown, and little progress is made in optimizing sperm freezing media, which are still based on old formulations. Bearing in mind strict regulations and quality assurance as imposed by the European Directives for Cell and Tissue Banks, ART centres no longer prepare cryomedia in house, but obtain ready-prepared media from commercial companies who subject their products to stringent quality assessment. Accordingly, the use of xeno products as egg yolk is avoided by different centres.

Use of permeable cryoprotectants, however, has also been associated with damage to sperm acrosomal membrane and nuclear integrity. Investigators have proposed that the high concentration of proteins/sugars and minimal fluid volume within sperm create a natural viscous intracellular matrix, eliminating the need for chemical cryoprotectants. Very promising results have been achieved with cryoprotectant-free sperm vitrification using only non-permeating

sugars such as sucrose along with human serum albumin (HSA) [54–56].

In order to counteract osmotic injury, it is advised to add the CPM rather slowly. Addition of a permeating cryoprotectant with high osmotic pressure initially causes dehydration and shrinkage of the cells due water efflux, followed by restoration of the volume due to influx of the cryoprotectant. Dropwise addition avoids abrupt shrinkage on the one hand. On the other hand, however, the exposure time to glycerol at room temperature should be minimized in order to limit cryoprotectant toxicity. Royere et al. [57] advocated a slow addition period within 10 min, while others showed acceptable loss in motility and viability after multistep addition of glycerol during 4 min with 1-minute intervals [58, 59]. More research is needed in order to find the optimal balance between minimizing hyperosmotic injury and reducing the chemical toxicity of the cryoprotectant.

Post-thaw removal of the cryoprotectant by dilution should equally be performed gradually. After initial swelling of the cells by influx of water, the cryoprotectant leaves the cells, after which a new equilibrium is reached. According to Morris et al. [60], there is no convincing evidence of intracellular ice formation in spermatozoa. In contrast with other cell types, spermatozoa show limited shrinkage or swelling due to a small volume of cytoplasm, which renders them relatively resistant to osmotic shocks.

To Wash or Not to Wash?

Sperm can be cryopreserved within the seminal plasma, i.e. for unwashed sperm, or it can be carried out after washing. The outcome of one method vs. the other is still a subject of debate. Proponents of freezing native semen claim that it allows the antioxidant protective effect of seminal plasma to guard against reactive oxygen species [61, 62]. While some researchers have reported better results with washed sperm [63], others have found no significant differences between freezing native and washed sperm samples [64].

The Packaging Container

Different aspects determine the efficiency of sperm freezing. The choice of the cryoprotectant, the cooling/freezing and warming rate and procedure, the packaging container and the storage conditions are important issues in order to guarantee optimal survival rates.

The optimal packaging container must fulfil a number of requirements. (1) It should be easy to use in terms of handling and labelling. (2) It should provide a large surface-to-volume ratio to allow uniform cooling or warming. (3) It must be resistant to extremely low temperatures, with minimal risk for breakage. (4) It must be safe, sterile and well sealed before storage in liquid nitrogen to avoid the potential risk of cross-contamination. (5) It should allow efficient and economic storage in liquid nitrogen tanks.

Different types of containers have been used in the past and up to now. The two main categories are straws and vials. Borosilicate glass vials or ampoules are nowadays completely abandoned because of their fragility and risk of post-thaw explosion. Screw-capped polypropylene cryovials are still widely used, especially in the United States, although they do not fulfil several of the above requirements. As well illustrated by Mortimer [65], the heat exchange is suboptimal as a temperature gradient is created towards the centre of the vial during the cooling or the warming process. Moreover, the screw cap does not provide effective sealing [66] and allows liquid nitrogen to enter into the vial with the potential risk of explosion at increasing temperature upon thawing [26] and the potential risk of contamination [67].

Straws have several advantages over vials regarding optimized heat exchange and economic storage. Until the late 1990s, plastic polyvinylchloride (PVC) straws, originally manufactured for animal use, were widely used for human sperm cryopreservation. Notwithstanding, they have been withdrawn for human use for different reasons. They were provided non-sterile and bulk packed. The material of the straw did not allow sterilization by irradiation without the risk of damage. Sealing was performed by use of

plastic spheres or plugs or by filling the open end with polyvinyl alcohol powder (PVA), which polymerizes upon contact with moisture. These plugs, however, were often ejected upon thawing or did not always provide effective sealing. Moreover, the PVC material is not resistant to mechanical shocks at the low temperature of liquid nitrogen which renders them very rigid and susceptible to breakage during manipulations in liquid nitrogen, increasing the risk of contamination. In the 1990s, these PVC straws have been replaced by high-security straws (Cryo Bio System CBS, France). These straws have significant advantages and fulfil all the above criteria. They are composed by ionomeric resin which has the benefit of mechanical resistance to ultralow temperatures. High-security straws are impermeable to viruses and bacteria and can easily and safely be heat-sealed using a special heat seal device. If properly sealed, microorganisms do not contaminate clean samples in the same tank, as extensively shown by Bielanski et al. [68]. There has never been evidence so far of any cross-contamination in the cryobank of a fertility clinic [26]. Furthermore, high-security straws are sterile-packed and provided with filling nozzles which allow clean handling and filling and creation of an air bubble for expansion during cooling. Identification labelling can securely be performed inside a separate compartment of the straw or by using sleeves or adhesive labels on the external surface of the straw. Because of its comprehensive properties for cryopreservation of human sperm, high-security straws have been approved for human application by the US Food and Drug Administration and are in compliance with the European Union Tissue and Cells Directive imposing strict regulations on safe identification and traceability.

The Cooling/Freezing Method

Stepwise or Gradual Cooling

Conventional cooling/freezing procedures for sperm can roughly be divided into two categories: static or stepwise vapour cooling and gradual computer-controlled cooling. Medeiros et al. [69] described optimal cooling rates for human sperm to be between 1 °C and 10 °C per minute. According to Mortimer [65], human spermatozoa are considered insensitive to cooling rates within the range of 1–25 °C/min. Suboptimal conditions regarding CPM formulations may, however, mask the important impact of the cooling rate.

Static vapour cooling has been introduced by Sherman in 1954 and widely used since then [70]. This procedure can only successfully be applied when sperm is packed in straws which are horizontally placed in liquid nitrogen vapour in order to guarantee uniform cooling rates along the length of the straw. Individual straws should be placed in a monolayer at the same level above the nitrogen surface and should not be collected in tubes during the cooling process as a temperature gradient will be created among the inner and outer straws. According to the diameter of the straws, one- or two-step cooling is preferred [71].

Computer-controlled freezing gained interest in the 1980s aiming to standardize the technique and increase the reproducibility of sperm freezing efficiency. While some groups observed that controlled freezing preserved sperm motility and viability better than vapour freezing [72–74], other investigators did not prove its advantage [43, 75] and advocated vapour freezing because of lower cost, time and practical implications. Recent research in order to optimize cooling rates for human spermatozoa is rather scarce which may be associated with the lower requirements for sperm quality and numbers if used for ICSI. Nowadays, different types of easy-to-use programmable freezers are commercially available, and companies provide optimized cooling programmes for both gametes and embryos.

Aiming for standardization and reproducibility, also for semen samples of extremely poor quality, more and more laboratories switch from vapour to computer-controlled sperm freezing.

Ultrarapid Cooling/Vitrification

Although slow or moderate to rapid cooling in the presence of glycerol as cryoprotectant has

been the method of choice for sperm freezing during several decades, other technologies gained more interest in order to increase cryoefficiency. Vitrification is solidification without ice formation resulting in the creation of a glass-like, i.e. vitreous, structure. The first successful results of vitrification of frog spermatozoa go back to 1938 [76]. However, it is only since 2002 that human sperm vitrification has been reconsidered for use in clinical sperm cryobanking.

Vitrification is the preferred method of oocyte and embryo freezing and requires addition of high concentration of permeable CPAs [45]. Spermatozoa cannot be frozen in the same mode due to the higher osmotic fragility [77].

The use of permeable cryoprotective agents, e.g. glycerol, ethylene glycol, propylene glycol and DMSO, in high concentrations is needed in order to ensure equilibrium intracellular vitrification [54]. However, the high concentrations of permeable cryoprotectants appeared toxic due to lethal osmotic imbalance and thus are not applicable for successful sperm vitrification. Nawroth et al. [78] introduced a new vitrification protocol involving rapid non-equilibrium freezing and thawing rates avoiding the use of permeable cryoprotectants and as such was named cryoprotectant-free vitrification (CPA-free) [78]. Direct plunging into liquid nitrogen of swim-up prepared sperm followed by very rapid warming showed higher motility than slow freezing with cryoprotectant and was more efficient than vitrification of native semen [78].

Recent data suggest that vitrification results in low overall DNA damage while maintaining sperm membrane integrity, mitochondrial activity and post-thaw survival rates [54, 56].

A number of pre-cooling techniques, carrier tools and thawing methods have been introduced to improve the efficacy of the sperm vitrification protocol [79], yet the results of studies on efficacy of sperm vitrification are conflicting [46].

Studies have shown that refinements in vitrification protocols specific to spermatozoa have eliminated the need for cryoprotectants, improving post-thaw vitality, motility and acrosomal preservation, as well as reducing the incidence of DNA fragmentation [80]. It may be possible that the vitrification could eliminate cryoprotectant-mediated (osmotic) damage.

Implementation of cryoadditives may improve efficacy. Antioxidant quercetin, catalase and brain-derived neurotrophic factor (BDNF) have been shown to produce improvements in post-thaw viability, motility and DNA damage [81–83].

Although a recent meta-analysis by Li et al. [46] concluded that vitrification is superior to conventional freezing methods in the preservation of spermatozoa, regarding total and progressive motility, the results need to be interpreted with caution. The efficacy of vitrification was limited by a small number of studies using different vitrification protocols and cryopreservation of different quality spermatozoa [46].

How to Store Sperm?

Storage Temperature

In order to stop all biological activity, sperm should be stored below −132 °C, the glass transition temperature of water [84]. Storage at higher temperatures as high as −80 °C (ultra-cold refrigerator) or − 79 °C (dry ice) for an extended period of time allows continuation of biological activity and degradation of sperm quality, manifested as a progressive decline in post-thaw motility [85]. Therefore, liquid nitrogen with its temperature of −196 °C has been the preferred storage surrounding for cells and tissues. During the last decade, several sperm banks and IVF centres switched to storage in liquid nitrogen vapour (−150 °C) in order to avoid direct contact with liquid nitrogen and counteract the potential risk of cross-contamination through liquid nitrogen, especially if open packaging systems are used (common for vitrification of oocytes and embryos). Although the quality of the frozen material is maintained at −150 °C, vapour storage is considered more critical when specimens are manipulated and concerns are raised regarding failure of automated filling systems. As long as temperatures as low as −132 °C are guaranteed, no problems are expected. Shipment of donor sperm by commercial sperm

banks is commonly performed in dry shippers which can support safe storage over several days, due to saturation of the inner wall with liquid nitrogen.

Storage in liquid nitrogen or in its vapour is still a subject of debate nowadays. The pros and cons of both systems should be weighed within each individual setting, based on the applied freezing methodologies. If properly closed straws/vials are used, storage in liquid nitrogen may be considered advantageous in terms of convenience and safety.

Storage Tanks and Facilities

The organization of the sperm bank highly differs from one unit to the other and largely depends on its size. Licensed commercial sperm banks have well-organized storage facilities according to accreditation criteria and provide safe freezing and storage. They use large dewars with a transparent inventory system, automatic nitrogen filling and continuous day/night monitoring of each individual tank by low-level liquid nitrogen or temperature control. In smaller sperm banks, however, different types of smaller storage tanks may be used. It is common practice to fill tanks manually, which is no problem if performed on a regular basis. Whatever the size of the bank, an alarm system using correctly installed low-level sensors or temperature sensors is advocated. The lifetime of a storage container is unpredictable, and failure can occur before or after the warranty period given by the manufacturer. In the case of alarm due to leakage, the specimens can be replaced in a "spare" tank before the quality of the material is compromised.

Vapour storage implying critical liquid nitrogen levels requires automatic filling and a conscientious monitoring system, which increases the investment cost and running cost of the installation. Therefore, a high proportion of small facilities continue storage in liquid nitrogen.

Recently, an automated robotic cryo-specimen management system designed specifically for in vitro fertilization (IVF) has been introduced [86].

How to Thaw Sperm?

Although sperm thawing is an important step, this procedure received less attention than cooling. Thawing of sperm at room temperature or at 37 °C seemed to preserve sperm quality [87, 88] and fertilizing ability [89] better than slower (in ice baths) or faster thawing (in warm-water baths). According to other groups, the optimal warming rate is dependent on the cooling rate [49, 75], slow cooling requiring slow thawing and vice versa. Even if sperm is frozen in a programmable freezer, thawing is generally performed manually in one or two temperature steps.

Pre-Freeze and Post-Thaw Preparation of Sperm

Pre-freeze and post-thaw preparations are not independent and are related to the type of ART treatment used. Ejaculated sperm is generally frozen without pre-freeze preparation, after addition of the CPM. Intra-cervical insemination (ICI) with unprepared semen after thawing is outdated and largely replaced by intrauterine insemination (IUI) with washed sperm because of higher success rates of the latter. In order to avoid uterine contractions after IUI, thawed semen is washed to remove seminal plasma and CPM compounds such as egg yolk. Sperm washing for IUI, IVF or ICSI is now commonly performed by density gradient centrifugation which enriches the final insemination fraction in terms of proportion of motile sperm and reduces the concentration of possible contaminants as leucocytes, microorganisms and debris. Commercial sperm banks provide sperm samples which have been prepared before freezing and are ready to use for IUI upon thawing. In that case, the use of egg yolk containing CPM should be avoided. The latter procedure is increasingly adopted in non-commercial sperm banks as well.

For sperm samples of very low quality designed for ICSI, either ejaculated sperm or surgically retrieved sperm, post-thaw double washing is still the method of choice, in order to recover the few available spermatozoa.

In the case of severe oligozoospermia, semen is preferably concentrated before addition of the CPM. Testicular sperm can be frozen as biopsy or as suspension. Crabbé et al. [90] showed that freezing testicular sperm in suspension after mechanical shredding preserved motility and viability significantly better than whole-biopsy freezing [90]. Suspension freezing has the advantage of using high-security straws which allow more efficient storage in the tanks. However, high-security vials (CBS) are available for secure storage of testicular biopsies.

Special Concerns of Sperm Cryopreservation

Cryopreservation of Surgically Retrieved Spermatozoa

Testicular Tissue Cryopreservation

The cryopreservation of testicular tissue (TT) as a safe and effective means to facilitate the treatment of couples with azoospermia has been used in clinical/laboratory practice for three decades. This has allowed storage of enough spermatozoa following one surgical procedure for their use in multiple ICSI cycles, thereby reducing patient costs and risks of repeated surgeries [91, 92]. The main purpose of early TT cryopreservation protocol was thus to preserve the most mature stages of spermiogenesis. The freezing media contained glycerol as this is used worldwide as the cryoprotectant of choice for mature spermatozoa, although it is not optimal for other cell types of TT [90].

Avarbock et al. showed in 1996 that spermatogenesis could be restored by transplanting cryopreserved spermatogonial stem cells (SSCs) in the seminiferous tubules of sterilized mice [93], paving the way for the TT cryopreservation in preserving the fertility of prepubertal boys who are undergoing gonadotoxic chemotherapy or radiotherapy [94].

Therefore, adapted TT cryopreservation protocols were needed with a focus on pre-serving SSCs and their supportive cells rather than mature germ cells. To find such, the rationale was to strive for minimal cryoinjury and maximal cell recovery. This posed a challenge, owing to the various cell types in TT, each differing in dimension, complexity and water permeability and hence requiring different optimal cryopreservation protocols.

Issues with TT Freezing

Tissue size: The macroscopic physical dimension of the tissue is a major point to be defined in a cryopreservation protocol. It is key to achieving equal distribution with rapid in- and out-diffusion of CPAs and uniform rates of temperature change to limit cryoinjury [95]. This is especially true for vitrification, as the sample size is a critical variable in the probability of successful solidification of the aqueous milieu of the cells/tissue into a non-crystalline glassy phase. It is also important in the prevention of devitrification which occurs during warming and is characterized by the formation of ice crystals [96]. Yet, few data exist on the effect of sample size on TT cryopreservation.

Storage vessel: Different TT freezing protocols have been developed over the years using either straws or vials to store cryopreserved TT. The choice of the vessel was mostly made based on previous literature and the logistic situation. Travers et al. [97] performed a comparative study in which they found that the morphology of immature rat TT was better protected using vials compared to straws [97].

In contrast to conventional slow freezing, studies dealing with vitrification carefully consider which vessel to be used during cryopreservation: an open or closed device. Open devices are devices allowing direct contact of the sample with the cooling solution, typically liquid nitrogen (LN2).

Using open devices, the cooling rates achieved are in general approximately 20,000–30,000 °C/min which favours good vitrification of the sample [98]. Indeed, open vitrification systems, i.e. open straw, have been successfully employed to preserve the integrity and activity in organotypic culture and long-term xenografting of immature mouse, monkey and human TT post-warming [99–101]. The problem is that direct contact with the cooling solution introduces a risk of pathogen

transmission to the sample during cooling and a high risk of cross-contamination in the container. In closed systems, on the other hand, the sample is not in contact with the cooling solution during freezing or storage, thereby tackling the problem of contamination. One drawback of a closed system is that the cooling rate is much lower and therefore requires higher concentrations of CPAs to prevent ice crystal formation. This makes the protocols potentially more dangerous for cells due to the cytotoxicity of CPAs [98].

Abrishami et al. [102] studied the feasibility of vitrifying mature human tissue with a protocol proven to preserve the functionality of immature porcine TT upon xenografting [102]. Signs of cryoinjury were observed to the human TT, and therefore, solid-surface vitrification needs further optimization for human applications [103].

Cryopreservation of Small Numbers of Human Spermatozoa

Various methods for cryopreservation of small numbers of human spermatozoa have been proposed. Cohen et al. [104] suggested an empty zona pellucida procedure. It failed to become mainstream because it depended on a biological carrier, with the potential risk of contamination and disease transmission ([104]).

Just et al. [105] developed spherical *V. globator* algae as a cryopreservation vehicle ([105]). Additional methods, such as agarose microspheres [106], straws [107], ICSI pipettes [108], cryoloops [109] and cell sleepers [110, 111], have also been tested.

A review, which included 30 reports on all the previous methods and techniques for cryopreservation of individual or small numbers of human spermatozoa, concluded that the ideal container or vessel/platform that could be used universally has yet to be developed. Novel cryopreservation technology specifically designed to handle small numbers of spermatozoa needed to be further explored [112].

A feasibility experiment has been carried out by Berkovitz et al. [113] of the novel sperm vitrification device (SpermVD) and a prospective cohort study of ICSI cycles in men suffering from non-obstructive azoospermia [113]. The authors have shown that SpermVD is an efficient and simple carrier method for freezing a small number of spermatozoa in low-volume droplets, reducing post-thaw search time from hours to minutes, allowing a 96% recovery rate and leading to successful use of sperm for fertilization. This device needs to be validated by larger studies.

Safety and Efficacy of Cryopreservation

Although frozen-thawed semen has great practical benefits for reproduction, it is widely reported that the cryopreservation process involving cooling, freezing and thawing induces serious detrimental changes in sperm functions [114]. It is well known that the process of sperm freezing and thawing not only results in adverse changes in membrane lipid composition, acrosome status and sperm motility and vitality but also causes an increase in sperm DNA damage, [54, 83, 115, 116]. In fact, approximately 50% of the spermatozoa in an ejaculate survive following freezing and thawing process [117]. The mechanism behind cryodamage may be related to osmotic stress, cold shock, intracellular ice crystal formation, excessive production of reactive oxygen species (ROS) and alteration in antioxidant defence systems [118].

Notwithstanding the risk of damage to sperm by freezing and thawing, frozen-thawed sperm is successfully used for IUI, IVF and ICSI. In IUI and IVF, sperm selection to fertilize the oocyte is a natural process. While spermatozoa for ICSI are selected by the lab operator on the basis of motility and morphology, individual sperm selection based on DNA integrity is not possible. Although IUI with frozen-thawed spermatozoa results in a lower pregnancy rate than insemination with fresh spermatozoa, the fertilization and pregnancy rates of ICSI using cryopreserved sperm parallel those of freshly obtained sperm. Cryopreserving sperm avoids the need for additional surgery and optimizes results in couples undergoing repeat IVF/ICSI cycles.

Nevertheless, the studies regarding the follow-up of children born following sperm cryopreservation are reassuring: data from robust meta-analyses indicate that there is no statistical

difference in clinical pregnancy or fertilization rates in couples undergoing ICSI or IVF using cryopreserved spermatozoa, when compared to fresh samples [119].

A large Australian sperm donation programme data demonstrated no statistically significant increase in adverse perinatal outcomes and congenital or chromosomal abnormalities when cryopreserved samples were compared to fresh [120–122].

The reassuring data on the outcome of cryopreserved sperm despite the damages induced by the cryopreservation and thawing need to be discussed with regard to the oocyte quality as well. The lack of correlation of DNA damage burden with clinical pregnancy rates achieved may be explained by work by Meseguer et al., who determined that the relative impact of sperm DNA fragmentation on pregnancy outcome was dependent on the quality of the oocyte available for fertilization [123] In other words, if spermatozoal DNA has sustained damage, then it may be possible for this damage to be repaired by the DNA repair machinery of the oocyte, but if the oocyte itself is of poor quality, this is less likely to occur. The oocyte quality remains the determining factor of the treatment outcome.

Conclusion

Sperm cryopreservation is one of techniques which has revolutionized the field of assisted reproduction. ICSI on the other hand has revolutionized the use of frozen sperm, ejaculated as well as non-ejaculated.

Semen cryopreservation is an established procedure in clinical practice, applied for a variety of indications. In contrast to oocytes and embryos, spermatozoa are usually available in high numbers, allowing for a certain loss of quality after thawing, which in turn may be the reason why freezing procedures for semen have not changed substantially over the past decades.

Glycerol remains the permeable cryoprotectant of choice for conventional sperm freezing with different cooling rates and programmes providing acceptable cryosurvival. Vitrification without permeable cryoprotectants as an alternative rapid procedure, especially for small numbers of spermatozoa, needs further investigation before being widely introduced.

With regard to safety aspects during handling, freezing and storage, sperm or germ cells should be packed in hermetically sealed high-security straws (or vials) and stored immersed in liquid nitrogen or in liquid nitrogen vapour. Storage tanks should be individually monitored with a day/night alarm. Legal requirements on quality and safety regarding donation, procurement, testing, processing, preservation storage and distribution should be respected during all the steps of the sperm cryopreservation process.

The data on the outcome of cryopreserved sperm are reassuring despite the damages induced by the cryopreservation.

Future research should focus on improving the post-thaw survival of sperm which is still suboptimal.

Take-Home Messages

- Indications for sperm cryopreservation include homologous and donor insemination, cryopreservation prior to/during/after and during surgical infertility treatment and fertility preservation prior to treatment for malignancies.
- Sperm cryopreservation avoids the need for additional surgery in couples undergoing multiple ICSI cycles.
- Sperm banking should be offered to all patients with malignancies who wish to have children.
- Regarding the safety aspects during handling, freezing and storage, sperm cells should be packed in hermetically sealed high-security straws (or vials) and stored immersed in liquid nitrogen or in liquid nitrogen vapour.
- Storage tanks should be individually monitored with a day/night alarm.
- Data regarding the follow-up of children born following sperm cryopreservation are reassuring.

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Indications and Methods of Epididymal or Testicular Sperm Retrieval for Cryopreservation

Caroline Kang, Nahid Punjani, and James A. Kashanian

Introduction

Approximately 50% of men will receive a cancer diagnosis during their lifetime [1]. With growing proportions of cancer survivors, fertility issues have become more prominent, and subsequently, a greater focus has been placed on fertility preservation. Additionally, men of reproductive age are increasingly being diagnosed with cancers necessitating early discussion of fertility preservation. Chemotherapy impacts spermatogenesis, is harmful to Leydig cells, and can result in hypogonadism [2]. Testicles are highly radiosensitive tissues, and radiation may result in irreversible testicular damage [3]. As a consequence of these anti-neoplastic treatments as well as radical cancer surgery, men may become infertile and no longer able to father children. For this reason, a multidisciplinary approach is needed to best manage cancer patients and their wishes regarding fertility preservation. Numerous professional organizations have implemented programs coordinating oncologic care with fertility preservation. The American Society of Reproductive Medicine (ASRM) and American Society of Clinical Oncology (ASCO) have recommended that all patients receive counseling and early referral for fertility preservation secondary to cancer treatments [4, 5]. Furthermore, treating providers must be aware of the significant impact the prescribed treatments have on future fertility of their patients. The focus of this chapter is to review the indications, evaluation, and methods used for fertility preservation in men.

Indication for Fertility Preservation

Various etiologies exist that place cancer patients at high risk for infertility. These etiologies include radiation therapy, systemic chemotherapeutic agents, and surgery.

Radiation

Radiation is used to treat a wide variety of malignancies in men. Since testicles are highly radiosensitive, ionizing radiation (either direct or indirect) may result in significant damage, including Leydig cell dysfunction and germ cell loss [6, 7]. Sperm production may be impacted by indirect radiation despite testicular shielding [3]. Some studies report radiation doses as low as 0.1 Gy inducing damage to the testicles, with irreversible damage occurring at 4 Gy [3,

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8]. At low doses (0.1 Gy), spermatogonia may be impacted, and slightly higher doses (2–3 Gy) cause spermatocyte and spermatid damage. High doses (>4 Gy) damage spermatozoa [3]. In addition to impairment of spermatogenesis, radiation also has been shown to have impacts on sperm DNA integrity. Damage may continue up to 2 years after radiation treatment, with higher rates of DNA damage in the immediate period after radiation treatment (i.e., 6 months after radiation) [8, 9]. Additional studies have reported that very high radiation doses (24–30 Gy) will result in impaired Leydig cell function, resulting in low testosterone production [10, 11].

Prostate cancer patients may receive either external beam radiotherapy (EBRT) or brachytherapy. Radiation to the prostate bed affects the testicles even though they are not the targeted organs. Radiation doses are reduced by advanced techniques including gold markers, which improve image guidance, and testicular shielding [12]. Brachytherapy involves placement of radiation seed pellets directly into the prostate. Although the seeds emit radiation over time, the effects of radiation on the testicles are less pronounced than EBRT, and testicular tissue is minimally impacted [13]. However, in addition to testicular damage, radiation effects on the seminal vesicles and prostate tissue may result in alterations in semen volume and sperm motility [12].

Childhood cancers such as lymphoma and leukemia may subject prepubescent males to total body irradiation. Patients receiving more than 10 Gy tend to have azoospermia [14]. Since treatment is required to the whole body, it is challenging to use strategies such as shielding to protect the testicles.

Chemotherapy

Chemotherapeutic agents are a mainstay for many cancer treatments. Men undergoing treatment are generally counseled to avoid conception due to the possible toxicity and subsequent damage to sperm DNA material while actively receiving treatment [3]. Chemotherapy exerts significant toxicity to germ cells resulting in up to a 100-fold decrease in sperm count within the first 2 months of therapy, with azoospermia occurring after 2 months [15]. Leydig cell damage also occurs from toxic chemotherapy which may result in hypogonadism in this patient population [2]. Different anti-neoplastic agents exert varying toxicity to the testicles and sperm, and alkylating agents, which are commonly given to males in many childhood cancer (i.e., testicular cancer and lymphoma/leukemia), have been reported as the most toxic [3]. Sperm DNA integrity also is impacted by chemotherapy and has been shown to recover over time, with return to baseline approximately 2 years after treatment [9].

Alkylating agents, such as cyclophosphamide, have been reported to produce permanent azoospermia in up to 90% of cases [16]. While these agents are commonly used in patients with Hodgkin's disease, non-alkylating chemotherapy regimens have been developed with similar therapeutic potential and decreased gonadotoxicity. With these new non-alkylating agents, improved sperm recovery is observed in the first 5 years (up to 90% of patients) [17]. For platinum-based agents, which are the mainstay for testicular germ cell tumors, sperm is recovered in 50% of patients after approximately 2 years and 80% patients after 8 years [18].

Surgery

Surgical resection of an entire organ, tumor, and/or associated lymph node dissection is a common part of cancer diagnosis and treatment. Testicular cancer remains one of the most common malignancies in young males which is treated initially with surgical intervention. Radical orchiectomy, or complete removal of the testicle containing the tumor along the spermatic cord up to the level of the internal inguinal ring, leaves men with a solitary remaining testicle. In the case of synchronous bilateral tumors or metachronous recurrence, patients can potentially be left with minimal or no testicular tissue. Following orchiectomy, sperm concentrations may decrease by up to 50% a few months after surgery, and up to 10% of these

patients may become azoospermic in the long term despite previously normal sperm counts [19]. Partial orchiectomy has been suggested by some groups to minimize the amount of testicular tissue removed, but is only recommended following strict criteria due to concerns for oncologic control and progression [19]. Men with advanced testicular cancer may require retroperitoneal lymph node dissection (RPLND), a procedure that commonly requires removal of lymphatic tissue that causes disruption of sympathetic ganglia and the hypogastric plexus resulting in ejaculatory dysfunction [20]. Retrograde ejaculation and failure of emission have been reported as high as 65% in patients who have undergone RPLND, but have declined to almost 10% with the advent of nerve-sparing procedures in appropriately selected cases [20]. Surgical intervention to other organs which impair the hypothalamic-pituitary axis may result in impaired spermatogenesis or testosterone production [21].

Prior pelvic surgery, including inguinal surgery, scrotal exploration, or prostate or bladder surgery, also may result in male reproductive tract obstruction [22]. Radical prostatectomy for prostate cancer always results in infertility secondary to obstruction since the vas deferens are ligated and seminal vesicles are removed for cancer prevention and grading. Additionally, nerves responsible for erectile function drape over the capsule of the prostate and may be affected at the time of prostate surgery removal. Similar to RPLND, nerve-sparing techniques have been developed to minimize the loss of erectile function due to nerve disruption after prostatectomy. Similarly, men who undergo radical cystoprostatectomy for bladder cancer have the prostate and seminal vesicles removed, which puts them at risk for erectile dysfunction and obstructive azoospermia. For patients with localized bladder cancer, transurethral resection of bladder tumor (TURBT) and intravesical immunotherapy or chemotherapy are the standard of care. Local surgical resection does not negatively impact spermatogenesis, but intravesical chemotherapy administered in conjunction with or following surgical resection may cause oligozoospermia [23].

Clinical Evaluation

Upon referral to a fertility specialist, patients should be carefully evaluated and their wishes for future fertility discussed. Evaluation of patients for fertility preservation includes obtaining a medical and surgical history, in addition to a fertility, sexual, developmental, and family history. Furthermore, cancer history and plans for treatment, including chemotherapeutic regimens and cycles, radiation dose totals, and targeted body sites, and radical surgery should be discussed. Table 1 summarizes the components of a thorough history during evaluation of men for fertility preservation. Next, a focused physical exam should be performed. Importantly, in pediatric patients, if there is uncertainty as to whether puberty has commenced (and spermatogenesis is occurring in the testicles), Tanner staging (discussed below, summarized in Table 2) can be used to determine sexual maturity and provide information useful in counseling the patient and family.

History

Past medical history, past surgical history, as well as developmental history should be obtained in patients interested in fertility preservation (Table 1). Family history to identify any possible fertility issues also is helpful. A fertility history should include a general review of systems followed by a set of focused questions related to fertility. Prior pregnancies with current or previous partners, and whether the pregnancies resulted in live births, should be noted. Prior issues with fertility or any infertility evaluations should be discussed. Relationship information also should be obtained, for example, whether the patient is partnered or single and whether they desire biological children in the future. Furthermore, sexual history including any issues with ejaculatory or erectile function should be elicited at this time.

Cancer treatment plans should be discussed with the patient and/or family (if the patient is a minor). In many instances, the patient and/or family may not fully understand or be aware of

Table 1 Components of history for the evaluation of men for fertility preservation

Past medical history	Example
Any medical comorbidities	Neurologic, cardiac, vascular, hepatic, renal diseases
Any current medications	Anti-depressants, opioids, steroids, testosterone
History of genitourinary tract infections	Orchitis, epididymitis, UTI, urethritis, STD
History of pelvic, perineal, genital trauma	Car accident, bike accident
Exposure to gonadotoxins	Medications, environmental exposures, dyes
Past surgical history	
History of inguinal or pelvic surgery	Inguinal hernia repair, prostatectomy, orchiectomy
History of prostate or bladder cancer surgery	Radical prostatectomy, TURBT, cystoprostatectomy
History of scrotal surgery	Orchiopexy, sperm retrieval
Fertility history	
Type of infertility	Primary vs. secondary
Prior pregnancies	Live births, termination
Prior infertility evaluation	Semen analyses, imaging
Planned future fertility	Number of biological children desired
Sexual history	
Partner information	Age, gynecologic history, prior infertility evaluation, previous pregnancies with patient or another partner
Erectile function	Problems with erections, medications for erections
Ejaculatory function	Premature ejaculation, anejaculation
Sexual drive/desire	Low libido, testosterone replacement therapy
Developmental history	
History of childhood illnesses	Mumps, orchitis, UTI, STD
History of childhood cancer	Cancer type, location, treatment, surgery
Genetic conditions	Klinefelter syndrome
Puberty	Age at puberty, development of secondary sex characteristics
History of undescended testis	Orchiopexy, resolution
Family history	
Siblings or other family with fertility issues	
Family history of genetic conditions	
Family history of cancer	
Cancer history	
Type of cancer	Location, grade, metastasis
Chemotherapy	Planned agent, dosing, timing, duration
Radiation treatment	Planned dose, location target, duration, shielding
Surgery	Site, organ/body part to be resected
Prior history of cancer	Type, location, grade, metastasis
Prior chemotherapy	Dose, duration, interval since treatment
Prior radiation treatment	Dose, duration, targeted site, interval since treatment, shielding
Other cancer treatments	Stem cell transplant, immunotherapy

Table 2 Tanner stages for assessment of sexual maturity in males [26]

Tanner stage	Pubic hair	External genitalia
1	No hair	Testis volume <4 ml or long axis <2.5 cm
2	Downy hair	Testis volume 4–8 ml or long axis 2.5–3.3 cm
3	Scant terminal hair	Testis volume 9–12 ml or long axis 3.4–4.0 cm
4	Terminal hair that fills the entire triangle overlying the pubic region	Testis volume 15–20 ml or long axis 4.1–4.5 cm
5	Terminal hair that extends beyond the inguinal crease onto the thigh	Testis volume >20 ml or long axis >4.5 cm

the treatment plans, and the fertility specialist should discuss the plans directly with the oncology provider(s). Important information to glean from the oncology team includes the type, dose, and duration of chemotherapeutic agent to be administered, along with any plans for radiation therapy (total cumulative dose, target site, shield-

ing). In additional to future cancer treatment plans, past treatments for cancer (including chemotherapy, radiation, and surgery) should be discussed with the patient and/or family.

Physical Exam

The physical exam is important in identifying patients who may be at an even higher-than-normal risk of infertility after cancer treatment. A general physical exam should be completed first, followed by a focused genitourinary exam. One should pay close attention to signs of androgen deficiency or other genitourinary anomalies [24, 25]. Components of a thorough physical exam are included below.

- General appearance (congenital abnormalities, syndromic features, secondary sex characteristics, frailty, body habitus).
- Masculinization (hair distribution, gynecomastia).
- Abdominal and inguinal exam for healed scars from prior surgery.
- Penile examination (appropriate development, circumcision status, meatal assessment).
- Spermatic cord examination (varicocele, hernia, presence of vas deferens).
- Testicular examination (size, consistency, masses).
- Epididymal examination (dilated or flat, epididymal cysts).
- Digital rectal exam (prostatic cysts, enlarged seminal vesicles).
- Location of radiation tattoos.
- Pediatric (Tanner staging to evaluate for sexual maturation) (Table 2) [26].

Obstructive azoospermia may be suspected in men without palpable vas deferens, dilated epididymides, or low volume and acidic ejaculates. Importantly, those without a palpable vas deferens should be screened for cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations and may require intra-abdominal imaging (renal ultrasound) to investigate for concurrent renal anomalies. In patients with absent vas deferens, partner screening for CFTR gene mutations and genetic counseling also is warranted. Men with obstructive azoospermia will generally have testis size >4.6 cm, whereas those with testicular failure have small and soft testicles (<4.6 cm) suggestive of non-obstructive azoospermia [27].

Laboratory Investigation

Laboratory investigations are not required in the evaluation of men for fertility preservation. However, because spermatogenesis is an intricate biologic process tightly regulated by hormones, obtaining serum hormone levels provides information to the patient and healthcare provider as to whether fertility issues exist prior to planned cancer treatment or fertility preservation. Spermatogenesis occurs in the testis and is dependent on adequate levels of circulating and intratesticular testosterone [28, 29]. The hypothalamic-pituitary-testis (HPT) axis is responsible for inducing both testosterone production and spermatogenesis. Gonadotropin-releasing hormone (GnRH) is released in a pulsatile fashion from the hypothalamus and stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland. LH then acts upon Leydig cells within the testis to produce testosterone, and FSH acts upon testicular Sertoli cells to promote spermatogenesis. Additionally, Sertoli cells also secrete inhibin, which negatively regulates the HPT axis and levels are positively correlated with testis volume, and androgen-binding protein, which increases testosterone concentrations in the seminiferous tubules necessary for spermatogenesis [30]. Perturbation of serum hormone levels can provide some information regarding the potential for spermatogenic failure and decreased testicular reserve.

In the evaluation of men with infertility, initial hormonal evaluation includes serum levels of testosterone (measured in the morning due to natural diurnal variation) and follicle-stimulating hormone (FSH) [24]. Additional adjunctive labora-

tory tests include luteinizing hormone (LH), thyroid-stimulating hormone (TSH), estradiol levels, and prolactin [31]. Greater than 90% of men with normal sperm production have FSH serum concentrations within the normal range [32]. If laboratory abnormalities are noted, medications can be provided to optimize the patient prior to sperm collection. For example, if tumor effects on the HPT axis result in lower LH and FSH levels, human chorionic gonadotropin (hCG) and recombinant FSH can be administered.

Medical Optimization

Various medical options exist to optimize intrinsic spermatogenesis and increase the chances of successful sperm retrieval in men with abnormal semen parameters. Hormonal optimization in non-obstructive azoospermic men has been supported by various reports in the literature [33]. Patients must be counseled as to the appropriate administration and side effects of these medications, and subsequent hormone levels should be followed closely. Various options are listed in Table 3 [34, 35]. It must be noted that many interventions aimed at improving male fertility may take 12–24 weeks for maximal benefit.

Semen Analysis and Cryopreservation

Healthcare providers should discuss fertility preservation at the earliest possible opportunity with their patients prior to initiation of gonadotoxic treatments [4, 5]. This discussion should encompass cryopreservation of sperm for future assisted reproductive technology. Specifically, for young adult male patients facing a new cancer diagnosis, the process of sperm banking for future fertility can be overwhelming [36]. Additional time and attention must be taken in counseling for this at-risk group [37]. Males who are planning to preserve sperm must first provide a semen sample for analysis. The World Health Organization (WHO) established values for normal semen parameters based on parameters observed in a cohort of men with documented fertility (Table 4) [38]. Although the majority of men with semen parameters within the established normal ranges are likely to be fertile, a man's semen parameters are not predictive of fertility. If sperm is present in the provided sample, semen parameters should be documented, and the sample can be cryopreserved. Ideally, these samples should be split into multiple portions to allow for individual thawing.

If sperm is not present in the sample, a repeat sample must be provided for analysis before a diagnosis of azoospermia, or no sperm in the ejaculate, can be given. According to the American Urological Association (AUA) Guidelines, azoospermia should be diagnosed only after no sperm is identified after examination of two centrifuged semen samples [24]. Semen specimens should be produced by self-stimulation and without the use of lubricants. For processing, provided samples are centrifuged at maximal speed (3000 *g* for 15 min at room temperature) followed by examination under a microscope by an experienced andrologist [27].

Table 3 Medications used to optimize spermatogenesis [35]

Medication	Indication	Mechanisms	Dose
Pseudoephedrine	Retrograde ejaculation	Closes bladder neck	60–120 mg prn
FSH	Hypogonadotropic hypogonadism	Stimulates Sertoli cells to promote spermatogenesis	100–1500 IU 2–3x/week
hCG	Hypogonadism	Stimulates Leydig cells to produce testosterone	1000–5000 IU 3x/week
Anastrozole Letrozole	Low serum testosterone-to-estradiol (T:E) ratio	Inhibits aromatase; inhibits peripheral conversion of testosterone to estradiol	1 mg/day 2.5 mg/day
Clomiphene citrate Tamoxifen	Hypogonadism	Selective estrogen receptor modulator; block negative feedback of estradiol on HPT axis	Up to 50 mg/day 20 mg/day

Table 4 WHO 2010 semen parameters [38]

Semen parameter	WHO parameters	Abnormality
Volume (ml)	1.5	Aspermia (volume = 0 ml)
Total sperm count (TSC) (million)	39	Azoospermia (TSC = 0) Severe oligozoospermia (TSC <five million) Oligozoospermia (TSC <15 million)
Concentration (million sperm/ml)	15	
Progressive motility (%)	32	Asthenospermia
Total motility (%)	40	
Normal morphology (%)	4	Teratozoospermia
Vitality (%)	58	
Leukocyte count (million/ml)	<1.0	

Samples demonstrating azoospermia are not suitable for cryopreservation. Sperm retrieval options are available for men with azoospermia, which are discussed below.

It is important to note that in addition to the absence of sperm, sperm cryopreservation may be challenging for a multitude of reasons including associated costs, concern that banking will delay urgent treatment, absence of adequate facilities, and psychological concerns regarding advanced disease and poor potential outcomes [39].

Types of Cryopreservation

Cryopreservation permits long-term preservation of biological samples. Low temperatures decrease the metabolism of the cellular component of the specimen, and all chemical and enzymatic reactions cease. Appropriate protocols and procedures are paramount for adequate tissue survival. Testicular tissue may be either frozen as whole tissue or processed in suspension, but post-thaw viabilities are similar for both methods [19]. Cryopreserved sperm has been used with assisted reproductive techniques with good fertility outcomes, and currently only mature sperm has been used successfully to achieve pregnancy [40, 41].

Sperm banking is the first-line method for fertility preservation in post-pubertal males since semen specimen collection is non-invasive and most post-pubertal males do not have issues with providing specimens for sperm cryopreservation. However, some post-pubertal males may have issues providing semen specimens for a host of reasons including pain from cancer, erectile or ejaculatory disorders, psychologic barriers, or religious beliefs. For individuals unable to provide a semen sample, which may be up to 15% of oncology patients, alternative sperm retrieval methods may be employed [42].

Prepubertal testicular tissue also may be harvested and cryopreserved. Currently, successful use of cryopreserved human testicular tissue to generate a pregnancy has not been achieved. However, promising results from animal models suggest autologous spermatogonial stem cell transplantation, grafting of the cryopreserved testicular tissue to the testis or other bodily area, or in vitro spermatogenesis may be successful in the future [43, 44]. Cryopreserved rhesus macaque testicular tissue has been used to obtain sperm after autologous, heterotopic engraftment [45]. The harvested sperm was functional and used in in vitro fertilization with intracytoplasmic injection (IVF/ICSI), and a baby rhesus macaque was born [45]. Thus, under proper counseling and with proper institutional review board oversight, testicular tissue may be harvested and cryopreserved in prepubertal males whose testicles do not yet have active spermatogenesis for potential future fertility use. However, it must be reinforced that at this time testicular tissue cryopreservation is entirely experimental.

Methods for Sperm Retrieval

Sperm can be retrieved in post-pubertal males from various locations in the reproductive tract. The different methods used for sperm retrieval are dependent on surgeon experience, presence of obstruction, location of obstruction, ejaculatory function, and cancer characteristics. Table 5 summarizes the various methods, indications for the procedure, and advantages and disadvantages of each method.

Table 5 Indications and sperm retrieval techniques and for fertility preservation

Technique	Indications	Advantage(s)	Disadvantage(s)
PESA	Vasal obstruction, CBAVD, EDO, anorgasmia	Can be performed in office, less invasive	Low yield, higher complication rates, additional procedures, low yield for cryopreservation
MESA	Vasal obstruction, CBAVD, EDO, anorgasmia	High sperm yield	General anesthesia/OR, microsurgical experience, cost
MIESA	Vasal obstruction, CBAVD, EDO, anorgasmia	High sperm yield, clinic procedure, local anesthetic, no microsurgical experience, low cost	No general anesthesia/OR, requires loupes (cost), decreased visualization of epididymal tubules
TESA	Vasal obstruction, CBAVD, EDO, anorgasmia, azoospermia	No microsurgical experience	Low sperm yield, cannot cryopreserve, higher complication rates
TESE	Azoospermia, CBAVD, EDO, anorgasmia	Can obtain testicular tissue for biopsy and cryopreservation, no microsurgical experience	Selection of testicular tissue random, cost
mTESE	Non-obstructive azoospermia	Can obtain testicular tissue for biopsy and cryopreservation	Microsurgical experience, cost
oncoTESE	Azoospermia, CBAVD, EDO, solitary testis, bilateral testicular tumors	Harvesting sperm from tissue that has been resected (single procedure)	Potential abnormalities in sperm, interference with pathologic analysis of tumor
PEU	Ejaculatory dysfunction (retrograde ejaculation)	Non-invasive	May need to be catheterized to obtain specimen
Percutaneous vasal aspiration	Ejaculatory dysfunction, vasal obstruction, EDO	Relatively non-invasive, office procedure	Small sample, additional procedures, low sperm yield
PVS	Ejaculatory dysfunction, anorgasmia, SCI	Non-invasive, easy to use device	May not be effective in producing specimen
EEJ	Ejaculatory dysfunction, anorgasmia, SCI	Low-risk procedure	General anesthesia/OR, possible sperm damage from probe, autonomic dysreflexia

PESA percutaneous epididymal sperm aspiration, *MESA* microsurgical epididymal sperm aspiration, *MIESA* minimally invasive epididymal sperm aspiration, *TESA* testicular sperm aspiration, *TESE* testicular sperm extraction, *mTESE* microsurgical testicular sperm extraction, *oncoTESE* onco-testicular sperm extraction, *PEU* post-ejaculatory urinalysis, *PVS* penile vibratory stimulation, *EEJ* electroejaculation, *CBAVD* congenital bilateral absence of the vas deferens, *EDO* ejaculatory duct obstruction, *SCI* spinal cord injury, *OR* operating room

Epididymal Sperm Retrieval

Epididymal sperm remains a reasonable option for sperm retrieval. The function of the epididymis is sperm maturation and transport, and therefore, epididymal sperm have more motility. Sperm may be retrieved through both percutaneous, minimally invasive, and open microsurgical options.

Percutaneous Epididymal Sperm Aspiration (PESA)

PESA is a procedure which can be completed under local anesthetic as an in-office procedure (Fig. 1). A spermatic cord block is performed using local anesthetic. The epididymis is securely isolated by the surgeon in order to avoid injury to nearby structures. A 21-gauge butterfly needle is inserted through the skin into the epididymis, and sperm is directly aspirated. The caput epididymis usually is chosen as this region of the epididymis is easiest to palpate and isolate. If necessary, multiple sites may be sampled in order to obtain an adequate sample. The specimen should then be examined microscopically to confirm presence of sperm. Some disadvantages of this technique include possible low yield, higher complication rates, and patients may require an open procedure if no sperm is aspirated. Because of low yield, it is preferred that any sperm retrieved be used immediately for in vitro fertilization instead of

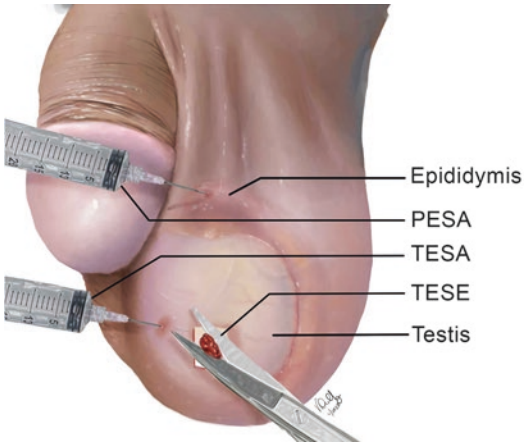

Fig. 1 *Minimally invasive techniques for sperm retrieval.* These techniques can be performed under local anesthetic and in the clinic setting. PESA, percutaneous epididymal sperm aspiration; TESA, testicular sperm aspiration; TESE, testicular sperm extraction

cryopreservation. Lastly, this procedure will often result in epididymal obstruction [46].

Microsurgical Epididymal Sperm Aspiration (MESA)

MESA is a more invasive form of epididymal sperm retrieval, is done in the operating room, and often requires general anesthetic. This procedure requires delivery of the testicle and epididymis through a scrotal incision with dissection through dartos fascia and entry into the tunica vaginalis (Fig. 2). The epididymis is isolated, and targeted epididymotomies are made with the assistance of a standard operating microscope. Sperm can be retrieved from all segments of the epididymis, if needed, as the entire epididymis is

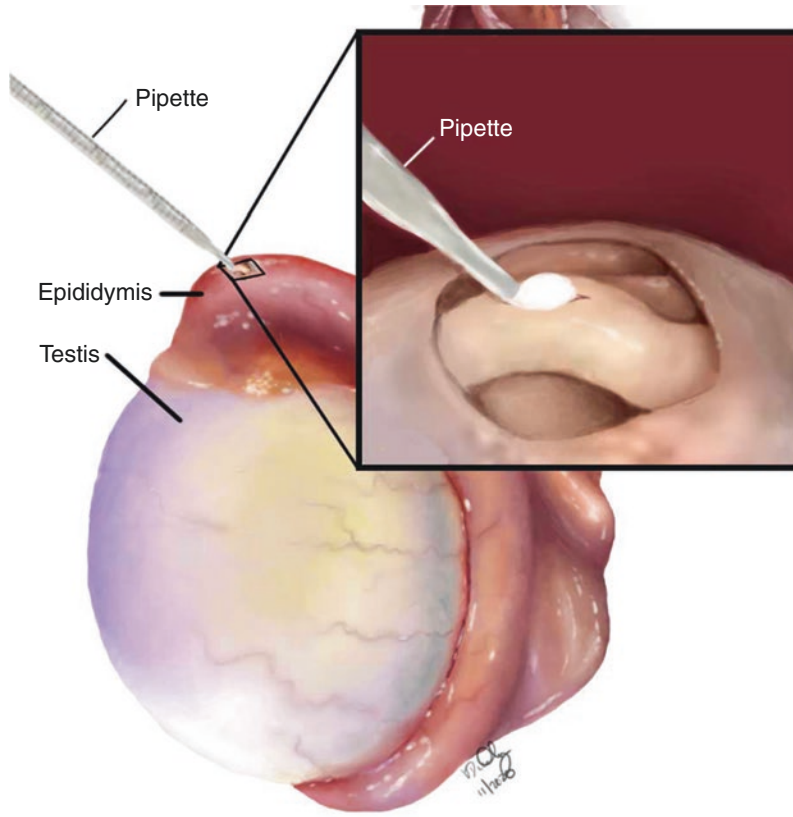

Fig. 2 *Microsurgical epididymal sperm aspiration.* After exposure of the epididymal tubules, an ultra-sharp ophthalmic blade is used to open a single tubule. A pipette is shown in the box aspirating fluid from the epididymal tubule. This fluid is examined for sperm under light or phase contrast microscopy

visualized. A suitable tubule is first identified, and an epididymotomy is formed using microsurgical scissors. Then using a 15-degree ultra-sharp ophthalmic knife, a targeted tubule is opened, and epididymal fluid is aspirated using a 25-gauge angiocath into a 1 cc syringe pre-filled with 0.1 ml buffered sperm media. Generally, there are high rates of sperm retrieval with large quantities of sperm obtained, and this sample can be easily cryopreserved. Unfortunately, the procedure is more expensive than in-office percutaneous approaches, requires a skilled microsurgeon, and similarly leads to epididymal obstruction [46].

Minimally Invasive Epididymal Sperm Aspiration (MIESA)

MIESA was introduced by Coward and Mills in 2017 [46]. This technique allows for sperm aspiration from the epididymis in a manner similar to MESA, but MIESA can be performed in the office (compared with being done in the OR with MESA), with loupe magnification (compared with use of the standard operating microscope in MESA), and with local anesthetic (compared with general anesthesia with MESA). Briefly, a small keyhole-sized incision is made in the scrotum, and the epididymis is exposed without full delivery of the testicle through the incision. Loupe magnification is used to identify epididymal tubules for sperm aspiration. Using MIESA, millions of sperm can be retrieved, and the procedure is low-risk and well-tolerated [46].

Testicular Sperm Retrieval

Testicular sperm also may be a reasonable option for sperm retrieval depending on the etiology of infertility. In those with non-obstructive azoospermia, epididymal retrieval is generally not feasible as these men have some component of testicular dysfunction. Testicular sperm may be retrieved in office via percutaneous aspiration or as a surgical procedure with tissue excision under a general anesthetic.

Testicular Sperm Aspiration (TESA)

TESA may be completed as an in-office procedure (Fig. 1). Patients receive local anesthetic with a spermatic cord block. The testicle is secured and the epididymis protected to avoid inadvertent damage. Using an 18-gauge needle and syringe pre-filled with buffered sperm media, fluid is aspirated from the testicle. Care should be taken to avoid known or suspected areas of vascularization. Multiple passes from the same needle entry may be completed. Yields of sperm are generally low, and thus, sperm retrieved is usually not cryopreserved. Since the procedure is completed without visualization of the testicle and seminiferous tubules, TESA has greater complication rates than open testicular sperm extraction procedures [46].

Testicular Sperm Extraction (TESE) and Microdissection Testicular Sperm Extraction (mTESE)

TESE is an open procedure that is performed with or without a standard operating microscope. Conventional TESE is typically done under a general anesthetic but may also be performed with local anesthesia and/or oral sedation. A small transverse incision (approximately 1 cm) is made on either side of the scrotum depending on the testicle that is being sampled, or a midline scrotal incision can be used (surgeon preference). Care should be taken to protect the epididymis by keeping this structure posterior. Dissection is carried down through dartos fascia toward the tunica vaginalis, which is then entered. The tunica albuginea is incised with an ultra-sharp knife, after which seminiferous tubules can be visualized. Gentle pressure on the testicle allows for delivery of these tubules, and using a sharp iris scissor or microsurgical forceps, testicular tissue or tubules are harvested and placed in buffered sperm media (Fig. 1). Tissue is processed by mincing the tissue with scissors and passage through a 25-gauge angiocath until large chunks of tissue have been eliminated, and a small sample of fluid is exam-

ined under the microscope for the presence of sperm. This procedure also may be completed with exposure of the entire testicle and a longer incision. The benefits of this procedure include a diagnostic benefit as well as therapeutic as testicular pathology can be examined to help determine the underlying histopathologic diagnosis. This does have risk of testicular scarring and injury to nearby structures and does require an open incision with sutures [46].

For men with non-obstructive azoospermia, the gold standard for sperm extraction is micro dissection TESE. This procedure requires an operating room, general anesthetic, and standard operating microscope. The scrotal incision is made along the midline or transversely over one hemiscrotum. Once the testicle is fully exposed, a bi-valving incision is made with an ultra-sharp knife and completed in an equatorial manner along the testicle with care to prevent avulsion of the blood supply. The testicular architecture is first examined without dissection to identify optimal and dilated seminiferous tubules. In a systematic fashion, the tubules are then assessed and bluntly dissected as needed with caution to not disrupt the overall architecture and blood supply (Fig. 3). Bipolar cautery should be used only as needed to prevent damage to tubules. Selected tubules are then processed in buffered sperm media. Processing is a critical component and may impact sperm yield. The specimens should then be examined under a microscope by a trained andrologist or embryologist.

Onco-Testicular Sperm Extraction (oncoTESE)

OncoTESE is a procedure where testicular tissue is harvested from a testis containing malignant tumor after radical orchiectomy [47, 48]. This should only be completed when the surgeon is confident that normal testicular tissue can be obtained a safe distance from the tumor. Testicular tissue can be processed in the operating room at time of radical orchiectomy to examine for sperm in the specimen. Briefly, the tunica albuginea is incised, revealing the underlying seminiferous tubules. As discussed, care is taken not to harvest tissue or seminiferous tubules associate with tumor. The harvested tissue is then processed similar to conventional TESE or mTESE depending on if testicular tissue or single seminiferous tubules are harvested. This method for fertility preservation is useful for patients who have bilateral testicular tumors or metachronous testicular tumors (i.e., radical orchiectomy in a patient with solitary testis) [47].

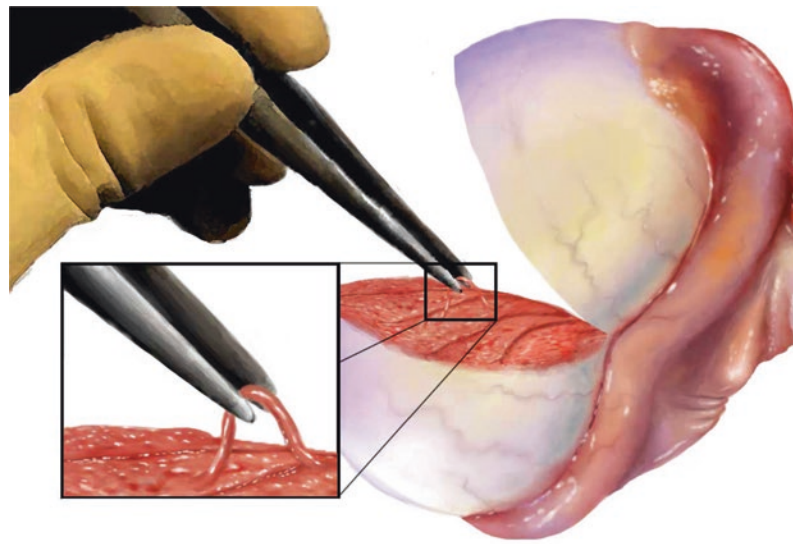

Fig. 3 *Microdissection testicular sperm extraction.* During this procedure, the testis is bivalved to expose the underlying seminiferous tubules. Individual tubules, as depicted, can be selected for processing and examination under the microscope for sperm

Percutaneous Vasal Retrieval

Retrieval of sperm from percutaneous vasal aspiration can be performed on patient with vasal obstruction (vasal aspiration would need to be more toward the testicular vas in relation to the obstruction), ejaculatory duct obstruction, or anejaculation. Vasal aspiration was first described in 1997 in patients with obstructive azoospermia [49]. Of the six individuals who underwent vasal aspiration, four had sperm successfully retrieved from the vas [49]. The procedure is typically performed using local anesthetic in a manner similar to when performing no-scalpel vasectomy. Through a small skin opening, the vas is isolated, and a 21-gauge sharp needle is used to pierce the vasal wall into the vas lumen [50]. A smaller 23-gauge blunt tip needle is introduced through the sharp needle into the lumen of the vas. A 10 ml syringe primed with 1 ml of sperm wash medium is attached to the blunt tip needle, a small amount of medium (0.2–0.3 ml) is injected into the vas, and the fluid is then aspirated into the syringe [50]. Alternatively, the vas can be hemisected with an ultra-sharp blade, vasal aspiration performed, and vasotomy closed with interrupted 10-0 and 9-0 nylon sutures using a standard operating microscope [51]. Although some success has been reported, obtaining high numbers of sperm with this method may be difficult [49]. However, being able to perform this procedure in the clinic setting is an appealing characteristic of the method and may be a more cost-effective alternative in the appropriately selected patient.

Other Sperm Retrieval Methods

Post-Ejaculatory Urinalysis

Retrograde ejaculation results from the improper closing of the bladder neck during ejaculation. A post-ejaculatory urinalysis is required to diagnose men with retrograde ejaculation. Sympathomimetic medications, such as pseudoephedrine, can be given to patients in an attempt to improve antegrade semen volume. These medications act to close the bladder neck by its alpha receptor agonist properties (Table 3). If an antegrade sample is unable to be produced, a retrograde semen sample can be attained and cryopreserved. For retrograde collection, the bladder is emptied immediately prior to semen sample production. Men then attempt to produce a sample by masturbation, and if antegrade ejaculate is produced, this specimen is collected. If no antegrade ejaculate is produced, the bladder is catheterized to retrieve the semen specimen. The sample is then centrifuged and washed. In order to minimize the harmful effects of urine on semen, sodium bicarbonate is often administered prior to sample collection in order to alkalinize the urine. Alternative options include pre-ejaculation instillation of buffered sperm media in the bladder via catheterization [52].

Penile Vibratory Stimulation (PVS)

PVS is a non-invasive technique to retrieve sperm from patients with ejaculatory dysfunction, which is typically characterized by anejaculation. Anejaculation can occur in men interested in fertility preservation due to psychological or physiological factors. No anesthesia is required, and PVS can typically be self-applied by the patient. The ease of use of the vibratory device even allows patients to perform this comfortably in their own homes, which may reduce stress and increase the likelihood of suitable sample collection.

The goal of PVS is to activate the ejaculatory reflex via the afferent penile dorsal nerve, and an intact spinal cord at the level of T11-S4 is required for PVS to be successful [53, 54]. The ejaculatory reflex ultimately stimulates the hypogastric (sympathetic) nerve resulting in seminal emission and the pudendal (somatic) and pelvic (parasympathetic) nerve resulting in projectile ejaculation.

To perform PVS, the individual is positioned in the supine or sitting position. The vibrating disc is placed on the frenulum for period of 2–3 min or until antegrade ejaculation occurs. There is a rest period of 1–2 min between stimu-

lation periods if ejaculation does not occur. Antegrade ejaculation is pulsatile and projectile, similar to normal ejaculation. Erection, muscle contractions, and muscle spasms can be observed during PVS [53]. Specimen should be collected in a similar fashion to semen analysis collection per the WHO standards.

Electroejaculation (EEJ)

EEJ is a relatively non-invasive technique of sperm retrieval for men with anejaculation. In contrast to PVS, EEJ requires general anesthesia and must be performed in an operating room. However, the procedure is relatively quick with minimal risks and generally well-tolerated. For these reasons, PVS often is recommended prior to EEJ, and EEJ is performed only if PVS is unsuccessful or patient prefers to undergo EEJ.

EEJ has been used in men with spinal cord injury and other ejaculatory disorders [55]. EEJ is performed under general anesthesia and involves insertion of a stimulation electrode into the rectum and applying voltage in a pulsatile pattern to the prostate and seminal vesicles. After general anesthesia is induced, the bladder is catheterized and emptied and then filled with a small amount of buffered sperm media. Buffered sperm media can be protective of retrograde ejaculated sperm from harmful effects of acidic urine [56, 57]. Patients are positioned in the left lateral decubitus position to allow for placement of the rectal probe. Rectal mucosa should always be assessed prior to manipulation and at the end of the procedure to rule out injury. Voltage is then applied to the probe as the electrode is in direct contact with the rectal mucosa in the region of the prostate and seminal vesicles. Voltage is applied in a pulsatile pattern and increased gradually (1–2 V increments) until ejaculation occurs [58]. While voltage is being applied, the antegrade fraction of the ejaculate is obtained by manual expression of the fluid along the urethra and then collected into a sterile container. For successful electroejaculation, reported voltage and current range from 5 to 25 V and 100 to 600 mA, respectively [59]. After the antegrade fraction is collected, the probe is removed, the patient is returned to supine position, and the bladder is catheterized for the retrograde fraction of the ejaculate. A small sample of the specimen is evaluated microscopically for sperm.

Sperm retrieval in anejaculatory men is typically successful after EEJ [60, 61]. However, despite the electroejaculate having normal sperm numbers, motility and morphology may be poor, and functional deficiencies may be noted [60]. The fertilization rate using sperm obtained by EEJ is generally low; however, using electroejaculated sperm with IVF and ICSI can result in fertilization rates of approximately 60–75% and clinical pregnancy rate 29–53% [60, 62, 63]. Reasons for low fertilization rates can be due to the procedure itself but also can be due to intrinsic sperm defects that are responsible for anejaculation. Another explanation is that many of these men have very long periods of abstinence and perhaps may have improved parameters with repeated procedures. Finally, heat and current from the EEJ probe may cause a reduction in the numbers of motile sperm, biochemical changes, and production of immature sperm or induce formation of anti-sperm antibodies [62, 64].

Special consideration must be taken in completing EEJ in those with spinal cord injury. EEJ can induce autonomic dysreflexia in men with injuries above T6 [65]. Men who are susceptible to autonomic dysreflexia can be pre-medicated with nifedipine and should be monitored closely and counseled pre-operatively.

Special Considerations in Prepubertal Males

An estimated 11,000 children under the age of 15 years will be diagnosed with cancer in 2020 [66]. The calculated 5-year survival rate for cancers in this age group is approximately 80% (measured from 2007 to 2013) [66]. Thus, although the overall incidence of cancer is increasing slightly in the pediatric population, improvements in cancer treatments are increasing the number of cancer survivors. And appropriately, the emphasis on short-term survival benefits has shifted to a focus

on survivorship and long-term effects of cancer treatments. Thus, fertility preservation among adolescent and young adult cancer patients has become a significant area of interest and research. ASCO recommends that healthcare providers initiate discussions regarding the possibility of infertility with parents or guardians and patients planning to undergo cancer treatment during their reproductive years [67]. In this discussion, fertility preservation options should be explained and/or a referral made to a reproductive/fertility specialist [67].

The onset of male puberty occurs around the age of 11 years and involves hormonal, physical, and cognitive changes. From a fertility standpoint, puberty is characterized by a rapid increase in spermatogonial density and testicular volume [68]. Tanner staging (Table 2), or sexual maturity assessment, is an easy and effective way to assess secondary sex characteristics that develop during puberty [26]. However, age and testicular size are the best predictors of active spermatogenesis [69]. Males younger than 12 years or with testis size less than 7 ml are unlikely to succeed in producing a semen sample [70]. In addition to physical barriers, psychological barriers also exist as prepubertal boys may feel embarrassment or anxiety in discussing fertility preservation with parents present. Allowing to collect at home may be helpful in reducing stress. Informed consent should be obtained from a parent or guardian, and instructions explained to the patient in private [71].

No current recommendations exist for fertility preservation in prepubertal boys. All current methods of assisted reproductive technology require mature spermatozoa which is not present in this cohort. Currently, the strategy for fertility preservation in prepubertal boys includes cryopreservation of immature testicular tissue, which remains experimental, with hopes that further research will allow for the future use of this tissue in fertility treatments [44]. And although harvesting immature testicular tissue requires general anesthetic, this procedure can be bundled with other potential procedures that need to be performed for cancer staging and treatment (e.g., lumbar puncture, bone marrow biopsy, port or central line placement).

Conclusion

As the number of cancer survivors increases secondary to improvements in cancer treatments, fertility preservation is an important future consideration that warrants early discussion prior to initiation of anti-neoplastic agents and treatments. Referral to a fertility specialist and/or discussion of fertility preservation by cryopreservation of sperm or testicular tissue is recommended. The indications for surgical and non-surgical sperm retrieval methods are patients with ejaculatory dysfunction and obstructive or non-obstructive azoospermia. Various methods exist to retrieve sperm or testicular tissue from cancer patients so that these samples can be used for future fertility use.

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Testis-Sparing Surgery

Eric Huyghe

Search Strategy and Selection Criteria

Data for this review were identified by searches of MEDLINE, PubMed and references from relevant articles using the key words 'testicular neoplasms' and 'testis-sparing surgery'. Only papers published in English between 1980 and 2020 were included. From 137 papers, we selected 50 articles devoted to testis-sparing surgery or giving new and additional information, retained for their methodological quality and their first importance results.

Introduction

Testicular cancer is the most common malignancy in young men under the age of 40, and its incidence has been increasing for several decades in nearly all industrialized countries [1, 2]. Malignant testicular tumours are almost exclusively germ cell tumours, which are divided into seminoma and non-seminomatous germ cell tumours. Advances in treatment over the last 50 years have improved life expectancy, and their 10-year cancer-specific survival is currently over 95% [3]. Germ cell tumours represent about 90% of all testicular tumours, and the remaining 10% are predominantly benign tumours, the most frequent type being Leydig cell tumour. In these patients, fertility is a major preoccupation as they are often young men, without children, or considering having children in the future [4]. Synchronous bilateral testicular tumours (which means that tumours are found in both testicles at the same time) are rare, accounting for only 0.5–1% of all testicular cancers [5]. Metachronous bilateral testicular cancers are more frequent, occurring in 5–6% of men with a unilateral tumour [6]. Literature reveals that most metachronous bilateral testicular cancers are seminomas and occur on an average after 4 years [7, 8]. In the case of germ cell tumour occurring in a solitary testicle or in both testicles, bilateral orchidectomy is no longer the standard treatment, as it results in lifelong androgenic insufficiency, azoospermia and psychological stress [9].

Whenever possible, testis-sparing surgery (TSS) should be considered, as it is the only way to avoid lifelong androgen replacement therapy and may allow to preserve a chance for the patient of fathering a child. Considering that non-palpable tumours are showed to be benign in nearly 80% in the general population [10, 11], and in infertile men [12, 13], assigning TSS as a first-line treatment in these cases is tempting. In this review, we will precise the indications of

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TSS, its technique, outcome, adjuvant treatments and follow-up after TSS.

Indications of Testis-Sparing Surgery

In the General Population

Choice of performing a testis-sparing surgery rather than radical orchiectomy should be a shared decision made between the physician and patient, after thorough information, notably concerning the risk of recurrence and the need for close long-term follow-up.

According to the European Association of Urology Guidelines, TSS can be performed when tumour volume is less than 30% of the testicular volume and when the following selection criteria are met: synchronous bilateral tumours, metachronous contralateral tumours or solitary testicular tumour with normal preoperative testosterone levels [14]. The proposed tumour size cutoff is 20 mm, as feasibility is not guaranteed above. Paffenholz et al. proposed the threshold of 2.8 cm^3 (about 17 mm) as a significant predictor for malignancy (sensitivity 83%, specificity 89%, OR 1.39 [95% CI 1.04–1.86], $p = 0.03$) [15]. Rather than the tumour diameter, it is certainly the percentage occupied by the lesion in the testis that matters as suggested by Weissbach et al. [16]. Additionally, TSS has been proven safe in Leydig cell tumours ranging from 13 to 16 mm, with no malignant long-term recurrence (up to 4 years) [17–19].

In Infertile Men

The aforementioned criteria remain valid in the infertile population. The population of infertile men is characterized by:

- A high risk of developing a testicular tumour, as shown by Bieniek et al. in a series of 4088 infertile men where 120 (2.9%) were holding a testicular tumour below 1 cm [20]. In this population, Leydig cell tumours (mostly benign) and germ cell tumours (malignant) are both relatively frequent, due to common risk factors between germ cell tumours and male infertility (cryptorchidism, microlithiasis, gonadal dysgenesis syndrome or Klinefelter syndrome) [21–23]. Raman et al. described that men with abnormal semen analysis have a 20-fold greater incidence of testicular cancer compared to the general population [24].
- A great variability of the proportion of malignant tumours in this population [12, 24–26]. A recent French multi-centre cohort study found that 25% of incidental testicular tumours were malignant, which is close to the 36% of germ cell tumours identified by Bojanic et al. [27].
- A majority of small non-palpable testicular masses, discovered incidentally during the evaluation of infertile men. The widespread use of high-frequency ultrasound has led to an increasing diagnosis of incidental small testicular masses [28]. On the contrary to palpable testicular tumours that are malignant in 95% of cases [29], a high percentage (about 80%) of these non-palpable testicular lesions are histologically benign [13, 30, 31], making TSS [12, 28] and active surveillance [20, 32, 33] two attractive options.

So far, in many cases, it is difficult even for the trained sonographer to say formally that the tumour is benign on a single exam [20]. It is often the evolution that guides the decision. Performing a TSS followed by a frozen section analysis is often preferred to ultrasonographic surveillance when tumour size is above 5 mm, in the case of tumours growth or ultrasound risk criteria.

A retrospective 11-year review was conducted by Li et al. [32] in 101 men diagnosed with a small testicular lesion on scrotal ultrasonography. Treatment was immediate surgery for 17 (16.8%) patients, delayed surgery after ultrasound follow-up ranging from 1 to 7 months for eight (7.9%) patients and surveillance only for 76 (75.3%) patients of 101 with ultrasound follow-up of 6–84 months. Logistic regression analysis showed that lesion size was the only independent risk factor for malignancy in hypoechoic small

testicular lesions ($p < 0.05$). Most of the small testicular lesions were stable on serial sonograms and likely benign.

Toren et al. [33] retrospectively analysed a series of 46 incidentally discovered small hypoechoic testicular lesions diagnosed at Mount Sinai Hospital Fertility Clinic from 2001 to 2008. Male infertility was diagnosed in 39 patients (85%) (azoospermia in 15, oligospermia in 18 and normospermia in seven patients). The mean follow-up was of 253 days, and the mean number of ultrasounds was 2.8. Mean lesion diameter was 4.3 mm (ranging from 1 to 10). The mean growth was of 0.5 mm per year (95% CI −2.2–3.3). Three patients underwent immediate surgery, and five delayed surgery after a period of ultrasound follow-up. Indications for surgery were tumour growth, patient choice, larger size and presence of vascular flow. Most excised masses were benign.

Of 4088 men in whom scrotal ultrasound was completed for male infertility evaluation, Bieniek et al. [20] found that 120 (2.9%) had a subcentimetre testicular mass. Average follow-up was 1.30 years (range 0.1–16.9). A total of 18 men (15%) proceeded to extirpative surgery, while 102 remained on surveillance at the last follow-up, with a mean lesion growth rate of 0.01 mm per year. Reasons for surgery included testicular exploration for infertility, mass growth, positive tumour markers, history of testis cancer, concerning imaging characteristics and patient choice. One third of men who underwent surgery were found to have malignancy, which was seminoma in all. All malignant lesions were greater than 5 mm on initial imaging and demonstrated vascularity. Despite this result, so far, the threshold size below which monitoring can be proposed remains imprecise.

Technique of Testis-Sparing Surgery

The technique of inguinal partial orchidectomy has been described in detail by Weissbach [16]:

1. Surgical approach. Through an incision in the groin, identical to that of the radical orchidectomy (Fig. 1), the external oblique fascia is incised. Then, the spermatic cord is isolated with a Penrose drain (Fig. 2). Dissection of testicular blood supply has to be meticulous. The testicle inside the vaginal tunic is then separated from the scrotal skin by carefully dissecting the gubernaculum to avoid tearing the scrotal skin. Sterile drapes are then placed around the skin incision to protect it from involuntary exposure of the tumour (Fig. 3).

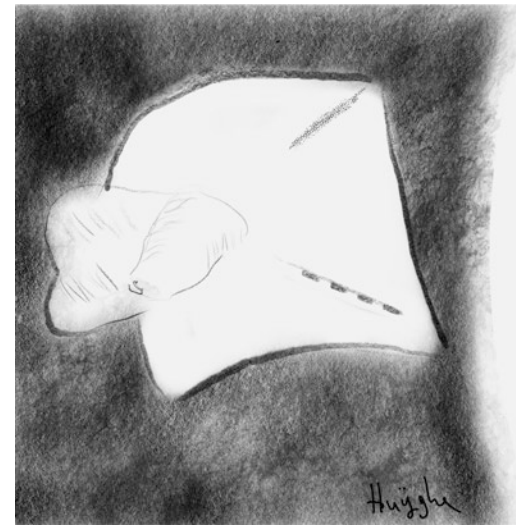

Fig. 1 Surgical incision in the groin

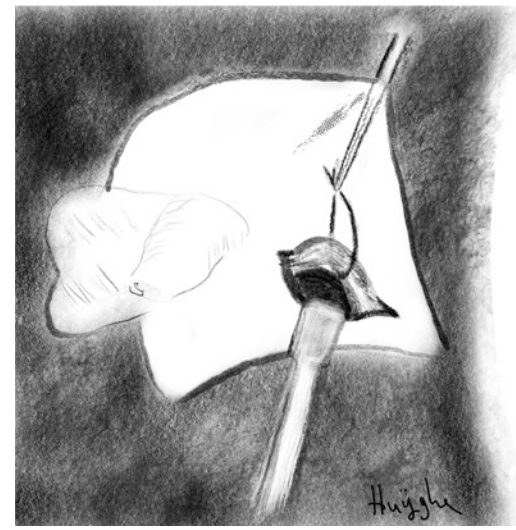

Fig. 2 Dissection of the spermatic cord and isolation with a Penrose drain

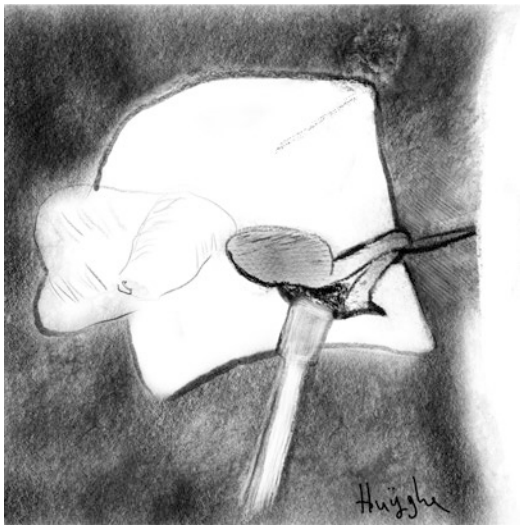

Fig. 3 Extraction of the testis through the inguinal incision after section of the gubernaculum testis

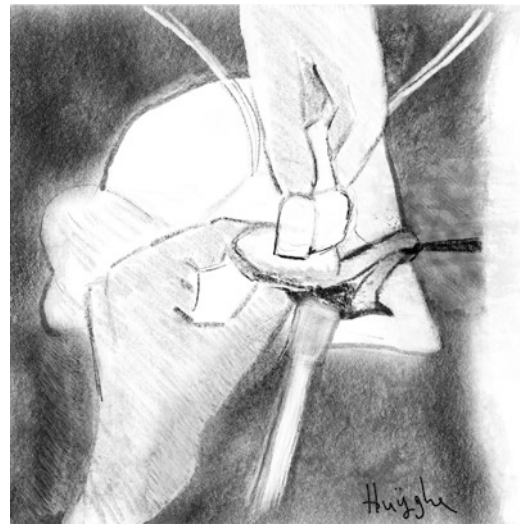

Fig. 5 Usage of the intraoperative Doppler ultrasound to localize the tumour and to preserve the intratesticular vessels

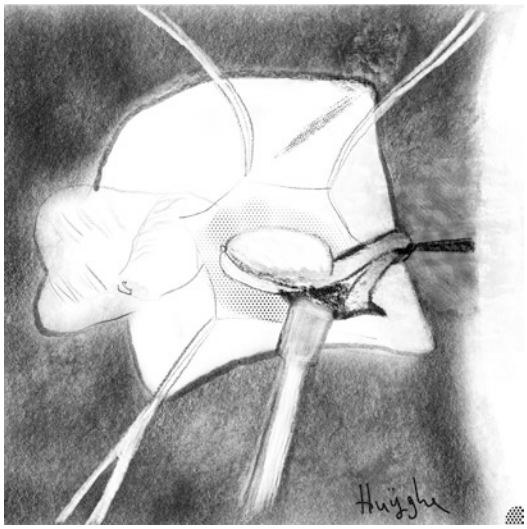

Fig. 4 Opening of the tunica vaginalis

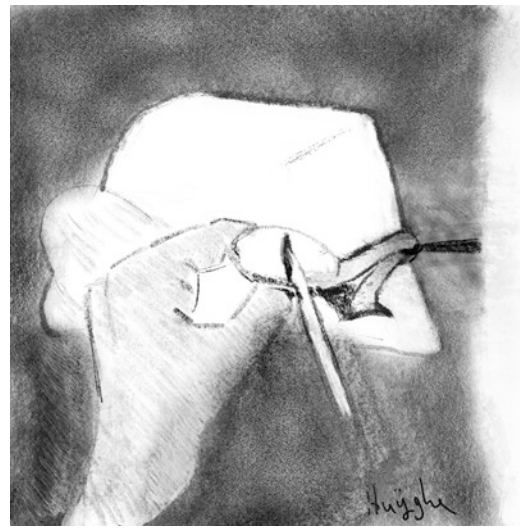

Fig. 6 Incision of the albuginea tunic

2. Exposure of the tumour and separation of the tumour from the surrounding healthy parenchyma. The tunica vaginalis is opened (Fig. 4), and the testis is exposed in the operating field. Intraoperative Doppler ultrasound is useful for planning the excision strategy to localize the tumour in the case of small, non-palpable lesions and to preserve the intratesticular vessels (Fig. 5). In the first case, it is possible to use hook wire guides for the detection of neoplasia [34]. Then, the tunica albuginea is incised (Fig. 6), and the tumour is isolated (Fig. 7). In the initial technique, the pedicle was clamped, and the testis was maintained in cold ischaemia while waiting the results of extemporaneous analysis. However, findings of a series of 33 testis-sparing surgeries without clamping, showing that all men were free of disease at a median follow-up of

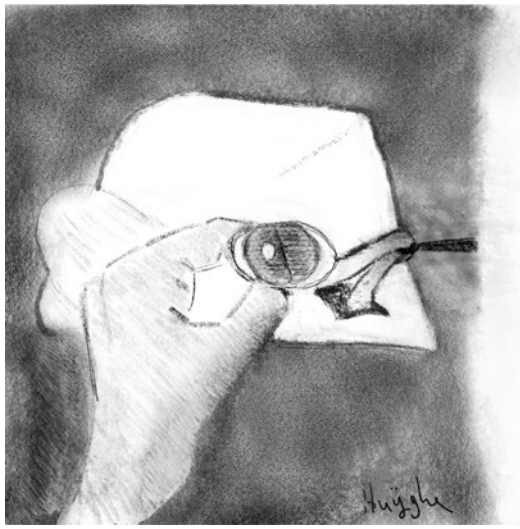

Fig. 7 Isolation of the tumour

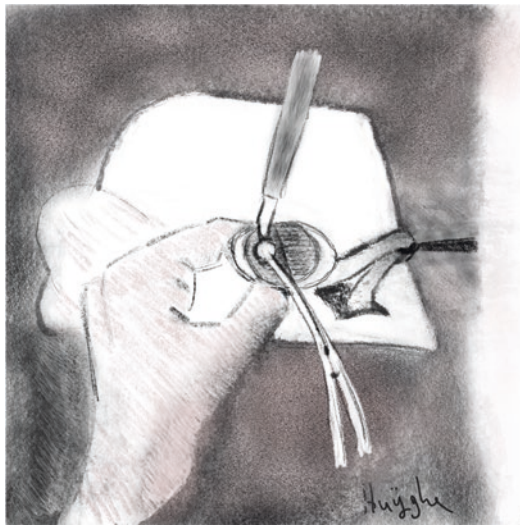

Fig. 8 Tumour enucleation

52.5 months, suggest that a 'no-clamping' OSS technique is safe and feasible [35].

Tumour enucleation (Fig. 8). The manoeuvre is facilitated by the presence, in most cases, of a pseudocapsule. It is important to avoid violating the tumour capsule because this can increase the risk of local recurrence. Extemporaneous histological examination is systematically performed to verify that the margins are negative and to know if the tumour is malignant or benign. In the French collaborative study [36], frozen section analysis exhibited a high positive predictive value (92%) and sensitivity (80%). Other series showed even higher sensitivity of about 100% [29, 37].

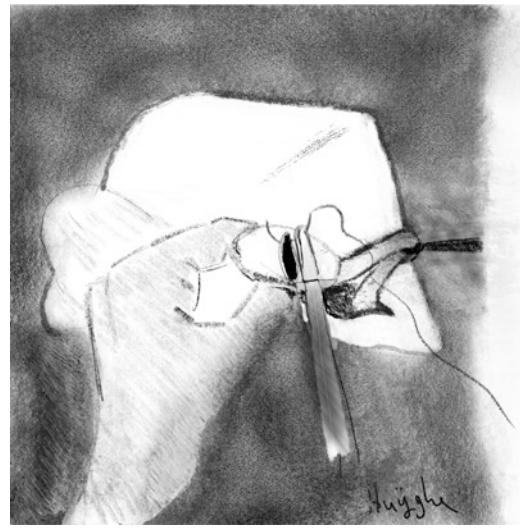

Fig. 9 Closure of the tunica albuginea with a reabsorbable suture

3. Biopsies of the resection bed. After removal of the tumour, biopsies of the resection bed are performed, due to the high incidence (80–90%) of areas of ITGCN (intra-tubular germ cell neoplasia) in the surrounding parenchyma [16, 38].
4. Surgical closure time. After ensuring that the resection bed is negative for neoplasia, the tunica albuginea is closed with a reabsorbable suture (Fig. 9), and the testis is relocated in the scrotum [39].

Oncological Outcome of Testis-Sparing Surgery

In Germ Cell Tumours

So far, only a few series of germ cell tumours treated by testis-sparing surgery have been published. In this population, given the high prevalence of ITGCN lesions, what literature evolves is mainly the outcome of a strategy combining conservative surgery followed by radiotherapy.

The German Testicular Cancer Study Group published a series of 101 men with GCT, including patients with GCT bilaterally or in solitary testicles with a tumour volume <75% of the testicle, who were treated by TSS in eight centres [38]. The average tumour size was 15 mm (range: 5–30 mm). During surgery, multiple biopsies of the surgical bed were performed to reveal concomitant ITGCN lesions, and local adjuvant radiotherapy with an 18 Gy dose was administered to 80 patients with ITGCN. After an average follow-up of 80 months, the cancer-specific survival was excellent (100/101), with a low rate of local recurrence (6 out of 101). Recurrence occurred in four patients who refused adjuvant radiotherapy.

With regard to ITGCN management, an American cohort [30] showed the outcome of a cohort in which a greater number of men choose not to receive radiotherapy compared to the German study. Seventeen men had malignancy, including seminoma in nine, teratoma in three, embryonal lesion in one, Leydig cell tumour in three and carcinoma in situ in one. Frozen section was accurate, no positive margins were reported, and all tumours were stage 1. Carcinoma in situ was found in nine patients (53%). No perioperative complications were recorded. Management after TSS was observation in 12 of 17 cases. Two patients underwent completion orchiectomy for local recurrence of carcinoma in situ only, including chemotherapy in one. A patient with seminoma elected radiation, and one required retroperitoneal lymph node dissection for teratoma. The remaining five patients with carcinoma in situ were surveilled. Of the men, 31% required testosterone substitution. All patients were disease-free at a median 5.7-year follow-up with no local recurrences. The authors recommended observation in patients at low risk of local recurrence, with high compliance to follow-up and with a postoperative negative ultrasound, and concluded that focal organ-conservative surgery remains a valid option for the reduction of morbidity but is potentially associated with oncological recurrence and requires adjuvant treatment and androgenic replacement therapy.

Outcome in Leydig Cell Tumours

Leonhartsberger et al. [31] presented the results of a series of 40 patients with Leydig cell tumour who were treated with testis-sparing surgery. At a median time of 63 months after treatment, 37 of them had no recurrence of disease, and three had Leydig cell tumour recurrence in the contralateral or ipsilateral testicle and therefore underwent conservative organ surgery again.

Another small series of 16 men with Leydig cell tumour treated between 1992 and 2008 also suggested that in patients with 25 mm Leydig cell tumour, testis-sparing surgery is a safe treatment option from an oncological point of view, if a reliable extemporaneous histological examination is available [18]. Accurate perioperative distinction between Leydig cell tumour and germ cell tumours is crucial and perhaps one of the most important limiting factors in the use of testis-sparing surgery in clinical practice. Finally, as Leydig cell tumours can rarely be malignant, with a high risk of metastases in this case, the follow-up of Leydig cell tumour patients should take into account the possible late onset of metastases.

Adjuvant Treatments After Testis-Sparing Surgery in Germ Cell Tumours

Radiotherapy

Radiotherapy for testicular ITGCN was first described in 1986 by von der Maase and colleagues [40]. After partial orchiectomy, the testicle is typically treated with a 20 Gy dose if one of the adjacent six quadrant biopsies demonstrates ITGCN unless the patient desires paternity [16, 41]. A lower radiation dose can compromise oncologic control, and, therefore, most studies have advocated 18–20 Gy [42], with few case reports of radiation therapy failure in the literature [43, 44].

Chemotherapy

Whether cisplatin-based chemotherapy may eradicate testicular ITGCN is controversial.

Kleinschmidt and colleagues [45] have studied the eradication of ITGCN after administering carboplatin or a combination of cisplatin, etoposide and bleomycin (PEB). At an average of 8.8 months post-treatment, biopsy revealed ITGCN in 7 of the 11 studied patients. Possible explanations may be the blood-testicular barrier, which hinders the spread of chemotherapy, or the possibility that ITGCN is resistant to chemotherapy.

In a Dutch population-based study, van Basten et al. noted that the incidence rate of contralateral testicular cancer was three times lower in the chemotherapy arm than in the surveillance arm [46]. In another study, Fossa et al. [47] also noted a lower incidence of contralateral testicular cancer attributed to the benefit of platinum-based chemotherapy. Anyway, after chemotherapy, careful follow-up remains strongly recommended [12].

Follow-Up

Active surveillance through repeated scrotal ultrasounds has been described and evaluated for TT $\leq$1 cm complemented by negative markers and appears to be safe (mean growth rate being identified as -0.01 ± 2.36 mm per year and orchiectomy was performed on growing masses) [19, 27]. However, long-term outcome data for recurrences in patients undergoing this testis-sparing surgery are few in number. In one small study of 73 partial orchiectomy patients with a mean follow-up time of 91 months, four patients who did not receive postsurgical radiation had a local recurrence of disease between 3 and 165 months after resection and were subsequently treated with a radical orchiectomy eliminating the disease [38]. One patient from this series had systemic recurrence and died of the disease. Therefore, the inability to reliably have long-term follow-up should be considered as a contraindication to providing testis-sparing surgery, especially if adjuvant radiation is not done after confirmation that it is a germ cell tumour.

Clinical Case

A 28-year-old patient had a left inguinal orchiectomy for a pure seminoma. Initial thoracoabdominal CT scan tumour markers (HCG, AFP, LDH) were negative, and surveillance was indicated. This patient conserved 12 semen straws usable in ICSI (semen analysis showed severe oligospermia at 0.06 million per mL and 35% progressive motile sperm).

Three years later, a second lesion was discovered at the lower pole of the contralateral testicle. This lesion was palpable on clinical examination. It measures $10 \times 9 \times 11$ mm. The patient was asymptomatic with a total testosterone level at 3.6 ng/mL. He was compliant with the terms of regular monitoring. Thus, the decision shared with the patient was to perform a testis-sparing surgery.

Frozen section analysis concluded that it was a germ cell tumour and that surgical line was not in contact with the tumour proliferation. Definitive anatomopathological examination concluded that it was a pure seminoma measuring 12-mm-long axis.

No vascular embolus was observed.

Stage pTNM was pT1a (within the limit of partial orchiectomy).

In the testicular parenchyma adjacent to the nodule, intra-tubular germ cell neoplasia (ITGCN) was present in a few seminiferous tubes.

Proposal of the multidisciplinary meeting was complementary treatment by testicular radiotherapy or chemotherapy in order to prevent the risk of subsequent evolution of ITGCN.

The patient refused this proposal, arguing that he feared the side effects of radiotherapy, notably impact on endocrine function.

Two years later, ultrasonography revealed three nodules, the largest measuring 5 mm.

Orchiectomy was performed, and anatomopathological examination (Fig. 10) concluded the existence of three

pure seminomas [48], measuring 5 mm, 3 mm and 3 mm, respectively, without vascular invasion. Healthy spermatic cord cut. Stage TNM [49]: UICC classification was pT1a R0.

Non-tumoural testicular parenchyma showed focal atrophy with tubal fibrosis, Leydig cell hyperplasia and presence of ITGCN.

Fig. 10 Macroscopic aspect of a seminomatous tumour

Conclusion

The potential benefits of testis-sparing surgery over radical orchiectomy are preservation of fertility and endocrine function, genital anatomical integrity and male body image.

A way to preserve fertility in patients with testicular cancer is to maximize the amount of viable tissue available for spermatogenesis, by considering partial orchiectomy in some cases.

However, so far, indications for partial orchiectomy remain limited:

- Tumour on a single testicle
- Synchronous bilateral tumours
- Strong suspicion of benign lesion

The procedure requires:

- Highly selected, motivated, monitorable patients with a single testicle (synchronous or metachronous bilateral lesion)
- Normal preoperative endocrine function
- Tumour volume less than 30% of gonadal volume
- A tumour diameter of less than 2 cm
- An extemporaneous analysis
- Cold ischaemia during the procedure
- Biopsies of adjacent testicular tissue to ensure negative margins

Criteria for safe testis-sparing surgery include: small tumours (<20 mm), ultrasound detected, non-palpable and limited risk factors such as cryptorchidism or microlithiases [11, 12, 25, 27].

The procedure is performed using the same inguinal approach as for a total orchiectomy. Intraoperative ultrasound can be used to help define the margins of the tumour for excision planning.

The discovery of a Cis on remote biopsies or on the periphery of the tumour should be treated with a postoperative scrotal irradiation. This adjuvant treatment will make it possible to preserve the endocrine function of the testicle but without maintaining spermatogenesis.

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Fertility Preservation in Hypogonadal Men

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Abbreviations

AAS	Anabolic-androgenic steroids
AI	Aromatase inhibitor
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
HH	Hypogonadotropic hypogonadism
ICSI	Intracytoplasmic sperm injection
IHH	Idiopathic hypogonadotropic hypogonadism
LH	Luteinizing hormone
NOA	Non-obstructive azoospermia
RCT	Randomized controlled trial
rhFSH	Recombinant human FSH
ROS	Reactive oxygen species
SERM	Selective estrogen receptor modulator
T	Testosterone
TST	Testosterone supplementation therapy

The following chapter contains definitions, clinical tips, final messages, and key readings providing a well-rounded approach to fertility preservation in hypogonadal men.

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Introduction

The post-millennial rise in the use of exogenous testosterone for symptoms related to hypogonadal symptoms of fatigue, depression, decreased libido, and erectile dysfunction has grown threefold in men over 40 years of age in the last two decades [1]. Exogenous testosterone therapy can alter the natural regulation of the hypothalamic-pituitary-gonadal axis leading to impaired spermatogenesis with azoospermia being a serious possible result, thus rendering the individual infertile. Beyond prescribed testosterone supplementation treatment (TST), anabolic steroids are frequently used by young high school athletes. In the United States, estimates of individuals who use anabolic steroids range from one to three million people [2]. This combined rise in the illicit use of anabolic steroids and the frequency with which TST supplements are prescribed have serious consequence on the future fertility issue of the given patients. In addition, the later age of fatherhood has resulted in a paradigm shift away from universal testosterone prescriptions for all patients with symptoms of hypogonadism without an initial serious conversation about the interest of the patient in his future fertility [3]. This chapter reviews the epidemiology of hypogonadism and then subdivides the treatment strategies into those men with hypogonadism unrelated to androgen usage, and the second part addresses what is known about treatment options in lieu of

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testosterone for men who had been on androgen. There are some newer testosterone therapies that may allow hypogonadal men to concurrently maintain spermatogenesis as well as receive testosterone supplementation therapy and thus preserve the ability to initiate a pregnancy.

Epidemiology and Pathogenesis of Male Hypogonadism

Ninety-five percent of the serum testosterone (T) in males is synthesized by the Leydig cells of the testis under the influence of luteinizing hormone (LH) secreted from the pituitary gland. Testicular failure is defined as the impairment or loss of both endocrine function of the testis (production of testosterone or T) and exocrine function (production of spermatozoa). Male hypogonadism, the clinical syndrome with variable symptoms associated with low testosterone, can affect men of all ages. Male hypogonadism is the clinical condition representing a constellation of symptoms along with a measured decreased T. The normal range for morning T in a male is between 300 and 1000 ng/dL [4] with hypogonadism being traditionally defined as total T <300 ng/dL [5]. Hypogonadism can be subdivided into primary or secondary etiologies. Primary testicular failure is characterized by normal/low T in the presence of elevated follicle-stimulating hormone (FSH) indicating intact feedback loops to promote spermatogenesis and testosterone production in the central nervous system. The age-related decrease in testosterone apparently reflects adulthood cellular degeneration, reduced number of functional Leydig cells, and atherosclerosis of testicular arterioles [6]. The difference between the decline of TT and free testosterone over the aging process is explained by the age-related increase in circulating concentration of sex hormone-binding globulin (SHBG), which reduces the proportion of free testosterone [7]. In healthy men, the age-related decline of testosterone coupled with an increase in LH supports a diagnosis of primary testicular failure compensated for by an increased LH secretion [8]. The age-related decrease in testosterone apparently reflects general age-related cellular degeneration, reduced number of functional Leydig cells, and atherosclerosis of testicular arterioles [6].

The prevalence of androgen deficiency in the United States is substantial. In men 20–45 years of age, male hypogonadism occurs in approximately 3–8%; however, the incidence approaches 39% in men aged 45 years or older in a multicenter study of men presenting to primary care centers [9, 10]. In addition, the Massachusetts Male Aging Study estimated that approximately 2.4 million 40- to 69-year-old men in the United States have androgen deficiency and concluded that the rate increased significantly with age [11]. Based on large-scale population studies, the use of exogenous testosterone in this same age group has increased exponentially in the last decade, as much as threefold [1]. The widespread use of TST on the part of urologists and primary care providers in men with hypogonadism for the treatment of symptoms including decreased libido, fatigue and exercise capacity, depression, and erectile dysfunction has significant implications on potential fertility. Due to the known negative feedback inhibition of exogenous testosterone on the normal intratesticular testosterone production, spermatogenesis is appreciably diminished in this cohort. The same phenomenon is observed in active individuals and athletes using anabolic steroids.

In contradistinction, in younger males and adolescents, the etiology of hypogonadism is a result of congenital or acquired conditions that disrupt the testis production of testosterone or signaling from the hypothalamic-pituitary-gonadal axis. These etiologies may be acquired or congenital, though congenital perturbations are more common. Conversely, low serum FSH, luteinizing hormone (LH), and T correspond to a state of hypogonadotropic hypogonadism. This state may arise as a result of congenital gonadotropin-releasing hormone (GnRH) deficiency, an array of central nervous system disorders, or systemic diseases impacting the normal hypothalamic-pituitary axis and in many cases is

idiopathic. However, in the developing world, a number of increasing conditions may be shifting the prevalence of hypogonadism away from traditional central explanations to a population healthcare crisis.

Beyond the senescent testis and the escalating use of steroids, the growing rise of obesity in the young adult population is of significant concern not just for the obvious deleterious effects on the cardiovascular system but the entire microvasculature and late effects on testicular blood flow. The prevalence of obesity in young adults is increasing at a staggering rate and is anticipated to triple within the next decade [12]. A study looking at the adolescent and young population utilizing the National Health and Nutrition Examination Survey (NHANES) demonstrated a statistically significant increase in BMI from 1999 to 2016 [12]. In the European Male Ageing Study, 73% of men with reduced testosterone were overweight or obese, and serum testosterone in men with a BMI >30 kg/m^2 was on average 5 nmol/L lower than those with normal weight [13]. The exact mechanism for the hormonal reduction in obesity is not completely understood but may relate to the rise in the adipose peptide leptin which is needed for the normal function of the hypothalamic-pituitary gland [12]. Often accompanying obesity is the presence of diabetes. Type II diabetes has gone from a condition seen in grandparents to that in our grandchildren. Among pre-adolescents and adolescent aged 10–19, the rate of new diagnosed cases of type II diabetes rose most sharply in Native Americans (8.9%), Asian Americans/Pacific Islanders (8.5%), and non-Hispanic blacks (6.3%) [14]. A recent study by Chosich and colleagues that hyperinsulinemia in conjunction with elevated serum lipid levels suppress gonadotropin release at the pituitary level provides a mechanistic explanation to the low androgen levels in this cadre of patients [15]. With the rising epidemic of adolescent and young adult obesity and type II diabetes, it is highly plausible that these conditions in isolation or in tandem may explain lower than normal androgen levels in patients 20–40 years of age [16].

Diagnosis

The symptoms of low testosterone in males can be very challenging to diagnose. Given the numerous pathways within the pituitary-hypothalamic-gonadal axis and the slow changes to the hormonal levels, the signs and symptoms suggestive of androgen deficiency take time to clinically manifest. The signs and symptoms of low androgen levels include reduced sexual desire and activity, erectile dysfunction, decreased spontaneous erections, incomplete or delayed sexual development, eunuchoidism, small testes, gynecomastia, loss of body hair/reduced shaving, subfertility, reduced bone mass, and hot flushes/sweats. Less specific symptoms and signs in the older population are decreased energy and motivation, reduced physical performance, depressed mood, poor concentration and memory, sleep disturbances, anemia, reduced muscle mass and strength, and increased body fat. This is clinically different than in younger males where the more likely clinical complaint is that of low energy.

The confirmatory laboratory test required to support the clinical suspicion should be a fasting serum testosterone level between 7:00 and 11:00 AM. While consensus lacks for the exact biochemical level at which to ascribe the diagnosis of hypogonadism, a recent publication by the Endocrine Society with support from the US Centers for Disease Control and Prevention has a level <264 ng/dL in non-obese males as diagnostic for androgen deficiency [5]. According to the European Male Ageing Study, at least three clinical sexual symptoms should be present in conjunction with the laboratory abnormal values to confirm the diagnosis of androgen deficiency [8]. Symptoms of fatigue and lack of energy may be more specific in the younger adult cohort than lack of sexual symptoms. Following confirmation of low serum testosterone levels and concomitant signs and symptoms of hypogonadism, clinicians should use serum LH and FSH in conjunction with testosterone to differentiate between primary and secondary hypogonadism.

Treatment Options

Treatment of the underlying disorder, when identifiable, may allow for restoration of the normal hormonal axis with subsequent improvements in endogenous testosterone production and spermatogenesis. In general, patients with secondary hypogonadism have decreased GnRH, low or inappropriately normal FSH, and low LH levels with low TT. Patients with primary hypogonadism have elevated GnRH, high LH and FSH, and low TT. The remainder of this chapter focuses on contemporary treatments available for male hypogonadism and fertility preservation. Pharmacologic and dietary modifications to reduce the incidence of obesity and diabetes should obviously be addressed as the initial management strategy in those with an elevated BMI or HgBA1C. Outside of these modifying identifiable risk factors, the treatment of hypogonadism is rather extensive with some newer therapies coming to the bedside. The following sections distinguish between men with hypogonadism interested in fertility preservation NOT on androgen supplementation and those patients with androgen deficiency on androgen therapy.

Fertility Preservation in the Hypogonadal Man *Naïve* to Androgen Supplementation

Hypergonadotropic Hypogonadism (Primary Testicular Failure)

Sample Case

JR is a 26-year-old male with known pediatric history of cryptorchidism who presents with the chief complaint of infertility. His labs demonstrate low testosterone, high FSH, and high LH. Sperm analysis reveals azoospermia. Clinical diagnosis of Klinefelter syndrome confirmed with karyotype. This case represents hypergonadotropic hypogonadism, a disease of primary testicular failure.

There are various causes of primary testicular failure, and these include gonadotoxins (e.g., alcohol, nicotine, anabolic steroids, marijuana, alkylating agents, radiation), genetic abnormalities (e.g., Klinefelter syndrome), and absent testes and non-functioning testes (cryptorchidism, bilateral atrophy, bilateral torsion). Currently, there is no endocrine therapy available for the treatment of infertility in men with primary testicular failure. The options available to aid in our patient's fertility are limited and include artificial insemination with donor semen adoption, in vitro fertilization (IVF), or intracytoplasmic sperm injection (ICSI). Primary testicular failure, as well as those with normal hormones but oligospermia, may benefit from recombinant follicle-stimulating hormone (FSH) therapy prior to IVF/ICSI [17]. Furthermore, the sperm retrieval rate in Klinefelter patients with profoundly low serum testosterone levels <15.6 nmol/L had a 72% sperm retrieval rate using testicular sperm extraction after receiving aromatase inhibitors (*discussed below*), and 69% had adequate sperm for ICSI [18]. Patients with primary testicular failure are limited in their treatment options, and although expensive, they have some promising outcomes in aiding these patients with fertility.

Secondary Hypogonadism (Hypogonadotropic/Normogonadotropic Hypogonadism) Low Testosterone/Low or Normal FSH/LH

Sample Case

JR is a 28-year-old male with no past medical history and is being evaluated for infertility. He underwent normal pubertal development. Physical exam reveals normal

testicular size. Labs reveal a testosterone less than 30 ng/dL. FSH and LH are normal. Sperm analysis reveals less than five million sperm/ejaculate. MRI reveals normal appearance of the hypothalamus and pituitary regions. The diagnosis of idiopathic hypogonadotropic hypogonadism is made. Hypogonadotropic hypogonadism may be idiopathic or due to congenital deficiency of GnRH (Kallman's syndrome), central nervous system neoplasm, systemic disease such as sarcoidosis or hemochromatosis, and acquired causes that lead to the suppression of gonadotropins or damage to gonadotroph cells. Unlike primary testicular failure, hypogonadotropic hypogonadism is amenable to medical treatments, which are described in detail below.

GnRH

GnRH is one option if the patient has a hypothalamic cause for hypogonadism. Patients with a pituitary etiology for their hypogonadism will require gonadotropins (*discussed below*) to stimulate the testes. It is understood that the pulsatile release of GnRH from the hypothalamus will in turn stimulate release of gonadotropins (LH and FSH) from the anterior pituitary promoting testosterone production in the testis and spermatogenesis. This pulsatile secretion can be recapitulated by the use of GnRH subcutaneous infusion pump at a dose of 5–20 μg every 1–2 h, but given the inherent inconvenience, it is largely only available at specialty centers for clinical trials [19]. Pulsatile GnRH replacement therapy is initiated with a starting dose of 25 ng/kg/pulse every 2 h subcutaneously via portable infusion pump with dose adjustment to obtain mid-normal testosterone. Doses up to 200 ng/kg may be needed to induce virilization, at which point it may be reduced [20]. GnRH failure is more likely in those patients with GnRH gene receptor mutation as well as in those who develop antibodies formed during the chronic intravenous infusions. While pulsatile GnRH replacement and recombinant gonadotropins appear equivalent in improving semen analysis parameters and pregnancy rates [21, 22], the mainstay of therapy tends to be gonadotropin replacement. GnRH is not favored in many institutions because of the cumbersome nature of the infusion as well as the costs associated with GnRH.

Gonadotropins

Gonadotropins are hormones released from the pituitary which stimulate the activity of gonads; thus, they are used mainly in cases of pituitary causes of hypogonadism and GnRH receptor gene defects. While gonadotropins were previously extracted from urine, high-quality recombinant human chorionic gonadotropin (hCG), FSH, and LH as well as purified urinary gonadotropins are available for use with no differences in safety or clinical efficacy observed among them [23].

- hCG, a hormone similar in chemical structure to LH, has been used as treatment in male hypogonadism to induce testosterone production and spermatogenesis. hCG can stimulate Leydig cells to produce testosterone and maintain adequate levels of intratesticular testosterone. Conventional therapy for gonadotropin deficiency involves the subcutaneous administration of hCG to replace physiologic LH at 1500–3000 IU two or three times weekly, with or without menopausal FSH (75 IU two or three times weekly) or recombinant human FSH (100–150 IU two or three times weekly) (rhFSH). hCG is first administered to correct LH deficiency, and the dose is adjusted to achieve nadir T at 48 h post-injection in the normal range. Following administration of hCG for 4–6 months, if no sperm are detected on semen analysis, recombinant or purified FSH can be co-administered, with improvement in semen parameters taking up to 1–2 years [24].

The efficacy of combined hCG and recombinant human follicle-stimulating hormone (rhFSH) has been established, and a prospective observational study by Saleh and Agarwal demonstrated an increase in average testicular vol-

ume from 4.1 to 12.4 mL and total motile sperm count from 0 to 4.8 million [25]. Another study of men with HH treated initially with hCG identified 81 men who had responded in regard to testosterone level but remain azoospermic. Of these, 84% achieved spermatogenesis, and 69% achieved a sperm concentration >15 million/mL after the addition of rhFSH [24]. A multi-institutional phase III randomized efficacy and safety study confirmed that weekly rhFSH of 450 IU dosing, in combination with hCG, was adequate to induce spermatogenesis in hypogonadotropic hypogonadal (HH) men and azoospermia who had failed on hCG alone [26]. Predictors of a good response to gonadotropin therapy included post-pubertal onset of gonadotropin deficiency and testicular volume >8 mL indicating less severe gonadotropin deficiency [27, 28]. The addition of FSH to hCG is shown to be most efficacious in the restoration of spermatogenesis in patients with pre-pubertal onset HH, whereas in men with post-pubertal onset, hCG alone appears to be sufficient [36]. Additionally, cryptorchidism requiring orchidopexy is a known negative prognostic indicator of spermatogenic induction in men with idiopathic HH [28].

There is little evidence for the use of gonadotropins in men with idiopathic infertility in the absence of HH; however, there is preliminary evidence suggestive that rhFSH may be of clinical benefit in limited circumstances. One clinical trial randomized 112 men with idiopathic oligozoospermia to treatment with 100 IU of rhFSH every other day for 3 months versus no treatment. The treatment cohort overall showed no benefit, but on subgroup analysis, 30 men (48.4%) with cytologic evidence for hypospermatogenesis without maturation defect on fine needle aspiration demonstrated improvement in semen parameters and a significantly higher spontaneous pregnancy rate compared to non-responders and non-treated patients (5/30 [16.7%] versus 1/32 [3.1%] and 2/50 [4.0%], respectively) [17]. Early evidence also suggests a specific role for rhFSH therapy in men with primary spermatogenic failure who also harbor certain FSH receptor polymorphisms. In one study, patients were randomized to 3 months of rhFSH at 150 IU three times weekly ($n = 70$) and no treatment ($n = 30$). When the 70 treated subjects were divided by genotype, only those men with a serine at position 680 demonstrated a statistically improvement in seminal parameters [29]. Further studies are certainly needed to validate the clinical use of rhFSH in these circumstances.

Treatment with hCG has shown the ability not only to reverse azoospermia brought on by testosterone supplementation therapy but also to help maintain spermatogenesis and elevated intratesticular testosterone levels. In addition, often used concomitantly are selective estrogen receptor modulators (*discussed below*) [30]. In the past few years, a number of genetic loci (ANOS1, FGFR1, KISS1, KISS1R, TAC3) have recently been discovered that encode proteins that are involved in the development and migration of GnRH or the synthesis and secretion of GnRH itself. These genes have been implicated in rare forms of GnRH deficiencies such as congenital hypogonadotropic hypogonadism and early-onset puberty forms. Many novel investigations are looking into genetic treatments at these specific loci to help preclude fertility issues that would occur later in life [31]. It should be noted that both HCG and clomiphene citrate are not without their side effects, notably bone mineral density loss and decreased libido.

Selective Estrogen Receptor Modulators (SERMs)

SERMs are a class of agents with estrogen receptor agonist or antagonist activity, such as clomiphene citrate and tamoxifen. While their use has been well established in the stimulation of ovulatory cycles, treatment of osteoporosis, and breast cancer in women, the utility of these agents for the treatment of male infertility in the setting of secondary hypogonadism remains off-label. They are an attractive method of treatment given their low cost, east of administration, and favorable side effect profile.

Clomiphene Citrate

Clomiphene citrate (CC) has anti-estrogenic effects on the hypothalamus and pituitary, block-

ing the negative feedback inhibition of estrogen in the central nervous system and promoting increased LH and FSH secretion which drives endogenous testosterone production and spermatogenesis in the testis [30]. Based on this, treating hypogonadotropic hypogonadism men with clomiphene might be expected to have the same biological effect compared with gonadotropins. Its safety and efficacy have previously been established. In 2012, Katz et al. published their prospective study on the efficacy of clomiphene citrate in men with confirmed hypogonadism and baseline serum testosterone <300 ng/dL [32]. Eighty-six men with an average age of 29 years were treated with 25 mg of clomiphene citrate administered every other day over an average period of 19 months to a goal serum testosterone range of 500–600 ng/dL. As needed to meet this goal, the dose was uptitrated to 50 mg. Their results confirmed an increase in serum testosterone and gonadotropins [32]. Both the Katz study and a subsequent study by Ramasamy in 2014 found an increase in the aging men satisfaction score based on a validated questionnaire for the assessment of male hypogonadism (ADAM questionnaire) with CC treatment [32, 33]. No major side effects were reported in these two studies [32, 33]. In a 2019 study of 400 patients treated with CC for a mean duration of 25.5 ± 20.5 months, side effects were reported in 8% and included changes in mood, blurred vision, and/or breast tenderness. Of men on clomiphene citrate for more than 3 years, 88% achieved eugonadism, and 77% reported improved symptoms. This was a retrospective study carried out between 2010 and 2018. The results did not significantly differ between patients treated for more than three or fewer than three years exploring the possibility of improving testosterone in a shorter duration of time [34]. A recent retrospective study by Mazzola et al. showed that a testicular volume <14 mL and LH level >6 IU/mL were significant predictors of improvement in serum testosterone in hypogonadal men treated with clomiphene citrate [35]. In theory, clomiphene citrate can increase the level of estrogen and estrogenic side effects, yet Ramasamy showed no significant rise in serum estradiol in CC-treated men when compared with testosterone replacement gels or placebo [33]. While small sample studies have shown improvements in erectile function with CC, other similar small studies have not found improvements in nocturnal penile tumescence and rigidity [36, 37]. This is an area that should be considered for future research. While measures of testosterone levels are noteworthy, ultimately what many men want to know is will it improve fertility rates? In a study of 190 infertile couples (normal female) by the World Health Organization, men received 6 months of 25 mg/day of CC compared to placebo with no clear demonstrative change in the pregnancy rate [38]. Future multicenter prospective studies are needed to look at not only testosterone levels, symptoms, and potentially improved fertility numbers.

Clomiphene Citrate and Tamoxifen Combination

With regard to infertility, the efficacy of clomiphene and tamoxifen has been assessed in multiple clinical trials in conjunction with other agents, yet the efficacy of these drugs alone remains undetermined. A 2010 randomized controlled trial of daily clomiphene citrate with the antioxidant vitamin E (25 mg and 400 mg, respectively) in men with idiopathic oligoasthenozoospermia showed superiority of this regimen over placebo in improving semen analysis parameters including total count, progressive motility, and rates of unassisted pregnancy (36.7% versus 13.3%, $p = 0.04$) [39]. Hussein et al. published a multicenter case series of 42 men with non-obstructive azoospermia (NOA) treated with dose-titrated clomiphene citrate to achieve serum testosterone between 600 and 800 ng/dL, with periodic semen analyses during the treatment period [40]. With treatment, 64% of patients produced sperm in numbers sufficient for intracytoplasmic sperm injection (ICSI), ranging 1–16 million/mL (mean density 3.8 million/mL) [40]. Notably, the lack of a control group in this study limits the ability to attribute a treatment-related effect on fertility. A 2013 meta-analysis of recent randomized controlled trials investigating the use of either clomiphene citrate or tamoxifen for the treatment of

idiopathic male infertility with oligo- and/or asthenoteratozoospermia demonstrated a statistically significant increase in pregnancy rates compared to controls (pooled OR 2.42, 95% CI 1.47–3.94, $p = 0.00004$) as well as sperm concentration by a mean difference of 5.24 million ($p = 0.001$) and motility by a mean difference of 4.55 ($p = 0.03$) [41].

Enclomiphene

Enclomiphene, which is the trans-stereoisomer of clomiphene citrate, similarly has the potential to increase serum testosterone levels in men with secondary hypogonadism by restoring physiological endogenous testosterone secretion while maintaining testicular volume and potentially spermatogenesis. Phase II studies have shown a significant increase in serum testosterone levels with enclomiphene while preserving or improving spermatogenesis [42]. A recent parallel randomized placebo-controlled multicenter study comparing use of enclomiphene citrate and topical testosterone (AndroGel® 1.62%) in overweight men aged 18–60 years with secondary hypogonadism demonstrated an increase in serum T and serum gonadotropins, as well as normalization of sperm concentration in the enclomiphene citrate group. An expected increase in serum T and decrease in gonadotropins and sperm concentrations were seen in the topical testosterone group [43]. Enclomiphene citrate has yet to receive FDA approval owing to concerns that phase III studies were not adequately designed as far as study entry criteria, titration, and method validation to show clinical benefit in the target population of men with secondary hypogonadism and oligospermia.

Aromatase Inhibitors

The aromatase inhibitors (AI), such as anastrozole, testolactone, or letrozole, increase endogenous T levels by inhibiting the peripheral conversion of androgens to estrogens by blocking the enzyme aromatase which functions at a P-450 enzyme. Through this mechanism, there is a reduction in estradiol trough less feedback inhibition and indirectly raise the levels of FSH and LH [44]. The negative consequences of elevated serum estrogen in combination with low serum testosterone on spermatogenesis have been demonstrated in vivo [45]. The administration of AI can restore a normal T/E_2 ratio and has been shown to improve sperm concentration and motility in oligozoospermic men, though these studies were not placebo-controlled and randomized by design [44, 46, 47]. The mean serum T/E_2 ratio in fertile men is 14.5 ± 1.2; conversely, in men with NOA and Klinefelter syndrome, the ratio is 6.9 ± 0.6 and 4.4 ± 0.5, respectively [44, 46]. The patients who benefit most from such therapy carry a diagnosis of NOA or idiopathic oligoasthenospermia and low T and have a T/E_2 ratio of <10 [44, 46, 48, 49]. While the use of AI in this indication remains off-label and testolactone is commercially unavailable in the United States, a subset of infertile men with elevated serum estradiol appear to benefit from the use of AI (anastrozole 1 mg daily or letrozole 2.5 mg daily). These medications are generally well tolerated with rare side effects including nausea, decreased libido, and decreased bone mineral density [50, 51]. As suppression of estradiol production to near undetectable levels with daily dosing may have consequences for bone health and sex drive, we recommend dosing of anastrozole at 1 mg twice weekly for a pretreatment estradiol level between 60 and 80 pg/mL and 1 mg thrice weekly for a pretreatment estradiol level >80 pg/mL.

Antioxidants

The presence of reactive oxygen species (ROS) in seminal fluid has been associated with sperm dysfunction, sperm DNA damage, and impaired fertility, prompting clinicians to offer men antioxidant supplementation [52, 53]. Few clinical trials have suggested antioxidant therapy may confer improvements in sperm function and DNA integrity. A recent Cochrane Database systematic review analyzed data from 48 randomized controlled trials (RCT) comparing single and combined antioxidants with placebo, no treatment, or other antioxidant in a total population of 4179 men with infertility [54]. The duration of trials ranged 3–26 weeks with follow-up

ranging 3 weeks to 2 years, and the age of men enrolled ranged from 20 to 52 years. Most men had low motility and sperm concentration. The authors indicated the review was limited by the fact that 25 of the 48 trials reported on sperm parameters as their primary outcome with only three of those trials also reporting on live birth or clinical pregnancy, as well as poor reporting of and inconsistency of study design, imprecision, small sample size of many of the trials included, and lack of adverse event reporting resulting in a designation of the evidence in favor of antioxidant therapy as "very low" to "low." The authors concluded that antioxidants may increase live birth rates (OR 4.21, 95% CI 2.08–8.51, $p < 0.0001$, from four RCTs with 277 men) but this was based on only 44 live births from 277 couples in fur small studies. As for clinical pregnancy, they suggested that antioxidants may increase pregnancy rates (OR 3.43, 95% CI 1.92–6.11, $p < 0.0001$, from seven RCTs with 522 men) but again the quality of the evidence was low [54]. There remain no specific recommendations on the use of antioxidants for the treatment of male infertility.

Dopamine Agonists

Men who present with infertility and hyperprolactinemia should be considered to harbor a prolactin-secreting micro- or macroadenoma until proven otherwise and diagnostic evaluation for pituitary adenoma should ensue. Elevated serum prolactin inhibits the pulsatile secretion of GnRH-inducing hypogonadotropic hypogonadism and infertility, and space-occupying tumors may also lead to symptomatology such as headache or visual field defects due to compression at the optic chiasm. In this context, dopamine agonists such as bromocriptine or cabergoline are indicated for the treatment of both the adenoma and infertility with some evidence suggesting that cabergoline is superior in suppressing prolactin production with normalization of prolactin in 70% of patients who are bromocriptine-resistant [55, 56]. Cabergoline is thus the preferred choice and administered at a dose of 0.25–1.0 mg twice weekly. Reversal of infertility is seen in 53% of cases, with those that fail therapy potentially dopamine agonist-resistant and thus candidates for surgical resection of the adenoma [57].

Medical Therapy to Optimize Surgical Sperm Retrieval

It is understood that spermatogenesis depends on a local hormonal milieu of high intratesticular T and FSH for Sertoli cell stimulation, and as up to 70% of men with NOA will harbor focal spermatogenesis, optimization of the hormonal profile can be beneficial for maximal surgical sperm retrieval [58]. As previously described in this section, the use of SERMs, AI, and gonadotropins can increase intratesticular T levels and normalize serum estrogen. A retrospective study of Klinefelter patients with NOA who received clomiphene, AI, or hCG prior to micro-TESE with a rebound of serum testosterone to 250 ng/dL or greater had a 22% higher sperm retrieval rate compared to patients who did not meet that threshold testosterone level [59]. Another study in men without Klinefelter syndrome but with NOA and hypogonadism demonstrated that these men do respond to medical therapy (SERM, AI, or gonadotropin) with an increase in T levels, but in this context, neither pre- nor posttreatment T levels appear to correlate with overall sperm retrieval, clinical pregnancy, or live birth rates [60]. Despite these findings and the lack of well-designed RCTs to assess the use of medical therapy to optimize sperm retrieval, limited data suggest a benefit. One prospective study on the use of clomiphene citrate showed a statistically significant increase in the likelihood of sperm retrieval and favorable testis biopsy patterns in men with maturation arrest or hypospermatogenesis on pretreatment biopsy [40]. Additionally, the use of hCG and rhFSH is documented to improve posttreatment sperm retrieval in men with NOA and who failed initial micro-TESE [61, 62] as well as in men who failed initial therapy with clomiphene to normalize serum T levels before micro-TESE [63]. Future RCTs will be needed to further clarify the benefit these drugs may provide in surgical sperm retrieval.

Fertility Preservation in the Hypogonadal Man on Androgen Supplementation

Testosterone Therapy and Alternatives to Testosterone

Sample Case

MZ is a 29-year-old male who is an athlete, reports a long-term use of androgenic steroids, and is having issues conceiving. He reports decreased energy and libido. Labs reveal decreased testosterone, normal FSH, and decreased LH. Sperm analysis reveals oligospermia with less than five million sperm.

Presenting symptoms of hypogonadism commonly include decreased energy, decreased libido, depressed mood, decreased muscle mass, and increased body fat. The most common treatment for men with hypogonadism is TST. Treatment with exogenous testosterone has demonstrated efficacy in the management of hypogonadism. Testosterone therapy can improve sexual function, muscle strength and bone density, and mood and cognition [64].

In North America, TDS (testosterone deficiency syndrome) can be treated with exogenous testosterone using one of a variety of therapeutic options [65, 66], including topical transdermal gels, oral and buccal agents, IM injections, subcutaneous injections, subcutaneous pellets, and more recently nasal products (see below). Patients and physicians weigh advantages and disadvantages of each option to select a treatment that best fits the therapeutic needs, preferences, safety, tolerances, and lifestyle of the patient. Factors may include convenience, cost, potential adverse local (e.g., irritation) or systemic (e.g., cardiovascular, hematocrit) reactions, transference, smell or odor, and physician recommendations [67–72].

The majority of prescriptions for testosterone supplements come from endocrinologists (23.73%), followed by general practitioners (16.95%), and thirdly urologists (15.25%) [73]. Perhaps more alarming is the finding that up to 25% of urologists surveyed by the American Urological Association reported using testosterone therapy as a treatment for the indication of infertility despite the known contraceptive effect of testosterone supplementation [74]. Furthermore, increased mainstream consumerism through advertising and marketing has become more visible [75]. Although these benefits can significantly improve the quality of life for hypogonadal men, testosterone therapy has some side effects. Exogenous testosterone replacement may result in side effects such as gynecomastia, mastodynia, acne, secondary polycythemia, and testicular atrophy. While the relation between testosterone therapy and cardiovascular risks remains incompletely determined [76], in 2015, the US Food and Drug Administration (FDA) issued a warning cautioning that testosterone might increase the risk of heart attack and stroke [77].

Exogenous testosterone induces negative feedback inhibition on the hypothalamic-pituitary-gonadal axis (reducing LH and FSH), thus leading to atrophy of the germinal epithelium in otherwise normal men and suppressing spermatogenesis, with azoospermia inducible by 10 weeks of testosterone use [78]. Testicular atrophy is common with loss in volume due to both suppressed spermatogenesis and decreased Leydig cell function. *Testosterone replacement is not recommended for hypogonadal men who wish to maintain fertility potential or who are actively attempting to impregnate a partner*. It is for this reason that many patients seek alternatives that can preserve fertility and testicular volume and purposefully delay the onset to which they would need to take testosterone replacement therapy.

Testosterone Nasal Gel

While otherwise healthy men may demonstrate rebound of spermatogenesis after 6–18 months of abstinence from exogenous testosterone [79], up to 4–10% of patients with impaired spermatogenesis prior to TST may remain azoospermic after cessation of therapy, with significant implications for their future fertility [80]. Therefore, as a general rule, testosterone therapy is not advocated for hypogonadal men with paternity in mind. However, there is a recently released short-acting 4.5% testosterone nasal gel Natesto® that has a uniquely pulsatile PK profile with limited impact on the hypothalamic-pituitary system. In so doing, LH, FSH, and endogenous testosterone production is unimpeded, and sperm counts are preserved [64, 80–82]. In a recent post hoc analysis of a phase III trial, patients with a pre-Natesto dose with serum TT <100 ng/dL had a similar response with regard to mood and erectile function (PANAS and IEEF data) as those whose predose TT was >100 ng/dL [83]. In November 2017, Ramasamy and colleagues, in a single-institution, prospective investigator-initiated study funded by Aytu BioPharma, launched a clinical trial on the role of Natesto in preserving semen parameters in men with low T. Enrollment criteria were men aged 18–55 years, with at least two T levels <350 ng/dL, and who have two semen analyses with total motile sperm count (TMSC) greater than five million [81]. All enrollees were naïve to testosterone supplementation prior to the study inception. Published preliminary results on the first 23 subjects (median age 35 years), with data available on 15 subjects at 1 month of treatment, showed T levels in 14 out of 15 men were above 300 ng/dL, with median of 423.5 (350.0–870.0) ng/dL. In six of the subjects who completed 3 months of treatment, median total motile sperm count changed from 37.5 (17.0–63.9) million at baseline to 24.8 (7.1–52.0) million (statistically insignificant) [81]. Although these are preliminary results, the observation that use of Natesto TID can improve testosterone levels while at the same time having an insignificant effect on the total motile sperm count may offer a new promising therapy for men suffering from clinical symptoms attributed to androgen deficiency while at the same time interested in maintaining the ability to have children in the future.

HCG and TST Combinations

In the previous section, hCG therapy was discussed as a means of replacing LH in hypogonadal men to promote restoration of intratesticular testosterone production. Intramuscular hCG has also been shown to reduce the impact of exogenous testosterone on intratesticular T levels, though data is scarce in its use in men previously on TST/AAS. A RCT was conducted with 29 healthy men receiving 200 mg per week of testosterone enanthate, who were also randomized to receive intramuscular saline placebo, 125, 250, or 500 IU hCG every other day for 3 weeks. Intratesticular testosterone levels and gonadotropins were assessed at Days 0 and 21. Intratesticular T levels were suppressed by 94% in the T enanthate/placebo group, 25% in the T enanthate/125 IU hCG treatment group, and 7% in the T enanthate/250 IU hCG treatment group and were actually increased 26% from baseline levels in the T enanthate/500 IU hCG treatment group [84]. Endogenous LH and FSH levels were not surprisingly suppressed to 5% and 3% of baseline, respectively. This demonstrated that even supraphysiologic doses of TST can be countered by low-dose hCG to maintain normal levels of intratesticular testosterone.

Although testosterone alone inhibits spermatogenesis, hCG can stimulate spermatogenesis given its direct positive effects on the testis and can be considered in lieu of or as an adjunct treatment to TTh to simulate or maintain spermatogenesis.

The effect on spermatogenesis was shown in a retrospective study conducted on 26 hypogonadal men treated with TST via transdermal patches or intramuscular injections, as well as low-dose hCG. Serum total and free T, serum estradiol, semen parameters, and pregnancy rates were assessed. Pretreatment semen parameters included an average volume of 2.9 mL, concentration of 35.2 million/mL, motility of 49.0%, and forward progression of 2.3. There were no observed changes in semen parameters regardless of T formulation over more than 1 year of

follow-up, none of the men became azoospermic during the treatment course, and 9 of 26 contributed to a pregnancy with their partners [85]. A recent multi-institutional series of men previously on TST with subsequent azoospermia or severe oligospermia were treated with hCG 3000 IU every other day and supplemented with either FSH, clomiphene citrate, tamoxifen, or anastrozole [86]. Patients on these hCG-based combination therapies demonstrated a recovery of spermatogenesis to a mean density of 22 million/mL in 4 months [86]. These studies suggest a beneficial role for hCG therapy in hypogonadal men who desire both symptomatic relief via TST and preservation of fertility potential during their reproductive years [87]. Data is even more limited on the use of hCG therapy for men with hypogonadism secondary to AAS use. Case reports have documented hCG alone at doses of 2000 IU three times weekly to 10,000 IU once weekly can restore spermatogenesis and lead to clinical pregnancy [88–90]. hCG and FSH combination therapy (10,000 IU weekly and 75 IU daily, respectively) has also been reported with clinical success in restoring spermatogenesis [91]. The long-term effects of hCG in combination with TST are currently unknown.

Selective Estrogen Receptor Modulators (SERMs)

The role of SERMs was previously discussed in the treatment of symptomatic hypogonadism via suppression of estrogenic negative feedback inhibition on the hypothalamic-pituitary-gonadal axis, thus promoting increased gonadotropins and downstream intratesticular testosterone production. Data on the use of clomiphene citrate for restoration of spermatogenesis is scarce. Case reports on the use of high-dose clomiphene (100 mg daily) in men with AAS-induced hypogonadism documented restoration of the normal hormonal axis within 2–3 months, but spermatogenesis was not assessed [92, 93]. Also, as mentioned in the previous paragraph, clomiphene in combination with hCG has demonstrated efficacy in the recovery of spermatogenesis in men previously on TST. Enclomiphene citrate is a more potent and shorter-acting trans-isomer of clomiphene citrate that was evaluated in a randomized, open-label, controlled, phase IIB study designed to assess fertility in 12 men with secondary hypogonadism previously treated with 1% testosterone gel for a minimum 6 months [94]. After cessation of TST, morning total T values averaged 165 ± 66 pg/dL. The treatment group was then given 25 mg enclomiphene citrate, and the control group received 1% testosterone gel with results compared at 3 and 6 months including serum total T, FSH, LH, and semen parameters [94]. In follow-up, only enclomiphene citrate therapy was observed to restore both serum T levels and sperm counts while also elevating LH and FSH in the treatment group [94]. A later randomized, phase IIB, placebo-controlled, parallel, multicenter study of 73 men with secondary hypogonadism was conducted using two oral doses of enclomiphene citrate versus 1.62% topical T gel. All men had either discontinued prior TST for at least 6 months or had never been treated. This particular study population was notable for more severe hypogonadism and lower baseline serum T levels than prior studies. Again, enclomiphene was demonstrated to reverse low serum T and gonadotropins compared to placebo while preserving sperm production compared to the TST treatment group [95]. These findings have since been further validated in a phase III RCT [43]. As of this time, enclomiphene is not yet FDA approved for the treatment of male hypogonadism, and further phase III studies are pending.

Aromatase Inhibitors

There are no prospective trials in the literature evaluating the use of aromatase inhibitors in men with hypogonadism secondary to TST or AAS use. The previously mentioned retrospective series by Wenker et al. evaluating hCG-based combination therapies (including AI) in men with azoo- or oligospermia following TST demonstrated a 98% success rate at restoring spermatogenesis with no differences noted between supplemental therapy administered with hCG and the type of TST used [86]. Patients who stand to benefit most from therapy with these agents will have low serum T and have a T/E_2 ratio of <10

[44, 46, 48, 49]; thus, their role in the restoration and maintenance of spermatogenesis in men previously on TST/AAS or who wish to continue TST/AAS will be limited and likely adjunctive.

Varicoceles as Cause of Hypogonadism and Treatment Options to Help Fertility

Sample Case

A 25-year-old male presents due to infertility of unknown cause. He denies any urological symptoms. Physical exam is positive for minimal left-sided scrotal fullness that expands with Valsalva and disappears when the patient lies down. Testosterone, FSH, and LH are within normal limits. Sperm analysis reveals decreased sperm concentration, decreased total sperm count, and decreased total motile sperm count.

Varicocele is caused by dilation of the pampiniform plexus and is generally left-sided. This is due to the anatomical layout of the left gonadal vein entering the left renal vein at a perpendicular angle leading to increased intravascular pressure in the left gonadal vein that ultimately leads to retrograde blood flow. Varicocele repair can offer a unique approach to improving testosterone levels for a subset of hypogonadal men. Varicoceles can be associated with testicular dysfunction, testicular atrophy, and even azoospermia in some men. The etiology and pathophysiology of a varicocele are complex and multifactorial. Varicoceles not only reduce the quantity of Leydig cells but qualitatively impair Leydig cell function, resulting in reduced serum testosterone levels and testicular volume loss compared to control subjects. The exact mechanism by which a varicocele leads to impaired testicular function is unknown but likely relates to either altered testicular blood flow, increased scrotal temperatures, or redox imbalance. In a histologic study, Abdelrahim et al. evaluated bilateral testicular biopsies from 30 infertile men with varicocele taken both during varicocelectomies and postoperatively. Compared to healthy control subjects, preoperative biopsies showed reduced spermatogenesis with maturation arrest, dead spermatogenic epithelium, and a decrease in the volume of Leydig cells. After treatment, spermatogenesis improved in 22 (73%) of the patients. These patients also exhibited regeneration of the epithelium with the quantity of Leydig cells normalized in 18 patients (60%) [96]. Thus, improvement in serum testosterone levels following varicocele repair appears to be associated with improvements at the microstructural level. Given that the presence of a palpable varicocele has a progressive and deleterious effect on spermatogenesis and testosterone production, there is growing evidence that early repair of varicoceles may prevent future infertility and androgen deficiency. Tanrikut et al. conducted a case-control study and found that varicocele repair significantly increased testosterone levels (358 ± 126 ng/dL at baseline to 454 ± 168 ng/dL after repair; $p < 0.001$) [97]. A Cochrane review in 2012 analyzed data involving 814 men in ten different studies and found improvement in spontaneous pregnancy rates after varicocele repair from 26% to 40% [98]. They found that mean serum testosterone significantly increased by an average of 97.48 ng/dL after varicocelectomy. Although most data remain retrospective, evidence supporting a potentially beneficial effect of varicocelectomy on serum testosterone levels in hypogonadal men is growing. Thus, improvement in serum testosterone levels following varicocele repair appears to be associated with improvements at the microstructural level. Given that the presence of a palpable varicocele has a progressive and deleterious effect on spermatogenesis and testosterone production, there is growing evidence that early repair of varicoceles may prevent future infertility and androgen deficiency [99–101].

Lifestyle Modifications

Chronic health conditions such as obesity, metabolic syndrome, and obstructive sleep apnea are becoming more prevalent in Western countries and decrease serum testosterone levels. High fasting glucose, waist circumference, and elevated triglycerides have been associated with lower than normal circulating serum testosterone levels [102, 103]. Small case studies have shown that regular aerobic exercise and weight loss can have positive effects on serum testosterone levels [104, 105]. In a meta-analysis of 11 studies on the topic of diet and weight loss and the relationship to serum testosterone, Corona et al. observed that a Mediterranean diet of low fat, dairy, eggs, poultry, fish, and vegetables produced a positive result in sperm counts, while a Western diet of high-fat dairy processed foods and refined grains carried an inverse relation on sperm count [106].

Other lifestyle modifications such as improved sleep patterns and stress reduction can increase testosterone levels. Singer observed that men with high stress levels had significantly lower serum testosterone levels compared with controls [107]. They evaluated seven internal medicine residents (stressed) who had a mean serum testosterone level of 11.8 ± 1.06 nmol/L, significantly lower than testosterone levels of non-physician male healthcare personnel (controls; 20.6 ± 5.28 nmol/L; $p < 0.0005$). Before embarking on a pharmacologic treatment plan to boost serum testosterone, men who are overweight and have poorly controlled diabetes, obstructive sleep apnea, poor sleep patterns, or profoundly stressful jobs should consider an exercise plan, weight loss reduction, and work-life balance adjustments to boost their own internal testosterone level (Table 1).

Table 1 Summary of the reviewed available medical treatments for fertility preservation

Medication	Administration	Dosage/frequency	Special considerations
Selective estrogen receptor modulators (SERM)	Oral	Clomiphene citrate 25–50 mg daily	Generally well tolerated. Off-label use for male infertility. More potent isomer enclomiphene citrate currently in phase III trials
		Tamoxifen 20 mg daily	
Aromatase inhibitors (AI)	Oral	Anastrozole 1 mg daily	Indicated for men with T/E_2 ratio of <10. Consider twice- or thrice-weekly dosing for bone health and libido. Side effects include nausea, decreased libido, and bone demineralization. Off-label use for male infertility
		Letrozole 2.5 mg daily	
GnRH	Subcutaneous infusion pump	25–200 ng/kg per pulse every 2 h	Not commonly used outside of clinical trials due to inconvenience of administration
Human chorionic gonadotropin (hCG)	Subcutaneous/ intramuscular	1500–3000 IU two to three times per week	FDA approved for fertility preservation in secondary hypogonadism
Recombinant human follicle-stimulating hormone (rhFSH)	Subcutaneous/ intramuscular	75 IU two to three times per week	FDA approved for fertility preservation in secondary hypogonadism
Dopamine agonists	Oral	Cabergoline 0.25–1 mg two times per week	Cabergoline is preferred. Surgical resection of pituitary adenoma indicated for dopamine agonist resistance. Off-label use for male infertility
		Bromocriptine 2.5–5.0 mg two times per week	

Practical Clinical Tips and Take-Home Message

Low testosterone affects a significant and growing percentage of the male population. With increasing longevity as well as a desire to start a family later in life, physicians are faced with the challenge of managing the clinical symptoms attributed to low androgen levels while at the same time balancing one's desire for fertility/paternity. The goals of therapy are based on our knowledge of the hypothalamic-pituitary-gonadal axis and the importance of optimizing serum LH for endogenous testosterone production, serum FSH for spermatogenesis, and reduction of serum estrogens. While testosterone replacement seems a logical and easy "fix" to address the low androgen levels and the symptoms of decreased energy, libido, and erectile dysfunction, it seriously jeopardizes one's ability to successfully procreate. Withdrawal of testosterone replacement may cause rebound spermatogenesis that is not an absolute. Clomiphene citrate, anastrozole, and human chorionic gonadotropin have been used to increase intratesticular testosterone while simultaneously maintaining spermatogenesis. However, they are not without side effects, such as decreased bone mineral density and libido; additionally, they are not FDA approved for treating low T in men. There is some promising initial data with Natesto to increase serum testosterone while at the same time maintaining FSH, LH, and semen parameters. Lastly, lifestyle modifications should be discussed with the patient before embarking on a strategy to treat androgen deficiency.

Key Readings

Selected below from the references are vital readings for the audience: [3, 16, 25, 40, 82]**.**

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Genital Affirmation Surgery for Patients Assigned Male at Birth

Cecile A. Ferrando

Introduction

Vaginoplasty is a surgery that aims at creating a female vulva and vaginal canal in transgender women (individuals assigned male at birth who identify as women) seeking gender affirmation with genital surgery. The surgery involves deconstruction of the male external genitalia and creation of a neoclitoris, labia, vestibule, vagina, and urethra. The most commonly performed procedure is a modification of the penile inversion vaginoplasty. This procedure was first described in the 1950s by Drs. Gillies and Millard [1] and later popularized by Georges Burou, a French gynecologist who practiced in Casablanca [2]. In this chapter, the technique and outcomes for this procedure are reviewed. Perioperative, surgical considerations and postoperative outcomes are also discussed, and a section is dedicated to reviewing the technique of intestinal vaginoplasty, which is a less commonly performed procedure for gender affirmation, but remains an option for some patients.

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Preoperative Considerations

Before undergoing vaginoplasty surgery for gender affirmation, important perioperative considerations are necessary to ensure that outcomes are maximized for both the surgeon and the patient. Box 1 contains a list of these important considerations.

Box 1 Perioperative Considerations

History and physical and assessment of medical comorbidities
- Review of WPATH criteria for surgery
- Weight criteria
- Genital hair removal
- Smoking cessation
- Blood glucose control in diabetics
- HIV status and viral load if HIV+
- Continuity of care with a mental health provider
- Social support at the time of surgery

At the time of consultation, a thorough history and physical examination is recommended before proceeding with surgery. At this time, medical comorbidities are assessed and ensured to be well managed. Inquiry into all medical conditions allows the surgeon to determine the need for any preoperative clearance or testing that will help to reduce any perioperative complications. Specific to this surgery, an important part of the history taking

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is related to the patient's history of prostate disease including any cancer-related treatments she has received. Understanding the patient's history allows the surgeon to better counsel the patient about possible risks associated with the surgery. Of importance is also pre-surgical discussion of any urinary symptoms such as incontinence or post-void dribbling as well as sexual dysfunction and the inability to achieve orgasm, as patients are usually counseled before surgery that these types of symptoms are usually unchanged after surgery, and patients should know what to expect.

In order to be considered candidates for surgery, patients must meet criteria set by the World Professional Association for Transgender Health (WPATH). These criteria are meant to be used as guidelines for practitioners who treat patients with gender dysphoria seeking transition care. Insurance companies have now also aligned themselves with these guidelines and require that patients meet all of the criteria in order pursue surgery. Box 2 displays these criteria. Patients must have a formal diagnosis of gender dysphoria made by a mental health professional who is experienced in the care of gender dysphoric individuals. They must present with letters of referral from two separate mental health professionals, one of whom needs to have a PhD or MD degree. Patients must also have good documented control of any comorbid psychiatric conditions and have lived 12 continuous months on hormone therapy and as their self-affirmed gender. Patients who meet these criteria are considered candidates for surgery as long as they meet the other criteria set by the surgeon performing their surgery.

Box 2 WPATH Criteria

Formal diagnosis of gender dysphoria by a qualified mental health provider

Two letters of referral by qualified mental health providers

Control of comorbid psychiatric conditions

Living full time in their self affirmed gender

Gender affirming hormone therapy for ≥1 year

Weight criteria differ by surgeon. In our practice, we require patients to have a BMI ≤ 34. Other practices are more conservative, while others will allow a BMI as high as 45. We also require that all patients cease tobacco use if they are smokers in order to ensure good wound healing and to reduce complications. Patients who are HIV positive must have adequate neutrophil counts and an undetectable viral load. In addition, glucose control in diabetics is very important in order to avoid wound-healing complications which can lead to poor postoperative cosmetic results. We require that patients have a preoperative hgbA1c ≤ 7.0 within 3 months of their surgery.

Some surgeons require genital hair removal. A portion of the scrotal and sometimes penile skin is often used to line the neovagina if the penile inversion vaginoplasty technique is used. If this is the case, laser hair removal and electrolysis are necessary to ensure that there is no vaginal hair growth, which can be bothersome postoperatively [3]. Intraoperative techniques to remove hair follicles exist to help mitigate this problem as well, but preoperative hair removal, while costly, is the best way to prevent it.

The last two, but very important, perioperative considerations are our practice's requirement that all patients have active contact with their mental health professional leading up to and after surgery as well as demonstration that they will have friend or family support with them during the perioperative period. In our experience, these two considerations are key to ensuring that patients do well after surgery.

Surgical Considerations

Surgical considerations are displayed in Box 3. During the history and physical portion of the initial consultation, patients should be asked about any previous surgeries, including orchiectomy surgery for any indication, including gender affirmation. If patients have undergone previous orchiectomy, they will not need to have this procedure done during their vaginoplasty. Patients should be asked if the procedure was performed

through a groin versus scrotal incision. In the case of a transscrotal incision, spermatic cord remnants may be present, and careful dissection should be done to not cause an intraoperative vascular injury. It is not uncommon for patients to undergo orchiectomy as a bridge to vaginoplasty surgery, whether it be to reduce their feminizing hormone regimens or because of access challenges to vaginoplasty [4]. Regardless, providers performing these surgeries should be aware and should acknowledge this during their initial encounter with the patient.

Box 3 Surgical Considerations
Previous orchiectomy
History of prostate surgery or radiation for prostate surgery
Full-depth versus zero-depth vaginoplasty
Penoscrotal hypoplasia

Prostatic surgery and/or radiation therapy damages and scars the peri-prostatic tissues and can make dissection in the vesicorectal space very challenging, placing the patient with a history of prostate cancer at risk for visceral injury and possible development of a genitourinary or rectovaginal fistula. Patients should be counseled thoroughly about this risk, and consideration should be given to performing a vulvoplasty only, also known as a "zero-depth" procedure or "shallow-depth" procedure, rather than a complete vaginoplasty where a neovaginal canal is created. A zero-depth procedure is available to all patients if they so choose. Some patients do not desire vaginal penetrative intercourse and opt for this surgical modification to reduce potential complications and to make the postoperative recovery a little easier.

The last important surgical consideration is whether or not there is enough skin to perform an adequate genital reconstruction. Prolonged hormone therapy can alter external genitalia and reduce the amount of available skin. Furthermore, we are seeing more and more patients who have begun their transition during the prepubertal years who have undergone puberty suppression, and their genitalia do not reach adult size, also reducing available tissue and skin for reconstruction. In all cases, alternative sources of reconstructive material are needed to achieve good cosmesis and functioning. Split-thickness skin grafts can be obtained from the patient intraoperatively, biologic xenografts are also available, and recently, some surgeons have begun to investigate the use of peritoneal (both free and pedicled) for creation of the neovagina in this patient population [5]. Alternatively, intestinal vaginoplasty, although not as common, is an option for these patients.

Preoperative Planning

Preoperative considerations are listed in Box 4. Patients undergo routine preoperative testing based on their age and risk stratification.

Box 4 Preoperative Planning
Routine preoperative testing
Reduction or cessation of exogenous estrogens
Bowel preparation
Thorough informed consent

We do not stop exogenous estrogens preoperatively, but we do lower the dose based on recommendations made for postmenopausal women on hormone therapy for menopausal symptoms. Patients are asked to lower their estradiol to 1 mg daily 3 weeks before surgery. They are advised that they may stay on their prescribed dose of spironolactone, and a basic metabolic panel is checked preoperatively to ensure that there is no hyperkalemia as a result of the medication.

Patients undergoing a full-depth vaginoplasty procedure are usually asked to do a bowel preparation the day before surgery. In our practice, we ask patients to take magnesium citrate and Dulcolax 12–24 h before their surgery and to stay on a clear liquid diet during that time.

The informed consent is one of the most important parts of the preoperative period. In this visit, patients are reminded of all of the risks associated with surgery. Included are the risk of bleeding, hemorrhage, and need for transfusion; injury to surround viscera and possible development of fistula; and postoperative wound issues and infection. Time should be spent discussing common minor events that may require revision surgery, such as poor cosmetic results and urinary symptoms. The informed consent process is a time to ensure that the patient's expectations are in line with realistic outcomes. Patients often have very high expectations for the appearance of the vulva but sometimes lack knowledge about what normal anatomy may look like. Setting realistic expectations by using pictures of previous patients as well as variations in natal female anatomy is helpful and an important part of the informed consent.

Surgical Technique

In Box 5, we present the steps of the surgical technique.

Box 5 Steps of Vaginoplasty

Skin markings
- Removal of scrotal graft
- Orchiectomy
- Creation of neovaginal cavity
- Penile deconstruction
- Creation of clitoral flap
- Creation of penile skin tube and lining of neovaginal cavity
- Vulvoplasty

After undergoing general anesthesia, patients are positioned in the dorsal supine high lithotomy position with care taken not to hyper-extend or flex the lower extremities. Perioperative antibiotics and chemoprophylaxis against venous thromboembolism (VTE) are administered. A rectal lavage can be down with Betadine to reduce the risk of intraoperative contamination. This is important, especially if rectal exams are done during the case. The patient is then prepped and draped in a sterile fashion.

The patient is then marked (Fig. 1). Markings are made depending upon the amount of skin available for grafting. The perineal body is identified and marked with an inverted V-shape to create a perineal skin flap, which is used to create the posterior opening of the neovagina. Lateral incisions are marked medial to the groin creases. The superior incision is marked beneath the penile shaft ensuring that the skin can be pulled down with minimal tension to the perineal body mark. Using the markings, a large portion of the scrotal skin is removed, creating a scrotal skin graft, to later be used to line the neovaginal cavity. This graft is prepped, creating a split-thickness graft. It is sewn along a stent the size of the intended cavity.

If testes are present, a bilateral orchiectomy is performed (Fig. 2). The spermatic cord is ligated at the level of the external inguinal ring. This is done bilaterally. If an orchiectomy has been performed, spermatic cord remnants may be encountered and be left or ligated depending on whether

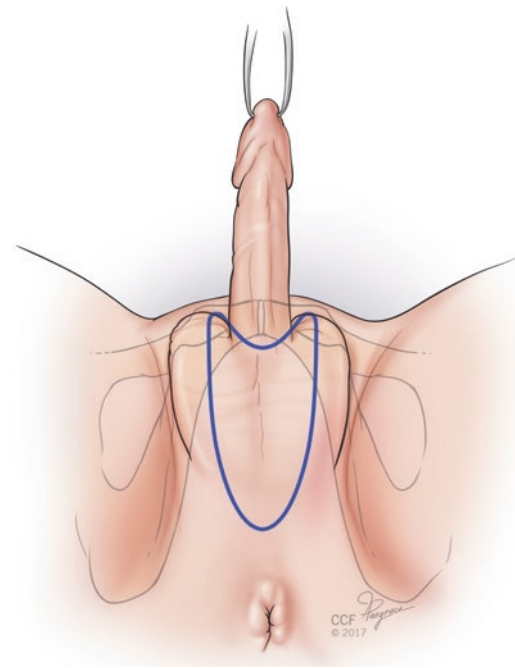

Fig. 1 Skin markings for scrotal skin graft

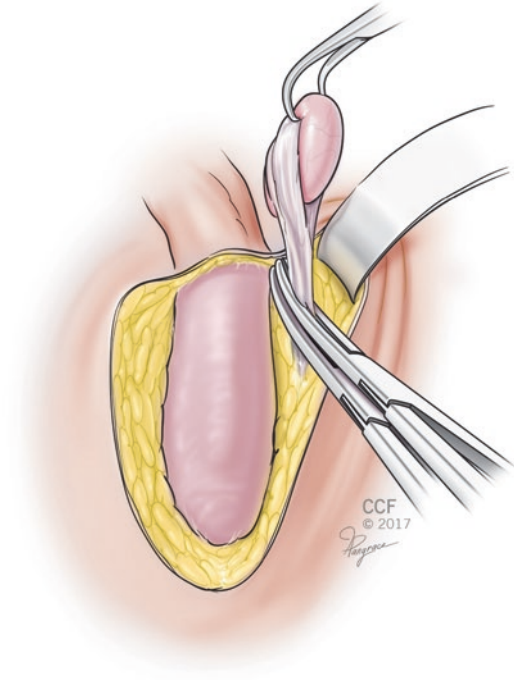

Fig. 2 Orchiectomy; the spermatic cord is clamped and ligated

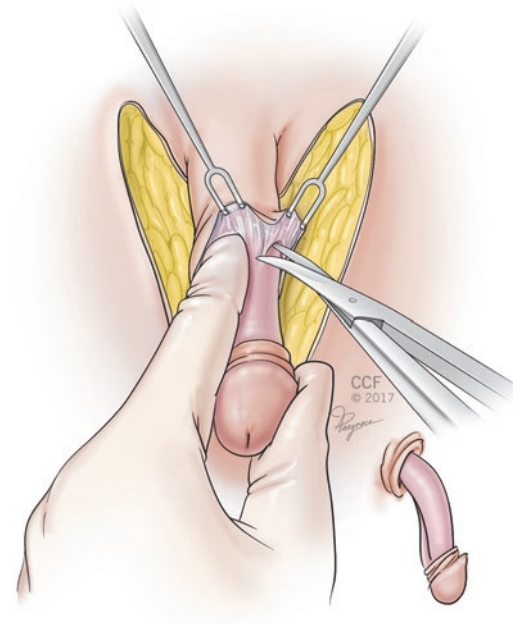

Fig. 3 Degloving of penile structures; creation of penile skin tube

they have caused the patient preoperatively or if they will change the outcome of the reconstruction. The inguinal ring can be closed with permanent suture. This theoretically reduces the risk of an inguinal hernia; however, there have been no documented cases of this post-vaginoplasty surgery.

The remaining periscrotal fat is then plicated in a symmetric way on each side in order to create the fad pads of the new labia majora. Next, the penile structures are skeletonized and degloved from the overlying penile skin (Fig. 3). The penile skin tube is preserved and used later during the reconstruction.

The neovaginal cavity can be created at any point during the surgery (Fig. 4a–c). In our practice, we perform this part of the surgery once the penis is degloved, before the penile structures are deconstructed. A Foley catheter is inserted, and the bladder is drained. The Foley bulb is used as a landmark to help guide the dissection. The central tendon of the perineum is identified by placing the bulbospongiosus of the penis on upward traction. It is transected sharply or with cautery. Dissection is then continued beneath the spongiosis using the technique that as commonly used to perform a perineal prostatectomy. Gentle traction is placed on the Foley, and the course of the urethra and body of the prostate are easily palpated. The levator ani muscles are partially divided laterally to create a large enough caliber for the canal. Eventually the ventral rectal fascia, also called *Denonvilliers* fascia, is encountered and provides a landmark to complete the dissection as the goal is to stay on top of the fascia. At this point, the dissection can be performed bluntly until adequate depth is reached, usually at the level of the vesicoperitoneal reflection. Once the cavity is created, it is temporarily packed until it is time to line it.

The penile structures are then deconstructed. The spongiosis and urethra are separated from the overlying cavernosa structures. The spongiosis is then excised in order to debulk the urethra. This is especially important at the level of the bulbous urethra. Next the clitoral flap is marked out on the glans of the penis. The goal is to maintain the dorsal neurovascular bundle within its

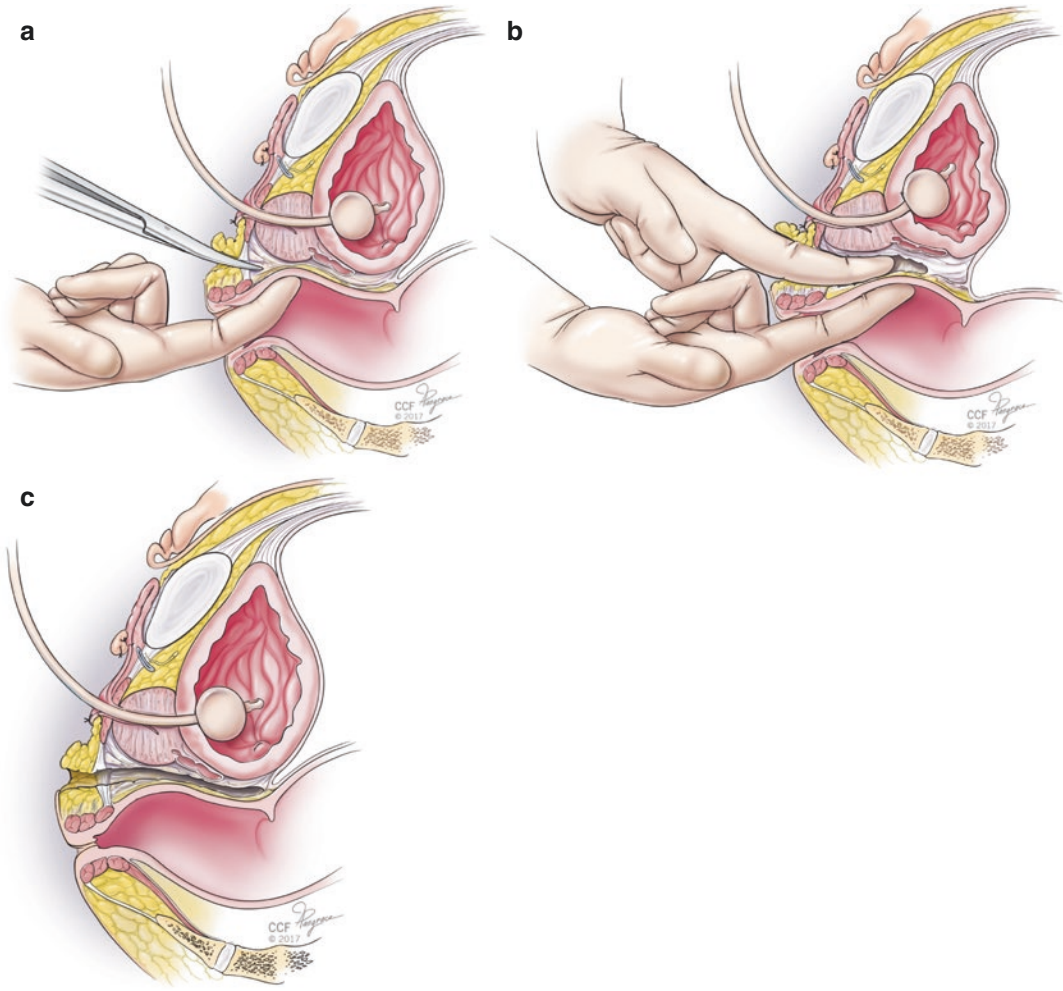

Fig. 4 (**a**–**c**) Creation of neovaginal cavity between the rectum and bladder using sharp and blunt dissection

tunica albuginea sheath. This sheath is dissected off of the corpora cavernosa, which are then amputated and discarded (Fig. 5). The crura of the cavernosa can also be excised, but this depends on surgeon preference and technique.

The clitoral flap is then folded on itself, creating a neoclitoris (Fig. 6). It is sutured to the underlying bony structures with the neoclitoris secured at the level of the insertion of the adductor longus tendon in the groin crease, which often correlates to the level of the clitoris in natal females. The urethra is then incised and opened ventrally over the catheter. The urethra is spatulated and secured, creating a new urethral meatus. The undersurface of the urethra is secured with the superior edge sutured to the neoclitoris, creating a new vestibule (Fig. 7).

Next the scrotal skin graft is sewn onto a stent and then anastomosed to the penile skin tube by passing the stent with the graft through the tube (Fig. 8a, b). A suprapubic drain is placed at this time. Alternatively, labial drains at the end of the procedure. The stent is then inverted into the neovaginal cavity. Care is taken to ensure that no tension is placed during the inversion process. In order to avoid introital stenosis and lack of definition of the vulvar structures, minimal tension between the opening of the penile tube and the perineal flap is necessary. Once the skin tube is inverted, the stent is removed, and the neovagina

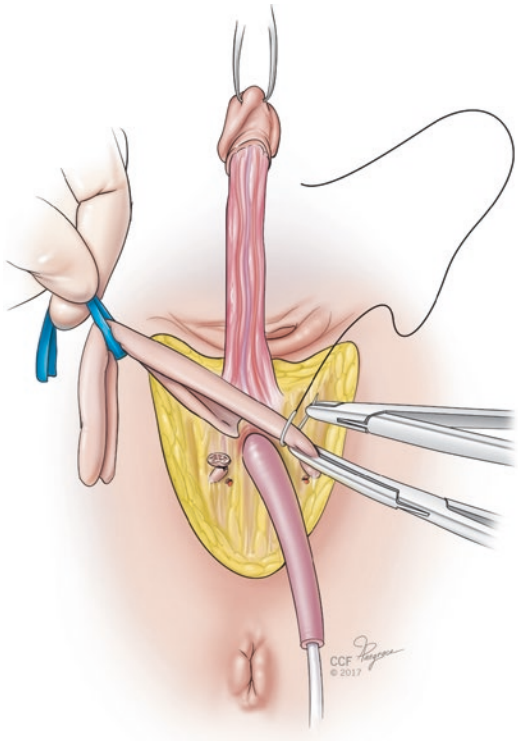

Fig. 5 Corpora cavernosa separated from overlying clitoral flap and removed

is packed tightly. The inferior margin of the neovaginal graft is secured.

The vulvar structures are created (Fig. 9a, b). The penile flap is incised in the midline, exposing the underlying vestibule (urethral flap) and clitoral flap. The skin edges are secured to the urethral flap, creating the labia minora and clitoral hood. Lastly, the labia majora incisions are reapproximated and closed. A pressure dressing is applied. It can be sewn into place for added pressure. The Foley catheter is left in place along with the vaginal packing.

Six-month results of two patients who have undergone vaginoplasty are shown (Fig. 10a, b).

Postoperative Care and Considerations

Length of stay and location of stay vary depending on the surgical practice. Our patients remain in the hospital for three nights and are discharged to a nearby hotel for an additional four nights where they receive daily care. Other practices use step-down units or keep their patients in the hospital the entire length of stay.

Patients are prescribed Bactrim twice daily for 7 days for prophylaxis against urinary tract infection and cellulitis. They also are given daily Lovenox for VTE prophylaxis during their stay at the hotel. They are also provided with oral analgesics.

Patients are allowed to have a regular diet and ambulate starting on postoperative Day 1. Their drain is removed on postoperative Day 2 or 3 depending on its output. The pressure dressing is removed on postoperative Day 3 before discharge. The Foley catheter and vaginal packing are removed on postoperative Day 6. On Day 7, patients are seen in the office, provided with dilators, and taught how to dilate properly. The dilation regimen is carefully reviewed, and all discharge precautions are given. Patients are allowed to return home after that visit.

Patients are seen on postoperative visit schedule to ensure that wound healing is adequate and that dilation is feasible. All patients are seen by a pelvic floor physical therapist 4 and 6 weeks after surgery to treat any pelvic floor dysfunction or levator ani pain from surgery and to help patients with dilation. Therapists also help with scar manipulation and healing. The incorporation of a postoperative pelvic floor physical therapy intervention is relatively new, and more centers are starting to employ this. In a retrospective analysis by Jiang et al., pre- and postoperative pelvic floor PT in patients who were post-vaginoplasty surgery identified a high incidence of pelvic floor and bowel dysfunction [6]. Of those patients who were found to have dysfunction preoperatively, the rates of resolution by the first postoperative visit of pelvic floor and bowel dysfunction were 69% and 73%, respectively. There were significantly lower rates of pelvic floor dysfunction postoperatively for those patients who attended therapy sessions.

Common postoperative complaints include difficulty with dilation, which is mitigated with the help of our pelvic floor therapists as mentioned. Patients also complain of vaginal dis-

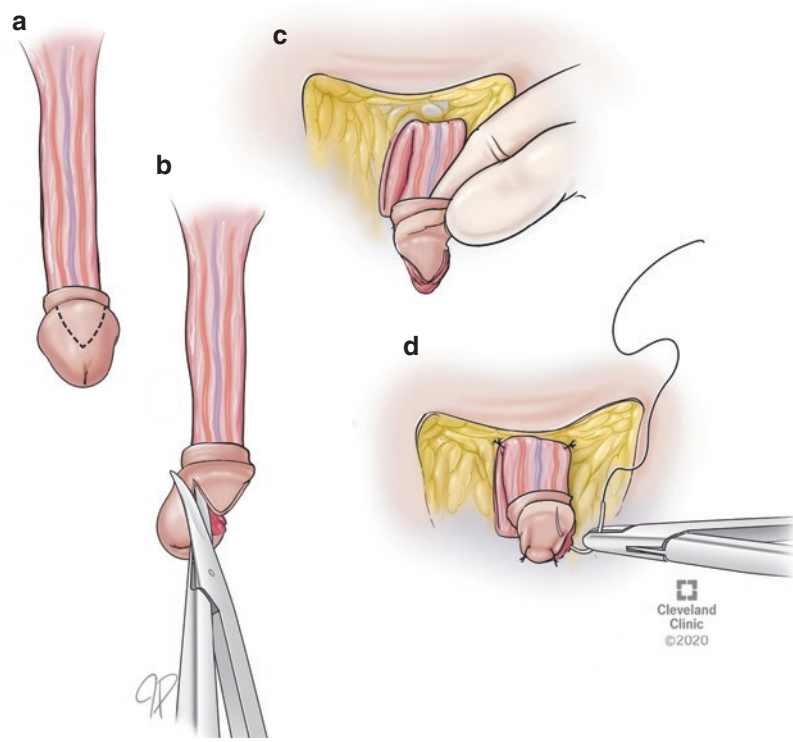

Fig. 6 (**a**–**d**) Creation of the clitoral flap and neoclitoris

charge and odor, which we address with varying douching regimens. Lastly, patients sometimes report vaginal bleeding with dilation, and this is usually from neovaginal granulation tissue that can be cauterized or excised in the office.

Postoperative Revision Surgery

Cosmetic revision surgery is common following vaginoplasty surgery. In one retrospective chart review looking at a single surgeon's experience with 117 patients who underwent penile inversion vaginoplasty, the authors found that 28 (23.9%) patients underwent revision labiaplasty and/or clitoroplasty [7]. Patients who required revision were significantly more likely to have problems with granulation tissue, intravaginal scarring, and complete vaginal stenosis following their index surgery. The majority of patients who underwent revision labiaplasty and/or clitoroplasty reported satisfaction with their final surgical outcome and resolution of their genital-related dysphoria.

Abnormal urinary stream is common following vaginoplasty. It is usually a result of periurethral scarring, obstructing the meatus, and deviating the urinary stream. Urethral revision can usually be done as outpatient surgery and requires very little postoperative recovery. Patients are usually highly satisfied with this type of revision surgery.

Introital scarring can lead to difficulty with dilation and penetrative intercourse. It is not as problematic is vaginal canal stenosis and can be repaired with a simple revision surgery. Often, excision of the scar with either a z-plasty closure or a distal vaginal advancement flap is sufficient to repair the scarred area and allow for satisfying function.

Postoperative Complications

Data are improving on outcomes following vaginoplasty. Until recently, most available data were retrospective case series of low to intermediate quality.

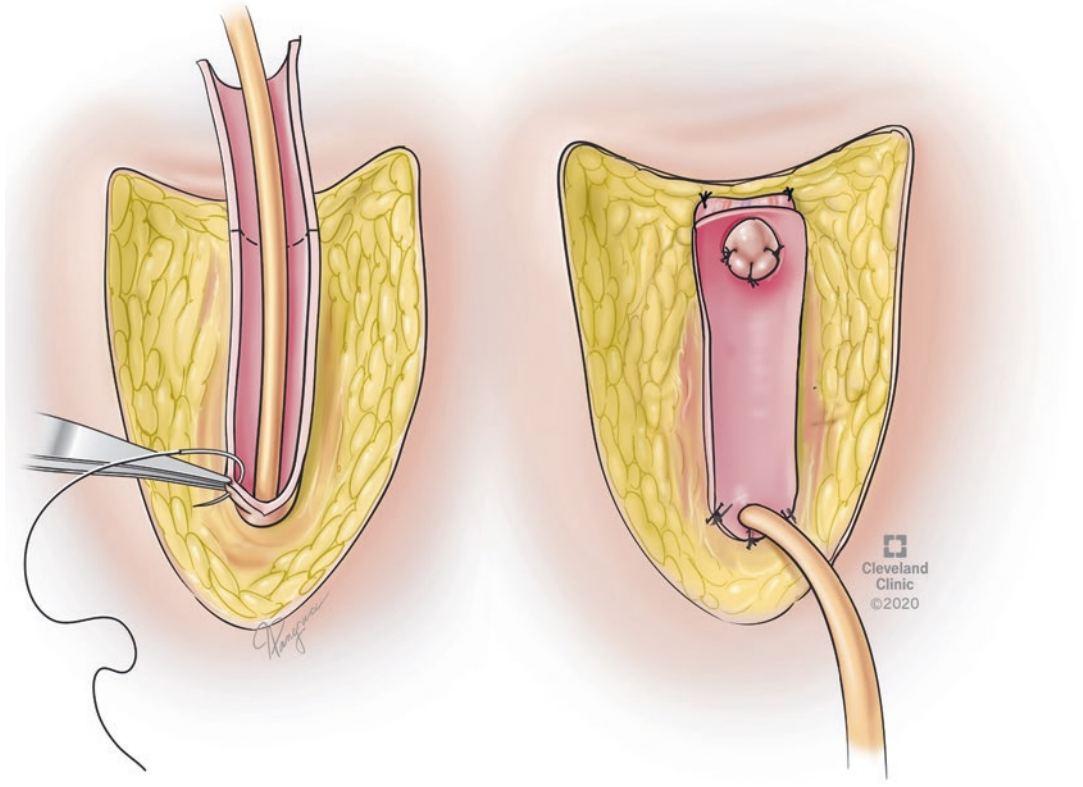

Fig. 7 Urethroplasty and creation of the vestibule

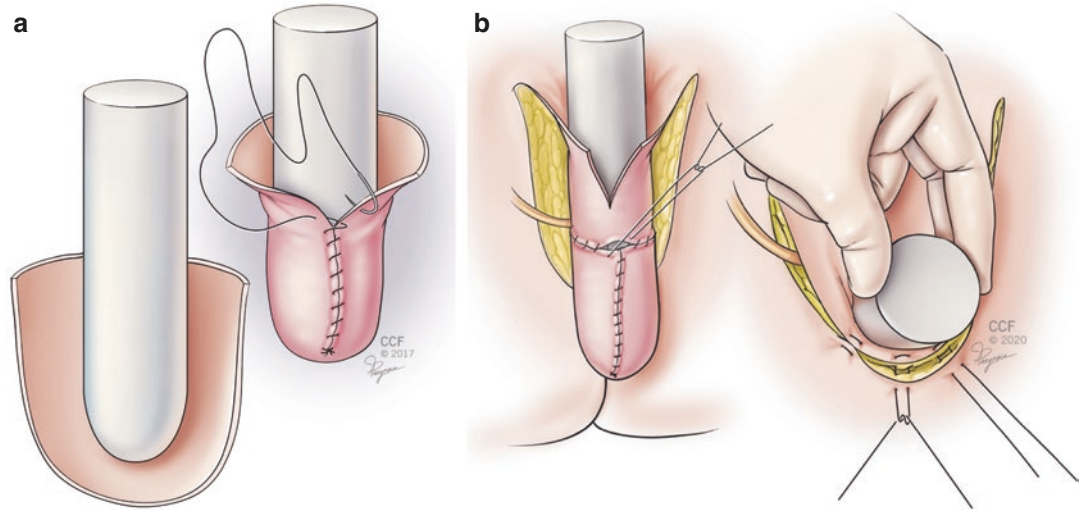

Fig. 8 (**a**, **b**) The scrotal skin graft is sewn on a stent and anastomosed to the penile skin tube by passing the stent with the graft through the tube

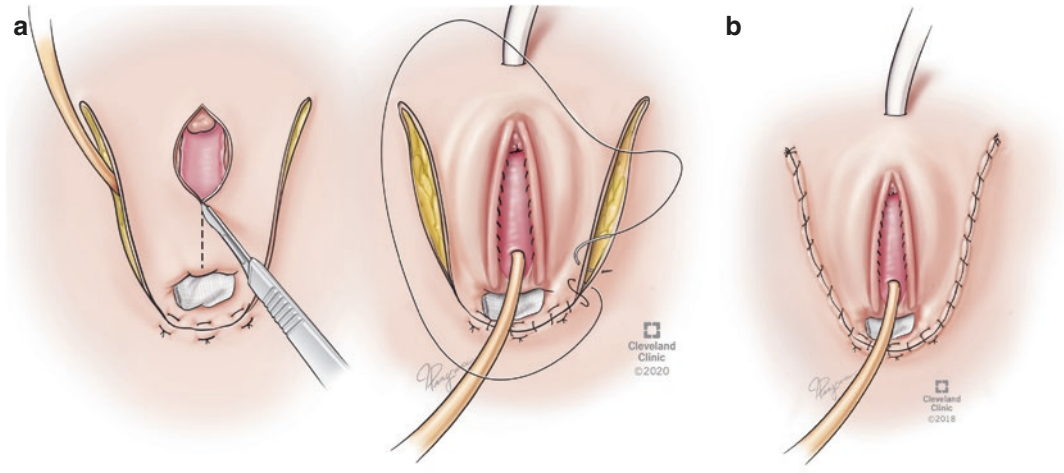

Fig. 9 (**a**, **b**) Vulvoplasty; the vulvar structures are created and there is completion of vulva

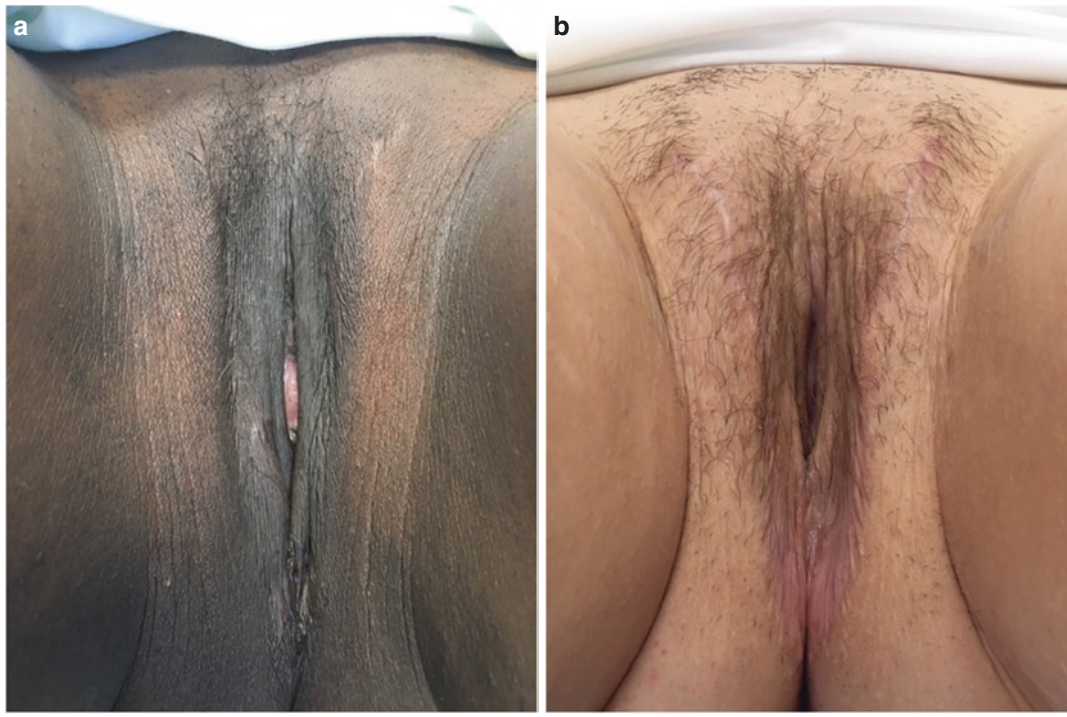

Fig. 10 (**a**, **b**) Patient results 6 months postoperatively

Vaginal stenosis is one of the most common serious complications. This problem leads to inadequate depth and dissatisfaction in patients. Revision of this type of complication can be achieved via a perineal approach. The neovaginal cavity is recreated surgically, all scarring is sharply excised, and the cavity is re-lined with another graft, which can be obtained from the patient. Xenografts are also available at some institutions. Patients must dilate regularly postoperatively to make sure the canal does not stenose again. Recently, techniques using peritoneal

flaps using a combined perineal and abdominal approach have been published, and outcomes thus far, in the hands of expert surgeons, have been favorable [8]. Bowel vaginoplasty is also an option for neovaginal revision surgery, and this technique is described later in this chapter.

The incidence of genitourinary fistula, specifically urethroneovaginal fistula, is reported to be 1.2% [9]. This type of fistula is usually a result of urethral injury at the time of neovaginal cavity creation. If a urethral injury is encountered during the dissection, the injury should be closed in two layers. Sometimes, a flap of bulbospongiosus tissue can be used to cover the defect before the neovaginal graft is placed to reduce the risk of postoperative fistulization. Prolonged postoperative catheterization is important in these patients.

Rectovaginal fistulae can also occur as a result of rectal injury (recognized and unrecognized) during creation of the neovaginal cavity. van der Sluis and colleagues published a very large retrospective analysis of transgender women who underwent vaginoplasty surgery and reported an overall incidence of rectoneovaginal fistula of 1.2% [9]. In this cohort, revision surgery was more likely to be associated with the development of a fistula: primary penile inversion surgery 0.8%, primary bowel vaginoplasty 0%, and revision bowel vaginoplasty 6.3%. In total, 23 patients (2.1%) had suffered an intraoperative rectal injury, and four of those patients (17.3%) developed a fistula as a result. The authors also reported that 38% of the patients requiring management of a fistula likely had an unrecognized rectal injury at the time of surgery, because they were diagnosed in the immediate postoperative period. Most of the patients in this study required surgery to repair their fistula. The median time to surgery was 3 months (range, 0.0–9.7 months). In most cases, fistulectomy with primary closure or local advancement flap was sufficient. Of those undergoing surgery, four patients underwent fecal diversion with direct or delayed fistula repair. In our practice, management of rectoneovaginal fistula, whether an immediate or delayed complication, is often managed with temporary fecal diversion and repair of the fistula in the interim with reversal of the diversion once the fistula is confirmed to be closed.

Intestinal Vaginoplasty

Intestinal vaginoplasty can be performed in patients with insufficient penoscrotal skin or in patients who have experienced vaginal stenosis and have been counseled about the advantages and disadvantages of the procedure. In intestinal vaginoplasty, an intestinal segment is isolated and anastomosed to the neovaginal cavity to form the neovaginal lining. The sigmoid and ileum are mostly common used, and the procedure can be performed through an open abdominal incision or through minimally invasive techniques [10].

The main advantages of this procedure are that it usually creates a neovagina with excellent depth and self-lubrication. The disadvantages are that it involves an abdominal procedure which carries its own morbidity, colitis in the neovagina can occur especially in the presence of known inflammatory bowel disease, risk of neovaginal malignancy, and introital stenosis and scarring.

Quality of Life After Vaginoplasty

Patient-reported outcome measures for patients undergoing vaginoplasty surgery do not currently exist. Work is under way to develop validated outcome measures that are sensitive to the unique urinary, sexual, and aesthetic results and potential complications of genital affirmation surgery. Systematic reviews looking at these kinds of data also are lacking.

In one retrospective review of 117 patients, 94% reported high satisfaction with their surgery, and 71% of patients reported resolution of their gender dysphoria [11]. The top predictors of patient dissatisfaction were intravaginal scarring, prolonged pain, excessive external scarring, loss of sensation, and postoperative hematoma/excessive bleeding. In another small study looking at patient satisfaction and quality of life after vaginoplasty, 91%

of patients experienced improvement in quality of life. All patients stated they would undergo SRS again and did not regret it at all. Anecdotally, in our practice, patients express high satisfaction after surgery and report good functioning. However, there is significant room for more research to close the knowledge gaps that currently exist with regard to patient-centered outcomes and quality of life following surgery.

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Testicular Tissue Transplantation

Dorien Van Saen and Ellen Goossens

Introduction

The transplantation of spermatogonial stem cells (SSCs) represents a potential approach to restore fertility in patients who are at risk of germ cell loss but do not have the option to cryopreserve a semen sample to preserve their fertility. SSCs are the founder cells of spermatogenesis and are thus indispensable for the production of sperm cells (90 million per day in the human). The function of SSCs is to maintain the stem cell population (self-renewal) in the testis on the one hand and to generate enough progenitor cells (differentiation) which will further differentiate in mature spermatozoa on the other hand [1]. To succeed in these functions, SSCs are positioned in a tightly regulated stem cell niche.

The interaction between Sertoli cells and the SSCs is of high importance for the regulation of spermatogenesis. The number of Sertoli cells also determines the number of SSCs in the testis and is thus responsible for the sperm output. They are connected to each other and to the germ cells by junctions allowing close communication between both cell types [2]. The Sertoli cells are assisted in their function to regulate the tight balance between SSC renewal and SSC differentiation by other somatic cells, including the Leydig cells, the peritubular myoid cells, other interstitial cell types and the vasculature [3]. Leydig cells support spermatogenesis by the synthesis of androgens which regulate spermatogenesis by interaction with androgen receptors present on the Sertoli cells and peritubular myoid cells. Increasing evidence shows that also the peritubular myoid cells play a role in the regulation of spermatogenesis. They provide structural support by forming the basement membrane together with the extracellular matrix and fibroblasts and stimulate attachment of Sertoli cells and SSCs to the basement membrane. Their myoid characteristics assist in the transport of mature spermatozoa in the seminiferous tubules toward the epididymis [4]. The close interaction between these different cell types ensures the tight regulation of spermatogenesis within the SSC niche (Fig. 1). It is obvious that any damage to this organ will eventually result in infertility.

A lot of the knowledge that is gathered today about the organization of spermatogenesis, SSC renewal, and the testicular niche has been obtained by transplanting SSCs to a germ cell-depleted testis. The transplantation of a testicular cell suspension resulted in the restoration of spermatogenesis in an infertile mouse testis [5, 6]. These very first

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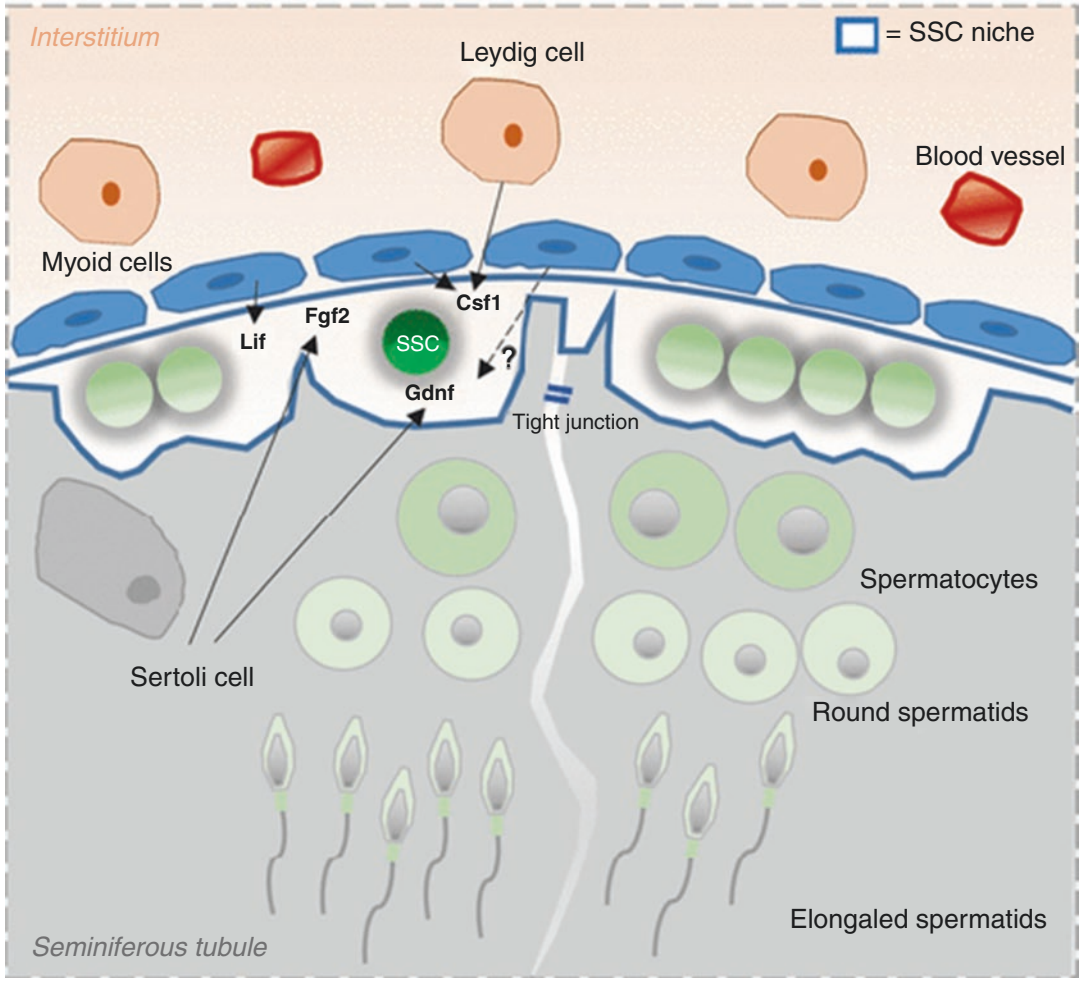


Fig. 1 Spermatogenesis is tightly regulated by the SSC niche, which includes several cell types (Sertoli cells, Leydig cells, peritubular myoid cells) and the vasculature

reports in 1994 paved the way to a new field of research: fertility preservation. The cryopreservation and transplantation of SSCs could become a means to preserve and restore fertility in patients facing SSC loss due to gonadotoxic treatments. Initially, testicular cell suspensions were transplanted, soon followed by the transplantation of testicular tissue [7]. Now, 25 years and many research papers later, a clinical application of this technique is within reach. However, although cryopreservation of testicular tissue is already offered worldwide, the transplantation procedure remains experimental, and the first clinical transplantation is still awaited [8].

This chapter provides an overview of the achievements and insights gathered by the transplantation of SSCs and its possible applications.

The Transplantation of Spermatogonial Stem Cells in Animal Models

SSC transplantation was originally developed in 1994 by Brinster and Avarbock in a mouse model. Isolated mouse testicular cells could recolonize the testis from an infertile mouse and differentiate into fully matured sperm with fertilization capacity as proven by the birth of viable offspring

[5, 6]. This was the first proof of concept that genetic information could be transmitted to the next generation via SSCs. The identification of donor-derived cells and offspring could be realized by the transplantation of labeled donor cells. The first reported detection system was based on the transplantation of ZFLacZ donor cells that stain blue after staining with X-gal [5]. The second system and also the most common one involved donor cells from transgenic animals that express green-fluorescent protein (GFP) in all cells under control of the β-actin promoter [9]. Next to their use in the development of fertility preservation strategies, these models could also add valuable information to the knowledge of the fundamental aspects of SSCs and spermatogenesis or be used in the preservation of endangered animal species and the generation of transgenic animals. Moreover, in the search for the ultimate SSC population in the testis, SSC transplantation was, and still is, the only way to prove stem cell activity.

Alternatively, instead of isolating and transplanting SSCs which have to be followed by SSCs finding their way to the niche (homing), SSCs could be transplanted within their own niche (testicular tissue grafting). The first reports showing restoration of spermatogenesis in testicular tissue grafted to immunosuppressed mice date from 2002 [7, 10, 11].

During the past 25 years, transplantation of SSCs (either as cell suspension or tissue) has been investigated thoroughly.

Xenotransplantations

After the success of restoring spermatogenesis by the transplantation of SSCs in a mouse model, the efficiency of interspecies transplantations was evaluated. Mature spermatozoa could be isolated from the mouse epididymis after SSC transplantation with donor cells from adult rat and hamster. The presence of donor-derived spermatogenesis was confirmed by colorimetric staining (β-galactosidase/X-gal) [12] and/or by the identification of morphologically different spermatozoa in the mouse epididymis [13]. Although testicular cell suspensions including germ cells and somatic cells were transplanted to the mouse recipient testis, only SSCs were able to home to an empty niche and colonize the seminiferous tubules, while other cells were phagocytized by Sertoli cells [14]. This implies that the somatic environment from the recipient mouse was able to support rat and hamster spermatogenesis. However, the efficiency of the microenvironment in recipient seminiferous tubules to support spermatogenesis from transplanted SSCs seems to decrease with increasing phylogenetic distance between donor and host. After transplantation of testicular cell suspensions from immature rabbits and pigs, colonization and limited spermatogonial expansion were noticed, but differentiation did not proceed to meiosis. Colonization, albeit with a low efficiency, was observed when testicular cells from dog, bull, and horse were transplanted to the mouse testis, but no differentiation was observed [15, 16]. This indicates that the interactions between Sertoli cells and germ cells are only partially conserved between different species.

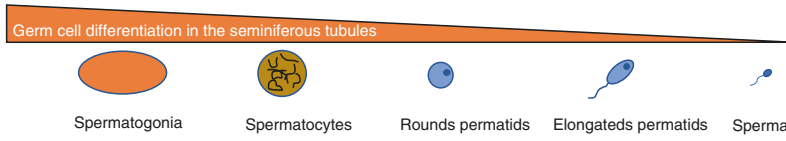
The problem of increased phylogenetic difference between niche and stem cell can be circumvented by transplanting testicular tissue. This gives the advantage of transplanting SSCs within their own niche and avoids the homing step which might result in low colonization efficiency [17]. Initially, grafting of immature testicular tissue was performed under the back skin of immunodeficient mice. Ectopic transplantation of immature tissue from hamster [18], pig, goat [7], cat [19], dog [20], horse [21], bovine [22], and rhesus macaques [7, 23] resulted in the production of complete spermatogenesis in the xenografts. The functionality of the ectopically produced sperm was confirmed by embryonic development with sperm isolated from mouse, goat [7], pig [24], and rhesus macaques [23]. Spermatozoa isolated from porcine xenografts could successfully activate embryonic development in porcine oocytes and support development up to the blastocyst stage [24]. Convincing evidence for the functionality of the spermatozoa generated in xenografts came with the birth of live offspring in pigs and monkeys [25, 26]. Initially, spermatozoa were retrieved

from fresh xenografts, but the same has been performed with spermatozoa isolated from xenografts that had been vitrified before transplantation [27]. The sexual maturation and fertility of piglets born from spermatozoa isolated from cryopreserved xenografts was evaluated. Male offspring showed normal testosterone concentration and testicular histology and were able to impregnate sows after natural mating resulting in the birth of piglets with normal litter size and birth weight. Female first-generation offspring gave birth to healthy pups after artificial insemination with sperm from conventional male pigs [28]. Sperm cells were also collected from monkey xenografts and were either directly used for ICSI or cryopreserved for later use. Transfer of in vitro fertilized oocytes resulted in the birth of seven monkeys [25].

Although successful sperm production in ectopic xenografts was reported for many species, the establishment of full spermatogenesis could not be achieved in human xenografts. The first reports about the xenografting of human tissue only showed limited survival of spermatogonia [10, 29, 30]. In these initial reports, adult testicular tissue was grafted, but this mostly resulted in degenerated and hyalinized tubules. In analogy to what was observed in hamster tissue, this limited success was attributed to the fact that adult tissue was used. The presence of ongoing spermatogenesis seems to have a negative influence on graft survival [10]. Transplantation of testicular tissue from immature human tissue (10 and 11 years) resulted in a better-preserved integrity of the seminiferous tubules, but with only limited survival of spermatogonia 4 and 9 months after transplantation [31]. Similar results were observed when newborn marmoset tissue was transplanted to the back skin of mice [10]. The weight of the seminal vesicles collected after grafting indicated poor androgen production in human and marmoset grafts, while seminal vesicle weight in castrated mice receiving immature mouse or hamster tissue was recovered to values in control mice [10, 30]. The capacity of marmoset and human ectopic tissue grafts to respond to mouse gonadotrophins and initiate testosterone production was lower compared to other species. Meiotic arrest was also observed in rat xenografts, which is exceptional since incomplete spermatogenesis is mostly observed in xenografts from larger species. Spermatogenesis in rat xenografts did not proceed beyond pachytene spermatocytes [32, 33]. Seminal vesicle weight in transplanted mice was restored to a size similar than intact control male mice. Luteinizing hormone (LH) levels were also similar to intact controls, while follicle-stimulating hormone (FSH) levels remained elevated compared to non-castrated mice. Expression of FSH receptor was lacking and could be responsible for the elevated FSH concentrations. Dysregulated expression of Sertoli cell transcripts was also observed, indicating that altered protein expression in the somatic compartment could have played a role in the spermatogenic arrest in rat xenografts [33]. This shows that the functional establishment of spermatogenesis in xenografts is species dependent.

Alternative to the ectopic location, donor testicular tissue can also be transplanted to an orthotopic location, i.e., the scrotum or the most natural place for testicular tissue, the testis itself. Human testicular tissue was transplanted to the scrotum after castration of the recipient mouse. Short-term evaluation (3 weeks after grafting immature testicular tissue from cryptorchid boys) showed a well-preserved integrity of the tubular structures, no fibrosis, and survival of spermatogonia, albeit with a significant loss of germ cells. The number of spermatogonia decreased from 0.55 spermatogonia per tubule in fresh tissue to only 0.08 after the freezing and grafting procedure [34]. Long-term evaluation (6 months after grafting) confirmed the survival of human spermatogonia in scrotal grafts from prepubertal boys. Although sperm cell-like cells were identified in the grafted tissue, these cells did not express meiotic or postmeiotic markers [35].

Intratesticular grafting was first reported by Shinohara et al. Testicular tissue from mouse and rabbit was transplanted under the tunica of the testis from immune-deficient mice. Spermatogenesis was restored, and sperm cells isolated from the grafted piece were used to fertilize oocytes, which resulted in the birth of healthy offspring. Murine sperm cells could be identified by the expression of GFP since a GFP+



	SSC transplantation		Grafting			
			Ectopic		Orthotopic	
Donor species	Most advanced germ cell type	Reference	Most advanced germ cell type	Reference	Most advanced germ cell type	Reference
rat		Clouthier et al.,1996		Schlatt et al., 2010 Goel et al., 2019		
hamster		Ogawa et al.,1999		Ehmcke et al., 2008		
rabbit		Dobrinski et al.,1999		Shinohara et al., 2002		
dog		Dobrinski et al.,1999		Abrishami et al., 2010		
cat				Kim etal., 2007		
pig		Dobrinski et al., 2000		Honaramooz et al., 2002; 2008 Nakai et al., 2010		
bull		Dobrinski et al., 2000		Oatley et al., 2004		
horse		Dobrinski et al., 2000		Rathi et al., 2006		
goat				Honaramooz et al., 2002		
rhesus				Honaramooz et al.,2004		
marmoset				Schlatt et al., 2002 Wistuba et al., 2004		Ntemou et al., 2019
human		Mirzapour et al., 2014		Geens M et al., 2006 Schlatt S et al., 2006 Goossens et al., 2008		Wyns et al., 2007; 2008 Van Saen et al., 2011; 2013 Ntemou et al., 2019

Fig. 2 Visual representation of the most advanced germ cell type present after spermatogonial stem cell transplantation or immature testicular tissue grafting in different species after xenotransplantation in mice

donor was used, while rabbit sperm cells were identified based on their unique sperm head [11]. Full spermatogenesis has also been reported in intratesticular xenotransplants from marmoset [36], but complete spermatogenesis was not achieved in testicular xenografts from pre- and peripubertal boys [37–39]. An overview of the testicular tissue xenograft achievements in different species is presented in Fig. 2.

Allogeneic Transplantations

The feasibility of restoring spermatogenesis by the transplantation of SSCs to the testis has been evaluated in many species other than the mouse. Successful transplantation of SSCs and regeneration of spermatogenesis was reported in goat, pig, dog, sheep, bovine, and monkey [40–45]. The first successful transfer of germ cells into large species was reported by the group of Schlatt [46].

Allogeneic transplantations might have to deal with immunological response due to a different genetic background between donor cells and recipient. Xeno- or allogeneic transplantations in rodents are performed in immunosuppressed animals, but this is not always feasible in larger species. In rodents, allogeneic pups were born by natural mating when recipient mice were treated with immunosuppressive factors [47]. Although spermatogenesis was observed in the testes, no sperm cells were observed in the epididymis of untreated rodents. However, successful generation of sperm cells in the ejaculate could be achieved after transplantation of allogeneic germ cells without any immunosuppression of the recipients indicating that a certain level of immune tolerance is present in the testis [41–43]. Even the transplantation between unrelated immunocompetent animals of different sheep breeds resulted in the production of donor-derived spermatozoa. The functionality of these

spermatozoa was proven by the birth of live progeny after artificial insemination [44].

To guarantee empty niches for the SSCs to home to, recipients need to be depleted of endogenous germ cells. In analogy to the rodent model, this was achieved by treating the recipients with chemotherapeutic drugs [42] or local irradiation of the testes [43, 44]. Alternatively, transplantation was also performed to prepubertal animals which lack spermatogenesis [41]. However, spermatogonia are still present in these animals and might thus prevent efficient homing of transplanted SSCs. Optimal preparation of the recipients can improve the efficiency of the transplantation procedure and result in an increased proportion of donor spermatozoa in the ejaculate [44].

Identification of donor-derived spermatozoa and thus proof of successful SSC transplantation are hampered by the presence of endogenous spermatogenesis. To confirm the presence of donor-derived spermatogenesis, donor cells need to be distinguished from recipient cells. One option is to label the cells with a fluorescent dye which enables detection of donor spermatogonia after colonization. However, the fluorescent marker appeared to be diluted by cell divisions and thus does not permit the identification of fluorescent sperm cells [41]. A recessively inherited condition resulting in immotile and anatomically abnormal sperm was used to prove the success of SSC transplantation in pigs. Affected boars were transplanted with donor cells from young normal crossbred boars. Motile sperm was observed in the ejaculate between 13 and 59 weeks after SSC transplantation [42]. Genotyping epididymal sperm cells could also be a way to differentiate between donor and recipient sperm cells by the use of microsatellite markers [43, 44].

In some species, testicular tissue has been transplanted under the back skin. Sperm cells produced in ectopic murine allografts showed full capability of fertilization resulting in the birth of live offspring which were proven to be fertile as well as indicating the lack of major damage to the germ cell lineage. Although functional sperm cells were produced in these ectopic grafts, premature sloughing and enlargement of the luminal space were noticed already at week 4 after transplantation, probably as a result of the accumulation of fluid [48]. Ectopic testicular tissue grafting has been explored in rhesus monkey and marmoset, but as a model in nonhuman primate species, for a clinical application, grafting was mainly performed autologously and will thus be discussed in the next paragraph.

Autologous Transplantations

In allo- or xenogeneic transplants, problems with immune tolerance due to the different genetic background from donor and recipient have to be taken into account. In a clinical application, this problem does not arise since SSCs will be preserved during therapy with the aim of performing an autologous transplantation.

Autologous transplantation has been performed in the bovine. Injection of bovine SSCs resulted in the presence of spermatozoa in 15% of the seminiferous tubules, while only spermatogonia were present in 45% of the tubules in the non-transplanted control testis [45].

To investigate the potential of SSC transplantation in a human clinical application, the restoration of spermatogenesis after injection of SSCs was evaluated in nonhuman primates. A cell suspension generated from a hemi-orchidectomized cynomolgus monkey was injected via the rete testis, guided by ultrasonography, in the contralateral testis of the same monkey. Four weeks after transplantation, differentiated spermatogonia were present in the testis [46]. Similar experiments were performed in a cynomolgus monkey made sterile by X-ray irradiation. However, spontaneous recovery of spermatogenesis could be observed in the saline-injected testes, making it hard to form conclusions about the presence of donor-derived spermatogenesis, although a higher testicular weight was reported for the SSC-injected testis [49]. Proof that spermatogenesis originated from transplanted donor cells was generated in busulfan-treated macaques. The presence of donor sperm was evaluated by single nucleotide polymorphisms which enable the differentiation between donor and recipient sperm. Complete spermatogenesis was observed 15–63 weeks after transplantation. This study showed that restoration of spermato-

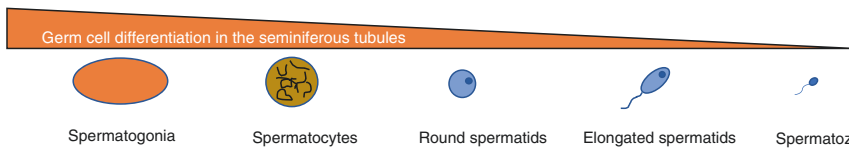
genesis is possible from SSCs which were transplanted to a chemotherapy-treated testis [40]. As testis biology, endocrine regulation, and immune function are similar between rhesus macaques and human, this is a reliable model for a human application.

The proof that spermatogenesis originated from the autologously transplanted donor cells and not from endogenous spermatogenesis is however difficult. Initially, BrdU labeling of the donor cells was used but seemed not efficient for long-term identification of donor cells after transplantation [46, 49]. The use of lentiviral vectors to induce GFP expression in the donor cells was efficient for detection of donor signal by polymerase chain reaction (PCR). However, differentiation between donor-derived and endogenous sperm cells was difficult since lentiviral fluorescence could not be distinguished from autofluorescence [40]. Stable transduction of SSCs can now be achieved as shown by the lentiviral transduction of goat SSCs with the green fluorescent protein. These transfected cells were able to colonize the seminiferous tubules of recipient mice [50].

Autologous grafting has been performed with testicular tissue from rhesus monkey [51, 52]. In 2012, complete spermatogenesis was only achieved in the orthotopic grafts, while ectopic grafts showed meiotic arrest [51]. However, in a recent report, the feasibility to achieve spermatogenesis in ectopic rhesus monkey grafts was shown. Four testicular tissue pieces were individually attached by sutures under the back skin or the scrotal skin. Grafts were collected 8–12 months after grafting, and individual grafts were found to be fused into one single large mass with a fivefold increase in weight. Complete spermatogenesis was present in both the ectopic and orthotopic grafts. Sperm cells were isolated and used for ICSI which resulted in the birth of a healthy monkey girl, Grady [52].

The generation of full spermatogenesis seemed more difficult to achieve in autologous grafts from marmoset. Full spermatogenesis was obtained but only in testicular tissue that was grafted to the scrotum, while spermatocytes were the most advanced germ cell type present in ectopic grafts [53, 54].

Although intratesticular tissue grafting was highly efficient in xenotransplantation studies, autologous transplantations to the testicular parenchyma have not been performed so far (Fig. 3).



	SSCT		Grafting			
			Ectopic		Orthopic	
Donor species	Most advanced germ cell type	Reference	Most advance germ cell type	Reference	Most advanced germ cell type	Reference
bull		Izadyar et al., 2003				
goat		Honaramooz et al., 2003				
sheep		Herrid et al., 2009				
dog		Kim et al., 2008				
pig		Mikkola et al., 2006				
rhesus		Jahnukainen et al., 2011 Hermann et al., 2012		Fayomi et al., 2019		Jahnukainen et al., 2012
marmoset				Wistuba et al., 2006 Luetjens et al., 2008		Luetjens et al., 2008

Fig. 3 Visual representation of the most advanced germ cell type present after spermatogonial stem cell transplantation or immature testicular tissue grafting from different large species after allogeneic or autologous transplantation

Applications of SSC Transplantation in Animals

Production of Transgenic Animals

The production of transgenic animals has practical applications in several fields. Transgenic mice have been produced for years, especially for their use in biomedical research. However, there is also an increasing interest and need for the production of transgenic livestock animals. The term livestock refers to domesticated animals which are kept in an agricultural setting for their use of animal products such as milk, beef, eggs, fur, wool, etc. They have their use in the production of biopharmaceutical products or agricultural products but could also serve as a biomedical model for human diseases [55]. The first genetically modified livestock animals were produced by pronuclear injection, which includes the injection of DNA in the pronucleus of a fertilized oocyte. Somatic cell nuclear transfer (referred to as cloning) is another technique to generate large transgenic animals. However, both techniques are technically challenging, costly, time-consuming, and inefficient. The SSC is unique in its ability to transmit genes to the subsequent generations, which makes it an ideal target for genetic manipulations. The transplantation of genetically modified germ cells can decrease the time to produce transgenic spermatozoa compared to somatic cell nuclear transfer. It also eliminates the labor-intensive outcross breeding to produce non-mosaic germline mutants. A single genetically modified SSC can result in the production of a large number of sperm cells to generate transgenic progeny. Moreover, spermatogenesis is a natural selection mechanism to eliminate spermatozoa with undesired mutations [56]. Goat SSCs were successfully transduced with eGFP by lentiviral transduction. These transduced cells were able to colonize the testis when transplanted to germ cell-depleted recipient mice [50].

Ectopic grafting could be an even better approach to generate transgenic animals. While the phylogenetic distance between donor and recipient is of more importance when SSC transplantation is performed, full spermatogenesis was achieved in ectopic xenografts for many species. The isolation of transgenic spermatozoa from these grafts is also easier since they can be retrieved from the subcutaneous grafts on the back side of a nude mouse. The isolation of transgenic spermatozoa after SSC transplantation will be more difficult since they have to be distinguished from endogenous spermatozoa. In a study where testicular tissue from a transgenic monkey was grafted, the birth of a donor-derived monkey was reported after ICSI using spermatozoa isolated from the ectopic xenografts. The presence of the transgene in the offspring was confirmed by RT-PCR and Western blot [25].

Preservation of Endangered Species

Due to human activities, such as habitat destruction, overhunting/fishing, and climate change, the extinction of several animal and plant species is occurring at a much higher rate than predicted [57]. Cryopreservation and subsequent SSC transplantation can be useful in the preservation of valuable or endangered species. Techniques involving the preservation of SSCs have several advantages over the preservation of mature spermatozoa. The cryopreservation of SSCs enables the preservation of the entire genetic potential of an individual species, since genetic recombination will occur in differentiated germ cells when transplanted. SSCs can be harvested from sexually immature males which is critical for species subject to high neonatal or juvenile mortality [58]. This can also be useful in the preservation of valuable species that undergo early castration (e.g., horses) but display superior traits later in life [59].

Fertility Preservation in Patients at Risk for Germ Cell Loss

A direct clinical application relies in the preservation of fertility in patients who are at risk of germ cell loss. The incidence of childhood cancers is continuously increasing. Luckily, mortality rates are at the same time decreasing [60]. In most industrialized countries, cancer is the most frequent non-accidental cause of death. The overall

5-year survival rate in children and adolescents diagnosed with cancer ranges from 72% up to 78% in Europe and the United States, with an individual difference between different types of cancer. The long-term survival rate of some cancer types can even be as high as 99% [61]. Unfortunately, the malignant cells are not the only cells that are targeted by chemo- and radiotherapy. The rapidly dividing cells in the testis are also destroyed by aggressive cancer treatments. This side effect from cancer treatment only becomes apparent many years later, when these childhood cancer survivors have a child wish. For many years, fertility of adult cancer patients can be preserved by the cryopreservation of sperm [62]. However, in prepubertal and young adolescent patients, spermatogenesis has not started yet making sperm collection impossible. The preservation and transplantation of SSCs provide hope for these patients. By isolating and storing the SSCs during therapy, the fertility potential of these patients could be safeguarded (Fig. 4).

Indications for Fertility Preservation

The inability to reproduce can have a major impact on the psychological aspects of life quality. The cryostorage of SSCs can offer hope to patients for having children after surviving childhood cancer. However, next to cancer patients, other patient groups might also benefit from SSC banking.

Gonadotoxic Treatments

The cancers diagnosed during childhood are of different nature than those in adults, and their occurrence is age dependent. While young children are mostly affected by tumors of embryonal origin such as neuroblastomas, the incidence of leukemia, central nervous tumors, and lymphomas (non-Hodgkin and Hodgkin) is higher in older children. The treatment regimens for these

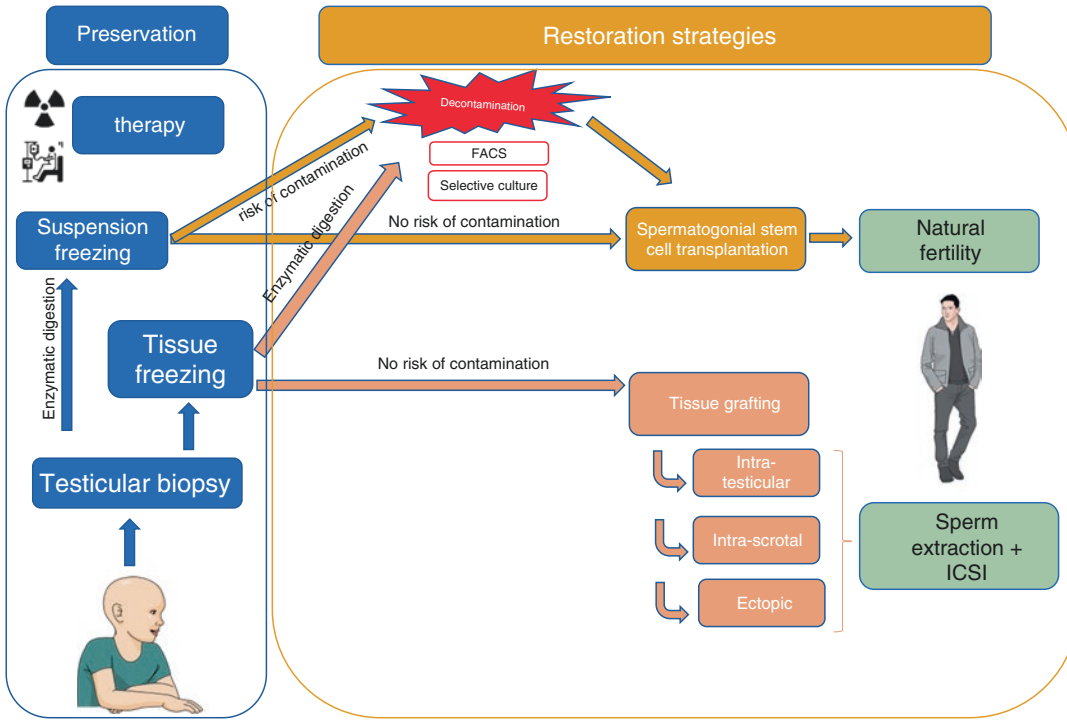


Fig. 4 Potential fertility preservation and restoration strategies for safeguarding fertility in patients at risk of germ cell loss by gonadotoxic therapies

kind of pediatric cancers often consist of chemotherapy with multiple agents [63]. Initially, it was thought that impact of childhood gonadotoxic treatments would be less severe compared to those given to adults since active spermatogenesis is lacking. However, germ cell proliferation and maturation of the somatic cells occur during childhood. From data in the marmoset testis, it is hypothesized that the transition toward the mature status of the testis occurs much earlier than at puberty [64]. The impact on fertility depends on the survival of two morphologically distinguishable stem cell populations present in the testis. The A_{dark} spermatogonia are considered to be the true testicular stem cells and function as testicular reserve. Their proliferation rate is low under normal circumstances but increases after a cytotoxic insult. The A_{pale} spermatogonia have a higher proliferation rate and serve as progenitor cells for the differentiating germ cells [65]. If only the A_{pale} spermatogonia are destroyed, restoration of fertility is possible due to replenishment of the progenitor pool by the A_{dark} spermatogonia. However, complete depletion of both stem cell populations will indefinitely lead to permanent infertility. Next to the survival of the germ cells, the damage to the somatic environment can have a high impact on the restoration of spermatogenesis [66]. Not only the kind of drug but also the dose, the duration of the treatment, and the age of the patient are influencing factors [63].

Treatment regimens can be subdivided in low-risk and high-risk treatments. Alkylating agents like cyclophosphamide, cisplatin, and busulfan are classified as high-risk treatments. These treatments have a high risk of both destroying the total stem cell population and affecting the somatic compartment reducing the chances of spontaneous recovery of spermatogenesis dramatically. Studies on the effect of these treatments on prepubertal testes are scarce. In a large cohort of childhood lymphoma survivors, a damaging effect on testicular function was observed in 42% of the patients. The damaging effects were mainly dependent on the cumulative doses and less related to the age or pubertal status [67, 68]. A significantly lower number of SSCs was observed in testicular biopsies from prepubertal boys having already received alkylating agents before testicular tissue banking, while no difference with control biopsies was observed when non-alkylating agents were received before banking [69].

Next to chemotherapy, irradiation can also induce damage to the germinal epithelium. Impaired spermatogenesis is already described after doses as low as 0.1 Gy, and irreversible gonadal damage occurs at 4 Gy. The damage not only depends on the dose, but also the fractionation of radiotherapy [70]. The long-term effects of irradiation before adulthood were studied in the rhesus monkey. Different doses ranging from 4.0 to 8.5 Gy were administered to monkeys which did not have reached full maturity yet. The long-term effects on testis development were studied 3–8 years after irradiation. Repopulation of the testes by SSCs was observed in most monkeys but did not reach full recovery even in monkeys receiving the lowest dose. Complete sterility was observed in 13% of the irradiated monkeys and occurred in monkeys receiving the highest doses of 8.0 and 8.5 Gy and in monkeys that received a fractionated dose of 6.0 Gy. A decrease in the Sertoli cell number was also reported. Since the number of Sertoli cells determines the number of germ cells, this also negatively impacts fertility [71].

Treatment regimens for nonmalignant conditions might also involve gonadotoxic treatments. Patients with hematological disorders like sickle cell disease or thalassemia often need hematopoietic stem cell transplantation requiring preconditioning treatments involving total body irradiation (10–13 Gy) to deplete the blood stem cell line [66, 72]. These patients also have a high risk of becoming infertile.

Pediatric oncologists/hematologists should inform patients about the risks of infertility and their options for fertility preservation. Since detailed knowledge about the risks of different treatments on infertility is lacking, there is a need to closely follow up fertility potential of these childhood cancer survivors.

Genetic Disorders

Azoospermia, defined as the absence of sperm in the ejaculate, is diagnosed in 10–15% of all infertile men [73]. Next to the idiopathic cases (40%) where the etiology remains unknown, genetic causes can be found in 20–25% of them [74]. Diagnosis only occurs at adult age leaving no option for these men to take precautionary measures to preserve their fertility. However, in about 14% of azoospermic patients, Klinefelter syndrome (KS) is diagnosed [74]. KS patients, having one or more extra copies of the X-chromosome, present with azoospermia resulting from massive germ cell loss which was thought to accelerate from puberty onward [75]. As KS patients are mainly diagnosed at adulthood, initial fertility preservation strategies focused on sperm cryopreservation. Sperm cell retrieval by testicular sperm extraction is feasible in about 40% of the patients [76, 77]. Since, now, more and more KS patients are diagnosed before adulthood [78], the idea was put forward to preserve germ cells already around adolescence (spermatozoa) or before puberty (SSCs). However, collecting spermatozoa at adolescent age did not prove to be beneficial [79–82]. Moreover, testicular biopsies from adolescent patients that were preserved for testicular tissue banking only showed a very low number of SSCs [83, 84]. Even in testicular biopsies that were cryopreserved at prepubertal age, SSC numbers were extremely low [85]. Therefore, testicular tissue banking is considered to have no additional benefit for fertility preservation in KS patients.

Cryopreservation of Spermatogonial Stem Cells

For testicular tissue transplantation to succeed, the testicular biopsy needs to be stored without affecting its functionality. Cryopreservation makes use of very low temperatures to preserve structurally intact living cells and tissues and thus enables the long-term storage of biological samples outside the body. The biological effects that occur when cooling below 0 °C are dominated by the freezing of water, which involves 80% of the tissue mass. Freezing converts liquid water into ice, resulting in the concentration of dissolved solutes in the remaining liquid phase and the precipitation of the solutes exceeding their solubility limit [86]. The formation of intracellular ice crystals must be avoided to assure cell survival during freezing and thawing procedures.

Cryopreservation damage can be controlled and diminished by the addition of cryoprotective agents (CPAs). Their cryoprotective action involves a decrease in the concentration of solutes and an increase in membrane stability during the dehydration and rehydration phases. Two classes of CPAs exist, permeating CPAs (e.g., dimethyl sulfoxide [DMSO], glycerol, formamide, and propanediol) and non-permeating CPAs including sugars (e.g., sucrose, trehalose, dextran, lactose, and D-mannitol) and high molecular weight compounds (e.g., polyethylene glycol and hydroxyethyl starch) [87]. The addition of CPAs to the freezing medium has enabled the storage of biomaterials at low temperature without losing functionality. However, the balance between CPA toxicity and their ability to protect cells from freezing damage needs to be carefully considered.

The cryopreservation of SSCs can be performed by either freezing the whole testicular tissue or freezing cell suspensions. Generally, freezing whole testicular tissue is more challenging since different cell types are involved, each cell type with its own characteristics and requirements. Tissue freezing is also hampered by the fact that rapid cooling and warming rates are not easy to achieve. Usually, cooling rates are not uniform across the tissue piece: the temperature in the center of the sample will change more slowly compared to the temperature at the surface of the tissue [88]. Although it is technically easier to cryopreserve cell suspensions, the viability and function of the SSCs can also be affected by the enzymatic dissociation. Besides that, freezing cell suspensions limits the options for transplantation after thawing [89].

Testicular Tissue Freezing

Freezing testicular tissue implies that not only the functionality of SSCs is maintained but also the integrity and functionality of the testicular stem cell niche. Testicular tissue freezing was already reported in the 1990s with the aim of cryopreserving testicular biopsies to avoid repetitive surgery in patients with azoospermia. Glycerol was used as CPA since this was the worldwide choice for the cryopreservation of spermatozoa and these protocols mainly aimed to recover the most mature stages of spermatogenesis [90, 91]. However, the use of glycerol did not seem to be beneficial for the storage of testicular tissue aimed at preservation of SSCs and testicular integrity. The same was observed when 1,2-propanediol was used. In contrast, DMSO was able to maintain testicular integrity and functional activity of Leydig cells as was evaluated by testosterone production in vitro [92]. A controlled slow-freezing (CSF) protocol using a programmable freezer was used to cryopreserve testicular tissue from immature nonhuman primates [93]. Frozen-thawed primate tissue retained the capacity to initiate spermatogenesis when xenografted in an adult mouse recipient, and, recently, sperm isolated from autologous grafts resulted in the birth of a healthy baby monkey [52]. Also, human spermatogonia survived in xenografted testicular tissue after CSF (with DMSO and sucrose as CPAs) [34, 35].

The previously mentioned protocol involves the use of an expensive freezing device and is rather time-consuming. Therefore, a simpler and more time-efficient protocol was developed, and, when evaluated in a mouse model, this proved to result in similar preservation of cellular and tubular integrity compared to CSF. In this single-step slow-freezing (SSF) protocol, vials are put in an isopropyl alcohol container for 24 h at −80 °C before plunging in liquid nitrogen [94]. This protocol was successfully used to cryopreserve immature human testicular tissue with survival of spermatogonia, maintenance of germ cell proliferation, and integrity of the seminiferous epithelium and the interstitial compartment [95]. Human immature SSCs were able to initiate differentiation after SSF in intratesticular tissue xenografts [37–39].

An alternative method to CSF and SSF is vitrification, which is an ultrarapid freezing method avoiding the formation of ice crystals. Vitrification might thus be beneficial for cell viability and preservation of tissue integrity. Vitrification of mouse prepubertal testicular tissue seemed efficient in preserving the tubular and cellular integrity [94, 96] and did not influence the graft survival and spermatogenic recovery in mouse intratesticular grafts [94]. Successful vitrification of testicular tissue has been reported in pig [27], cat [97], primate [98], and human [99, 100]. The birth of live offspring after vitrification of pig testicular tissue and ectopic xenografting proves that functional integrity is maintained [27].

Cell Suspension Freezing

The cryopreservation of testicular cell suspensions is studied less intensively and mostly applies to human adult cell suspensions [101–103]. Most fertility centers offering testicular tissue banking as a fertility preservation strategy to immature boys perform testicular tissue freezing with slow-freezing protocols. However, there might also be a need to store testicular cell suspensions when the stored testicular tissue is enzymatically digested for decontamination of malignant cells (see later) and subsequent SSC transplantation. During the safety testing procedure, the cell suspension might need to be cryopreserved again until the results of the decontamination procedure are available. When decontamination of the cell suspension is successful, the cell suspension can be thawed again to proceed to the SSC transplantation.

Optimization of a cryopreservation protocol was performed for immature mouse testicular cell suspensions. The initial protocol to cryopreserve a testicular cell suspension was an uncontrolled slow-freezing protocol with DMSO as CPA [104]. The use of a controlled protocol with the same CPA improved the recovery of viable cells after thawing. The use of EG as a CPA in the controlled protocol yielded similar recovery

rates. However, the reinitiation of spermatogenesis after SSC transplantation showed slightly better results with the DMSO protocol. Reinitiation of spermatogenesis from frozen-thawed cell suspension was limited compared to restoration of spermatogenesis after the injection of fresh cells. This indicates that the number of viable SSCs in these frozen-thawed suspensions might be very low and thus that cryopreservation protocols need further improvement [105]. The use of a vitrification protocol did not increase the recovery of viable cells compared to the controlled slow-freezing protocol. The storage in vials instead of straws and the addition of an anti-apoptotic factor (z-VAD[Oe]-FMK) were beneficial for cell survival and could restore spermatogenesis after SSC transplantation [106].

The Transplantation of Spermatogonial Stem Cells in a Clinical Situation

After 25 years of research involving the restoration of spermatogenesis by reintroducing SSCs to an infertile recipient, the clinical application of this technique is still awaited. Although many fertility centers already offer cryopreservation of prepubertal tissue before undergoing gonadotoxic therapies [8, 107], the transplantation of SSCs did not yet occur in a human clinical setting. The reported acceptance rate for cryopreservation of a testicular biopsy in boys diagnosed with cancer is 60% or higher as concluded from surveys in the United States and Europe [108–110]. Sooner or later, the wish for autotransplantation of the stored SSCs will be expressed in one of the centers offering testicular tissue banking. Several factors need to be put into consideration in the decision on how SSCs should be transplanted to the patient.

Tissue or Cells?

Since SSC cryopreservation is mostly offered as testicular tissue banking, both SSC transplantation and testicular tissue grafting are possible. Testicular tissue grafting offers the possibility to preserve the interaction between stem cells and their niche in the original state.

When SSC transplantation will be performed, SSCs have to pass the blood-testis barrier to colonize an empty niche. The efficiency of SSCs to successfully colonize an infertile mouse testis was reported to be only 12% [17]. In the adult testis, SSCs are very rare and barely constitute 0.01–0.03% of all cells [111]. While their proportion is higher in the prepubertal testis, merely small biopsies are available.

Although injection of single cells to the mouse testis was mostly performed via the efferent duct, translation of this technique to larger testes seemed difficult and inefficient. Injection via the rete testis, guided with ultrasound, seemed more promising [46, 112, 113]. The advantage of using ultrasound-guided rete testis injection is that this technique does not require surgery since the injection needle can be inserted through the scrotal skin [40]. Different injection sites were evaluated by injection of contrast liquid in human cadaver testes under ultrasound guidance and subsequent evaluation of the injected testes by micro-CT scan and histology. Infusions of the testicular parenchyma were not observed after injection through the efferent duct or through the head of the epididymis. Blind injections in the seminiferous tubules resulted in limited infusions, while clear filling of the testicular parenchyma was observed by rete testis infusions [112]. The feasibility of injecting a testicular cell suspension by the same injection method was evaluated by the injection of mouse GFP^+ testicular cells which were labeled with 99^m technetium for subsequent evaluation with single-photon-emission computerized tomography imaging [113]. Injection of the cells in the previous experiments was performed by hydrostatic pressure injection. However, high variation and leakage in the interstitial space were observed, which could be reduced by using an infusion pump [114].

The low colonization efficiency and the need for a challenging injection method suggest that testicular tissue transplantation might be easier to perform in a human application. When compared

in a mouse model, intratesticular tissue grafting resulted in a higher testis weight after transplantation and a higher graft recovery rate. In addition, SSC transplantation might fail, and, on top of that, not all successfully transplanted testes showed donor-derived spermatogenesis [9]. However, for patients who were diagnosed with blood cancer or a metastatic cancer, the autologous transplantation of cryopreserved testicular tissue involves a major risk in reintroducing malignant cells. Leukemic infiltrates in the testis of patients diagnosed with acute lymphocytic leukemia can be found in 21% of prepubertal patients [115]. This drawback implicates that SSC transplantation might be the only option for these patients. In contrast to testicular tissue, the removal of malignant cells from a cell suspension is theoretically possible. The efficiency in removing malignant cells from the testicular cell suspension needs to be high since it was reported that transplantation of as few as 20 leukemic cells can result in the transmission of leukemia in rats [116]. Decontamination strategies have been investigated by several research groups. Elimination of malignant cells by positive selection of germ cells and/or negative selection of cancer cells by magnetic-activated cell sorting (MACS) techniques did not seem to be sufficient to deplete all malignant cells since transmission of leukemia to the recipients occurred after transplantation of this MACS-decontaminated cell suspension [117–119]. Negative selection using two markers specific for malignant cells sorted by fluorescence-activated cell sorting (FACS) seemed to be sufficient to eliminate the risk of transmission of leukemic cells for at least 8 weeks after SSC transplantation [120]. However, these promising findings were not confirmed in another study where a combination of MACS and FACS was performed to selectively enrich for SSCs and decontaminate by negatively sorting out malignant cells. Analysis of the sorted fraction still showed the presence of malignant cells (0.4%) which was proven to be sufficient to induce transmission after transplantation in mice [117]. Total elimination of malignant cells is hampered by the similarities between antigens expressed on the membrane of malignant cells and SSCs. In addition, malignant cells can attach nonspecifically to germ cells which means that positive selection of germ cells still includes a risk of contamination. A combination of both negative and positive selection in two repeated FACS cycles did not induce leukemia for 120 days in transplanted mice [118]. The use of singlet discrimination to avoid the inappropriate sorting of cell clumps, together with positive and negative selection further improved the sorting efficiency. However, purity checks of the sorted fractions by FACS still detected remnant contamination [121]. Decontamination of human testicular cell suspensions was achieved using one surface marker for positive selection of SSCs and two markers for negative selection of leukemic cells. It was however shown that different leukemic cell lines required different sets of markers [122]. Immunephenotyping analysis of the malignant cells will thus be necessary to select the most effective markers for negative selection. Further improvement of the sorting technique in combination with individually determined marker selection will be necessary. Furthermore, depletion of malignant cells from a testicular cell suspension in a clinical application will have to be proven by PCR, since this is the only way to sensitively detect the smallest remnants of contaminating cells.

Although FACS seemed to be depleting single cell suspensions, a major concern associated with this is the loss of cells after sorting [118, 121]. The combination of this low cell recovery, with the low numbers of SSCs in testicular biopsies and the low colonization efficiency makes the expansion of SSCs indispensable for clinical application of SSC transplantation. Strategies to expand SSCs in vitro have been reported in rodents. However, translation of these culture methods to SSCs from larger animals did not give the expected results [123–125]. Recently, in vitro proliferation of porcine SSCs has been reported with preservation of their colonization capacity when xenotransplanted to the mouse testis [126]. It has been estimated that a 1300-fold increase in SSCs is necessary to achieve sufficient colonization in a clinical human application [127]. In vitro culture conditions for the propagation of

human SSCs have been reported for adult men [127] and prepubertal boys [128, 129]. The identity of these cultured cells was confirmed by the expression of SSC-specific markers shown by RT-PCR and immunohistochemistry. However, functional assays to prove SSC capacity of long-term cultured human cells are lacking since xenotransplantation of human SSCs is not efficient.

Transplantation Site

Obviously, when SSCs are transplanted as single cell suspension, the only possible option is to transplant them to the seminiferous tubules so they can colonize accessible niches. Healthy offspring has been produced after natural mating of transplanted males with females [130]. This would offer infertile patients the chance to impregnate their partner by natural conception.

The transplantation of testicular tissue has however been performed to different locations, such as the back skin (ectopic), the scrotum, and the testis (orthotopic). In most xenograft studies, testicular tissue was transplanted to the back skin of nude mice. In the model most closely related to the human, full spermatogenesis was achieved in ectopic grafts from rhesus monkeys. Full spermatogenesis could be observed in up to 15% of tubules in ectopic xenografts [23]. However, the same outcome could not be reached in the marmoset. In this species, the site of transplantation seemed to have a major effect on the outcome of the graft. The efficiency of inducing spermatogenesis in marmoset grafts was limited to spermatogonial survival and differentiation up to spermatocyte level [10]. Since androgens were not produced by the marmoset graft, it was hypothesized that failure of testosterone production caused the premeiotic arrest in these grafts. This hypothesis was supported by a deletion in exon 10 of the luteinizing hormone (LH) receptor gene, which makes this species sensitive to chorionic gonadotrophin (CG) instead of LH which is produced in the mouse recipient [131]. However, neither exogenous stimulation of the recipient with human CG nor the co-transplantation of hamster immature tissue to provide a high local testosterone level could overcome the meiotic blockage in the marmoset graft [10, 132]. When marmoset tissue was grafted to a suitable hormonal environment by performing autologous transplantation of immature marmoset testis pieces to the back skin of the same animal, induction of spermatogenesis was achieved but not beyond meiosis. The serum testosterone levels in these transplanted animals only showed a marginal increase which could explain the meiotic arrest. Next to this, the transplantation site was covered with fur resulting in an increased temperature [54]. This hyperthermia probably was the main reason for the failure of obtaining full spermatogenesis in ectopic grafts since the production of spermatozoa could be observed in autologous scrotal grafts in castrated marmosets [53]. Recently, full spermatogenesis was achieved in intratesticular xenografts without altering the hormonal milieu by castration or any exogenous gonadotrophin stimulation [36].

In a study in which cryopreserved rhesus monkey testicular tissue was grafted, the scrotal grafts showed the presence of full spermatogenesis, while pachytene spermatocytes were the most advanced cell type in autologous ectopic grafts. However, it should be mentioned that, in this study, testicular tissue from marmosets in which spermatogenesis was already initiated at the time of cryopreservation was used [51]. Indeed, no difference was observed between scrotal and back skin grafts when immature rhesus monkey testicular tissue was autologously grafted. Complete spermatogenesis was observed in more than 70% of the seminiferous tubules, regardless of the transplantation site. However, graft size was found to be larger in the scrotal grafts compared to the ectopic grafts [52]. The discrepancy between both studies could rely in the age of the donor or the cryopreservation protocol which was slightly different.

Adult human tissue grafted to the scrotal area also resulted in better integrity of the seminiferous tubules compared to ectopic grafts which were almost totally degenerated and fibrotic [34]. Although tubular integrity in ectopic human xenografts was also improved when immature human tissue was used, spermatogonial survival

seemed to be better in scrotal or intratesticular grafts [31, 35, 37].

Hormonal Environment

In xenotransplantations to the mouse, the endogenous gonadotrophins were sufficient to initiate spermatogenesis and to ensure its continuation in bovine grafts [22]. Elevated levels of LH and FSH were guaranteed by the castration of the host before transplantation [23]. The high FSH levels stimulate Sertoli cell proliferation and maturation, while LH triggers Leydig cells to mature and produce testosterone as evidenced by an increased weight of the seminal vesicles and increased levels of serum testosterone [7, 48]. Testosterone production by the ectopic graft will then exert negative feedback on gonadotrophin release in the castrated host resulting in decreasing LH and FSH levels [23, 48]. Increased weight of the seminal vesicles at the time of graft collection in castrated mice shows that testosterone production occurs in the grafted tissue. However, seminal vesicle weight, which is indicative for the production of bioactive testosterone, in grafted recipients is higher compared to castrated mice but still lower than in intact male mice. The production of less bioactive testosterone was attributed to the lower responsiveness of donor Leydig cells to murine gonadotrophins. In ectopic xenografts from horses, full spermatogenesis was shown, but could only be achieved in a very limited number of tubules. In most seminiferous tubules, progression up to meiosis was achieved. Treatment with exogenous human CG (LH-like) and pregnant mare's serum gonadotropin (FSH-like) resulted in improved germ cell differentiation in horse xenografts. This improvement was translated in the fact that elongated spermatids were observed in the grafts from an immature donor, while pachytene spermatocytes were the most advanced cell type in the nontreated controls. No improvement in the percentage of tubules with differentiated cells was noticed. Exogenous treatment with gonadotrophins did also not result in increased seminal vesicle weight [21]. The effect of administration of exogenous gonadotrophins to mice in which prepubertal human tissue was grafted to the testis was also evaluated but did not result in postmeiotic progression of spermatogenesis in these grafts. Meiotic activity was observed in the treated grafts as well as in the nontreated controls [38]. However, exogenous administration of gonadotropins resulted in complete spermatogenesis in rhesus monkey xenografts, while untreated grafts showed no germ cell differentiation. Sertoli cell maturation was observed in both treated and untreated grafts [133].

Postmeiotic progression of spermatogenesis is dependent on testosterone [134], and a low testosterone concentration in the grafts might thus explain the low efficiency of obtaining fully matured spermatozoa in xenografts. In ectopic grafts, testosterone production is solely coming from donor Leydig cells, which need to be stimulated by the mouse gonadotrophins. Since castrated mice were the prevalent model used for xenograft experiments, at the moment of transplantation, the ectopic xenografts are placed in an environment with a low testosterone concentration. This corresponds with the natural environment for neonatal and immature tissue. The high levels of FSH in castrated mice were thought to induce maturation of the somatic cells in the grafted tissue. The responsiveness of donor Leydig cells to the mouse gonadotrophins seems to be higher in immature or young tissue compared to adult tissue as observed by an increased seminal vesicle weight in mice receiving immature or young donor tissue grafts [20]. However, new insights came from a recent study which evaluated the effect of the host environment on the development of marmoset grafts. Grafts transplanted to non-castrated male mice achieved better tubular survival, seminiferous epithelial arrangement, and progression of spermatogenesis compared to female mice and castrated mice [135].

The Effect of Donor Age on the Maturation of Testicular Grafts

To understand the effect of the donor age is important to define the patient's age range for which the technique could be offered in order to

successfully restore spermatogenesis after autotransplantation. At least in xenograft experiments, when transplantation was performed to the back skin, a large variation in the success of achieving full spermatogenesis was noticed. Successful production of spermatozoa in ectopic xenografts has been achieved in many species, but the time needed to collect mature spermatozoa differed between species. In the pig and primate xenografts, spermatozoa production was observed at an earlier tissue age compared to age-matched controls [7, 23], while delayed spermatozoa production was noticed in the cat, horse, and bull [21, 22, 136]. The impact of donor age was evaluated in feline and canine xenografts. Initial acceleration of meiosis was observed in xenografts when young donor tissue was used, but mature spermatozoa were only recovered after the time point they normally appear in the feline testis. Ectopic tissue grafts with tissue from young adults did not develop but showed degeneration, while tissue from pubertal animals in which meiotic germ cells were already present at the time of grafting showed a lower recovery and less efficient production of mature spermatozoa compared to the prepubertal donor grafts [19]. Immature testicular tissue showed the highest potential to induce full spermatogenesis in the grafts. The same findings were observed in canine xenografts [20]. More extensive tubular damage was also observed in scrotal and intratesticular grafts from peripubertal human donors compared to grafts from prepubertal boys [37]. In general, it seems that the presence of spermatogenesis at the time of grafting increases the risk of tubular degeneration in the graft, and this risk increases with the level of spermatogenesis that is present in the donor tissue at the time of grafting [19, 20]. Adult testicular tissue has a low chance in surviving graft periods and mostly results in sclerotic grafts. This is reported in different species including mouse, hamster [10], horse [21], and human [29, 30] and can be explained by the fact that spermatogenesis demands high amounts of oxygen. Mature tissue is therefore more vulnerable to periods of ischemia, while immature tissue is more effective in restoring blood supply after grafting [10]. Also, seminal vesicle weight was higher in mice carrying grafts from immature or young donors compared to mice receiving adult donor tissue. This suggests that immature and young donor tissue is more responsive to the mouse gonadotrophins resulting in a higher production of testosterone in the graft, which in turn stimulates postmeiotic development [20].

Although there is a higher risk of tubular disintegration, donor tissue isolated at the onset of puberty shows the best potential to support the development of complete spermatogenesis. This is probably because the somatic environment and germ cells are at that point ready to start spermatogenesis. Nine months after transplantation, human peripubertal testicular tissue grafted to the mouse testis displayed better spermatogonial survival and showed spermatocytes entering meiosis, which was not the case in grafts from younger patients [37]. However, in a follow-up study, meiotic cells could also be observed in xenografts from prepubertal patients (2.5–12.5 years), but postmeiotic differentiation was not achieved [38].

Depending on the species, in vivo maturation of testicular tissue takes a few days (rodents), several months (pigs, sheep, goats), few years (horse, monkey, bovine), or, in case of the human, 9–15 years. This has of course important implications on the future clinical application. The maturation of the transplanted tissue cannot occur at the normal pace, since this could be more than 10 years if cryopreservation was performed during the neonatal period. Such a long incubation time will not only be negatively received by patients with a child wish but might also be too long for efficient graft survival. For testicular tissue that was cryopreserved at a young age and is transplanted during adulthood, accelerated maturation will thus be required for efficient restoration of fertility.

In animal models, accelerated maturation was achieved with [21, 133] as well as without [7, 23] exogenous stimulation of gonadotrophins. In human intratesticular xenografts, maturity was not achieved since Sertoli cells still showed expression of anti-Mullerian hormone. The lack of full maturation is probably the explanation why full spermatogenesis has not yet been

achieved in human prepubertal tissue grafts. In marmoset grafts, intratesticular grafting overcame the meiotic blockage which was observed in ectopic grafts, but this was not the case for human xenografts. Although human prepubertal tissue was grafted to the testis for a long period (12 months), no postmeiotic germ cells were achieved in these grafts [37–39]. The marmoset is the most accepted preclinical model for human testicular development, but, in the marmoset, testicular maturation is reached by 15–18 months [64], while it takes more than 10 years in the human. Unfortunately, this period cannot be covered in the life span of a mouse.

Delivery of Growth Factors

The first days after the transplantation of testicular tissue are crucial for further graft development. During this time, a connection between the transplant and the host needs to be established in order to reduce the ischemic period and provide the transplant of oxygen, nutrients, and factors supporting proliferation and differentiation of germ cells and maturation of somatic cells. A substantial loss of germ cells has been reported shortly after testicular tissue grafting [34, 37, 51]. Germ cell fate evaluated in bovine xenografts indicates that an initial loss of germ cells is one of the reasons for the low efficiency of generating full spermatogenesis in bovine xenografts. To limit this initial germ cell loss, it is important that blood supply to the transplant is established quickly and efficiently. Graft and germ cell survival may thus be increased by the stimulation of neovascularization. A higher number of blood vessels were detected in functional grafts compared to nonfunctional grafts, which highlight the importance of angiogenesis for the survival, development, and generation of spermatogenesis in grafted tissue [137]. It was shown that the connection of the capillary system between donor and host is established by a combination of outgrowing small capillaries from the graft and large blood vessels coming from the host [32].

With the aim to improve vascularization, the effect of treating grafts with vascular endothelium growth factor (VEGF) was evaluated. VEGF is an important regulator of blood vessel formation. During embryogenesis, it stimulates vasculogenesis and angiogenesis, but it is also known as a key mediator in neovascularization in cancer and other diseases [138]. In bovine xenografts, treatment with VEGF increased the number of seminiferous tubules with elongating spermatids. However, an increase in the number of blood vessels or microvascular density was not observed in these grafts [137]. Instead, the increased number of seminiferous tubules with complete spermatogenesis could be attributed to an increased survival of spermatogonia as a result of the production of the anti-apoptotic factor B-cell lymphoma 2 [139]. Although there was no effect on vascularity in these bovine xenografts, an increase in vascular surface and vessel density could be observed in human prepubertal xenografts. This resulted in a better tubular integrity and spermatogonial survival but did not stimulate postmeiotic differentiation [39]. VEGF treatment of the testicular transplants was either achieved by a direct and single injection at the transplantation site [137] or by treating the testicular tissue with VEGF in vitro for 5 days prior to grafting [39, 139]. Instead of a single injection or incubation of the testicular tissue before transplantation, testicular tissue transplants could benefit from a more prolonged delivery of VEGF to stimulate the connection with the vascular system of the recipient. However, systemic delivery of VEGF can result in uncontrolled effects at distant sites making it unsafe for use in a clinical context. Tissue engineering might be helpful to provide a sustained and controlled delivery of molecules. This involves the encapsulation in a three-dimensional environment, synthetically composed or derived from biological matrices to imitate the extracellular matrix [140]. A local and prolonged delivery of biomolecules might be achieved by the incorporation of testicular tissue transplants in biocompatible hydrogels, together with biomolecules which can be encapsulated in chitosan/dextran sulfate nanoparticles. The requirements for being biocompatible implicate that the used biomaterials should interact with the biological environment of the recipient with-

out inducing immune responses and allow full integration with the recipient. On top of that, products resulting from the biodegradation of the scaffold should be cleared from the body without interfering with other organs [140]. The use of supportive matrices to optimize testicular tissue grafting was evaluated in mouse autografts. Testicular tissue was encapsulated in two different hydrogels, alginate and fibrin. Encapsulation in alginate was superior to fibrin encapsulation and resulted in a higher vascular density and improved the recovery rate of scrotal grafts 5 days after grafting. Although the beneficial results observed few days after transplantation were not maintained at 21 days after transplantation, the additional value of tissue engineering to improve graft survival and differentiation should be explored further [141].

Fertility After Testicular Tissue Transplantation

Although natural conception might be possible when SSC transplantation is performed, this has never been proven for intratesticular grafting. It might be difficult for the grafted tissue to make a connection with the rete testis and allow spermatozoa produced in the grafted tissue to reach the epididymis and leave the testis at the time of ejaculation. Therefore, testicular spermatozoa will probably have to be collected through testicular sperm extraction. The most suitable time point to collect spermatozoa from the grafted tissue in a human application will need to be determined. It is not known how long it will take before mature spermatozoa are produced in the testis, and this might be dependent on the donor's age at the time of cryopreservation.

Spermatozoa isolated from ectopic porcine xenografts showed a lower fertilization rate compared to testicular, epididymal, and ejaculated spermatozoa. Ectopically produced spermatozoa do not undergo the final steps of maturation (which normally take place in the epididymis) but could be comparable to testicular sperm. However, in boars, a lower fertilization rate was observed for ectopically produced spermatozoa compared to testicular sperm from control boars. This lower fertilization rate could be caused by senescence of spermatozoa in the grafts. In vivo, a constant clearance of the produced spermatozoa from the seminiferous tubules is assured. However, this is not the case in grafts [24]. In theory, when testicular tissue is grafted to the testis, open seminiferous tubules in the grafted tissue could connect with existent seminiferous tubules. Although this could allow clearance of senescent spermatozoa from the grafted tissue, the number of sperm cells produced by the graft will probably be too low for natural conception. To avoid this problem, spermatozoa should be isolated from the graft as soon as possible.

Conclusion

Both SSC transplantation and testicular tissue transplantation seem to be promising strategies to preserve fertility in patients facing germ cell loss. Restoration of spermatogenesis from human spermatogonia has not yet been achieved in a xenotransplantation model. However, autologous transplantation of SSCs in nonhuman primates is able to restore fertility in the recipients. Also, spermatozoa produced in transplanted testicular tissue in nonhuman primates was able to fertilize an oocyte which resulted in the birth of healthy baby monkey. These promising results are reassuring for the translation of this techniques to the clinic. Careful evaluation of the efficiency and safety of these procedures will be necessary. Testicular tissue transplantation is believed to be the most efficient and easy method but holds too many risks when there is risk for contamination with malignant cells in the stored testicular tissue. Thorough evaluation of minimal residual disease will have to be implemented before transplantation to the patient can be performed. SSC transplantation might be an alternative on the condition that total elimination of malignant cells can be guaranteed.

Definitions

Spermatogonial stem cell Stem cell located at the basement membrane of the seminiferous tubules within the testis. Responsible for renewal of the stem cell pool in the testis and the founder cell of spermatogenesis.

Spermatogonial stem cell transplantation The transplantation of spermatogonial stem cells to the testis by injecting a testicular cell suspension through the efferent duct.

Testicular tissue grafting The transplantation of testicular tissue. The testicular tissue is inserted under the tunica albuginea, which is sutured after insertion of the testicular piece.

Practical Clinical Tips

Translation of the spermatogonial stem cell transplantation and testicular tissue transplantation to a clinical application needs to be performed. Testicular tissue freezing is already performed in several centers. All centers offering cryopreservation of testicular tissue use the slow-freezing protocol. Several factors need to be put into consideration in the decision on how SSCs should be transplanted to the patient before translation to the clinic:

- Should we transplant tissue or cells?
- If testicular tissue is transplanted, where should we transplant?
- Should we provide external support to the hormonal environment?
- Should we deliver growth factors to stimulate graft survival?

Clinical Cases

Clinical cases have not yet been performed.

Key Readings

- Picton HM, Wyns C, Anderson RA, Goossens E, Jahnukainen K, Kliesch S, et al. A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys. Hum Reprod. 2015;30(11):2463–75.
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Take Home Messages

- Restoration of spermatogenesis in infertile animals is possible by the transplantation of spermatogonial stem cells or testicular tissue.
- Healthy offspring have been born after transplantation of spermatogonial stem cells (cell suspension or tissue) in large animal species.
- Testicular tissue transplantation and spermatogonial stem cell transplantation are promising strategies to restore fertility in patients at risk of germ cell loss during puberty.
- Cryopreservation of testicular tissue is already offered in several fertility centers.
- Autotransplantation in a clinical setting has not yet been performed.

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Removal of Malignant Cells Before Autotransplantation of Spermatogonial Stem Cells

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This chapter evaluates the current knowledge about approaches to detect and remove malignant cells from testicular biopsy before spermatogonial stem cell (SSC) autotransplantation to restore fertility.

Introduction

Childhood cancer occurs in about 16 per 100,000 children in the United States [1]. Fortunately, there are increased success rates in treating cancers in children over the past few decades, and now approximately 80% of children get cured following treatments [2, 3]. Chemotherapy and radiotherapy for cancer patients can be gonadotoxic and cause permanent infertility [4, 5]. Infertility is an essential concern because it has a significant impact on the quality of life and emotional well-being of these patients [6].

Sperm cryopreservation is the gold standard option for adult males to preserve their fertility. Unfortunately, this is not an option for prepubertal boys who still do not have sperm. Testicular tissue cryopreservation holds the promise of fertility for these young cancer survivors. Several cells and tissue-based therapies in the research pipeline may allow for cryopreserved testicular tissues to generate sperm and produce biological children [7–9]. These promising technologies have prompted several centers worldwide to start cryopreserving testicular tissue for prepubertal cancer patients, pending that these new technologies will be available for them when they reach puberty [10].

One of the most critical hurdles facing the process of fertility preservation in prepubertal cancer boys is the possibility of malignant cell contamination in their testicular biopsies. Hematologic cancers as leukemia tend to infiltrate the testis [11]. The incidence of testicular leukemic infiltration is 5–40% in acute lymphoblastic leukemia (ALL) patients [12]. Supposedly, malignant cells in stored testicular tissue samples can reintroduce cancer to these patients after remission. Another study showed that even as

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few as 20 malignant cells can cause transmission of disease [13]. These facts raise concerns about the risks of testicular tissue or spermatogonial stem cell (SSC) autotransplantation using stored testicular tissue. There is an increased need to find a sensitive method to detect malignant presence in testicular tissue samples and an efficient way to purge these cells thoroughly.

The Risk of Malignant Cell Contamination in Testis Tissue

Jahnukainen and colleagues in 2001 showed that the transplantation of testicular cells from T-cell leukemic rats could cause transmission of leukemia to healthy rats [13]. Transplantation was done by injecting testicular cells into the rete testis or the interstitial testicular tissue. Recipient rats were first treated with busulfan to remove their spermatogonia. This study was divided into four groups; group one, injecting freshly isolated tubular cells from healthy and leukemic rats; group two, injecting frozen-thawed tubular cells from leukemic rats; group three, injecting frozen-thawed interstitial cells; and finally, group four, injecting cells from healthy donors mixed with different numbers of leukemic cells (2, 20, 200, or 6000 lymphoblasts from lymph node).

Results showed that all rats injected with freshly isolated tubular cells developed signs of terminal leukemia within 14 days; they were injected either via rete testis or into interstitial space. The group injected with frozen-thawed cells reached terminal leukemia 3–6 days later. In the group injected with interstitial cells, histological damages were more significant than those that received tubular cells. In the fourth group, which received healthy cells mixed with leukemic cells, rats showed terminal leukemia after 14 days and 19 days in the rats that received 6000 cells and 200 cells, respectively. Three of the five rats (60%) injected with only 20 lymphoblasts developed terminal leukemia 21 days after injection. The only rats that stayed healthy up to 42 days were the rats injected with two cells only.

This study showed that transplantation of testicular cells, either fresh or cryopreserved, from leukemic donors, carries a significant risk of transmitting leukemia to recipients. The small number of cells being as low as 20 cells did not eliminate that risk.

Over the past two decades, researchers have been trying to develop a strategy to detect and eliminate malignant cells within testicular tissue or testicular cell suspension. This chapter summarizes the previous studies, both on animals and human materials, to extrapolate the prospects to overcome this problem.

Animal Studies

Researchers have tried decontaminating testicular tissue in different animal species. Mice were the first to be experimented on, followed by rats and nonhuman primates (Table 1). The primary method that was used in the removal of malignant cells was cell sorting. Cell sorting means separating cells from a mixture according to their specific criteria. These criteria can differ according to size, morphology, or surface protein expression. The two main cell sorting methods being used in biologic research are magnetic-activated and fluorescence-activated cell sorting.

Magnetic-activated cell sorting (MACS) is a method that aims to enrich specific cell types from a mixed cell population (Fig. 1). This is made possible through functionalizing magnetic beads with the antibody against a particular surface marker on the target cells. These magnetic beads are composed of a magnetic core embedded in a polymer shell. The beads are biodegradable, and they do not affect cell functionality or structure. These beads are paramagnetic, meaning that they do not have permanent magnetism, but they become magnetized when applied to an external magnetic field.

The magnetic Isolation can be achieved through positive or negative selection. Positive selection means that the microbeads functionalized with the antibody against known cell antigen on the target cells will attach to the cell's surface. Then, when passing through the magnetic field, they will be isolated. Negative selection means that microbeads will be functionalized with the

Table 1 Summary and comparison of animal studies

Study	Year	Purging method	Malignant cells	Source of testicular cells	Quantity/percentage of malignant cells	Malignant cells markers	Germ cells markers	Detection method
Jahnukainen et al.	2001		Rat T-cell leukemia	PVG rats	2, 20, 200, or 6000 cells			Allotransplantation
Fujita et al.	2005	FACS	Murine C1498 leukemia	Mouse C57BL germ cells	Not determined	CD45, MHC-I ($H\text{-}2K_b$/$H\text{-}2D_b$)	TRA98	Allotransplantation
Hou et al. (a)	2007	FACS	Roser's T-cell leukemia	PVG rats	Used testicular cells from contaminated donors for sorting	CD4, MHC class I	Ep-CAM	Allotransplantation
Geens et al.	2007	MACS and FACS	Mouse EL4 T-lymphoma cells	B6CBAF/Juco mice	5% in mouse lymphoma experiment	H-2Kb (MHC-I)	α6 integrin (CD49f)	Flow cytometry, in vitro colony formation, allotransplantation
Hou et al. (b)	2007	Xenograft	Roser's T-cell leukemia	PVG rats	Not determined	CD4	CD90	Histology, flow cytometry, xenograft
Hou et al.	2009	MACS	Roser's T-cell leukemia	PVG rats	Not determined	CD4, CD90		Flow cytometry, tumor-forming in vivo immunocytochemistry
Fujita et al.	2008	Xenograft	Human Jurkat T-cell leukemia	SCID mice	100, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 cells		DAZL, Prm2	RT-PCR, xenograft
Hermann et al.	2011	FACS	Human MOLT4	Prepubertal nonhuman primate	10%	CD45	CD90	Immunocytochemistry, in vivo tumor formation (interstitial injection into the mouse testes)
Shabani et al.	2018	Drug carried on NPs	Mouse EL-4 ALL	Neonatal mouse SSC	5%	H-2Kb	α6 integrin	Flow cytometry, allotransplantation
Tani et al.	2019	Density gradient, MACS, and FACS	Human B-ALL	GFP and C57BL mouse germ cells	Not determined	CD20, CD38	CD90, α6 integrin (CD49f)	Allotransplantation, histology

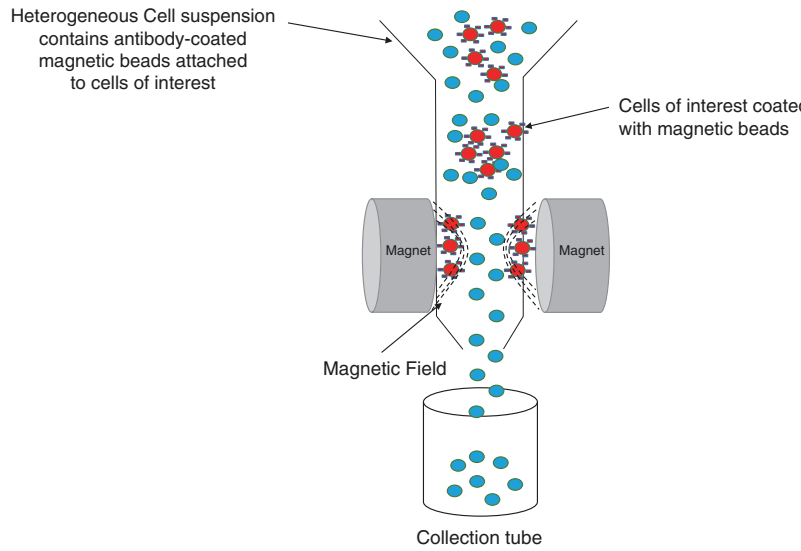

Fig. 1 Schematic diagram of Magnetic-activated cell sorting (MACS)

antibody against known cell antigen on the unwanted cells. When the cell mixture passes through the magnetic field, the unwanted cells will be removed, and the remaining solution will contain the enriched population of the desired cells. Negative selection is inferior to positive selection in terms of specificity and may require multiple sorting steps [14, 15].

Fluorescence-activated cell sorting (FACS) is a cell separation based on their fluorescence labeling (Fig. 2). Cells are stained with fluorophore-conjugated antibodies against specific cell surface markers. Individual cells from a heterogeneous cell population pass through the lasers, and they acquire an electric charge based on their fluorescence labeling. This charge causes deflecting of the cells into separate collection tubes. Each tube has an enriched population of either positively or negatively charged cells. As in MACS, FACS can also be done through positive or negative selection by staining either the wanted cells or the unwanted cells with a fluorescent-conjugated antibody. FACS is considered more stressful than MACS for cell viability and functionality; nevertheless, it is deemed superior to MACS in terms of specificity and purity of enriched cells [16].

Fujita and colleagues in 2005 [17] conducted a study using FACS to try to isolate the malignant cells from germ cells. They used the C1498 leukemia cell line, a murine leukemia cell line of C57L/6 origin. The cells were obtained from removed testes of C57L/6 mice 2 weeks after intraperitoneal injection with C1498 cells. CD45 and MHC class I heavy chain (H-2Kb/H-2Db) were used as surface markers for germ cells for isolation in FACS. C1498 cells were 99.7% $CD45^+$ and H-2Kb/H-$2Db^+$, while germ cells were 93.5% negative for both markers validated by flow cytometry. Cells that were H-2Kb/H-$2Db^-$ were confirmed to be germ cells by positive immunostaining for TRA98. TRA98 is a monoclonal antibody that stains germ cells' nucleus except for long spermatids and mature spermatozoa. Both enriched germ cell fraction and leukemic cell fraction were separately injected intraperitoneally into two groups, and each has 12 C57L/6 mice. All mice in the group injected with germ cell fraction survived until 300 days after injection with no leukemia signs. Their bone marrow and peritoneal exudates showed no signs of leukemia cells on histologic examination. On the contrary, the group injected with leukemic fraction showed leukemia and hemorrhagic ascites within 40 days.

Transplantation of germ cells was done from donor C1498-inoculated GFP mice into efferent ducts of recipient C57L/6 mice. Cells for transplantation were obtained from testes of GFP mice after 2 weeks of intraperitoneal injection with

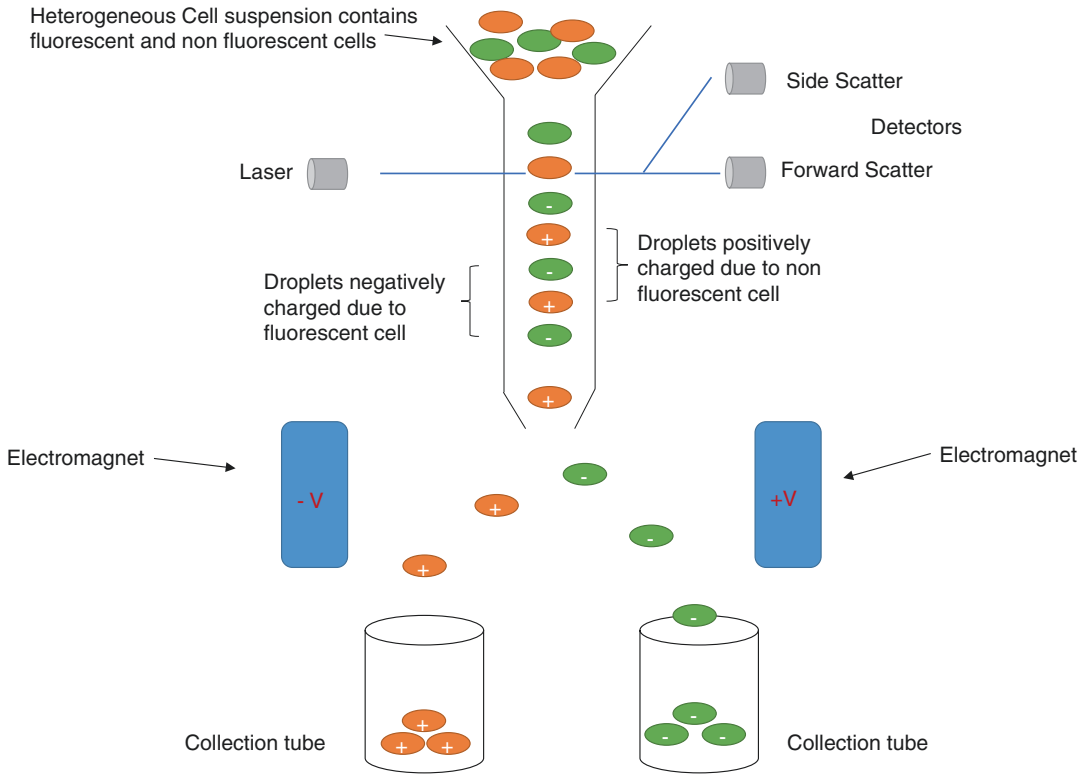

Fig. 2 Schematic diagram of Fluorescence-activated cell sorting (FACS)

C1498 cells. Recipients were rendered sterile by busulfan 4 weeks before injection to examine the sorted germ cells' capacity to undergo spermatogenesis. GFP mice were used as donors to differentiate between transplanted cells and recipient cells. All mice transplanted with enriched germ cell fraction remained alive for 8 weeks after transplantation. Their dissected testes showed a normal appearance. Besides, donor GFP cells were observed in the seminiferous tubules with normal spermatogenesis and producing mature sperms. On the contrary, all mice transplanted with unsorted germ cells developed terminal leukemia signs within 4 weeks. On examination their dissected testes showed leukemic infiltration 2 weeks after transplantation.

To investigate whether the GFP sperm obtained from recipient testes could support normal development, intracytoplasmic sperm injection was performed. Forty (95.2%) of the 42 embryos developed into two-cell stage within 24 h of culture. After transferring the embryos to female mice, 12 pups (28.6%) were born. Half of the pups showed green fluorescence under excitation light, indicating that sperms were derived from transplanted germ cells.

Hou and colleagues in 2007 [18] also conducted a study using FACS as a method of decontamination and enrichment of germ cells from testicular tissue samples. This study emphasized identifying surface markers expressed specifically by leukemic or germ cells in testicular specimens from rats with Roser's T-cell leukemia and examining cellular factors that affect the sorting process. A thorough immunophenotypic analysis of Roser's T-leukemic cells and testicular cells from leukemic and non-leukemic rats was done. Based on the findings in this analysis, markers CD4 and MHC-I were employed to identify Roser's T-cell lymphoblasts in testicular samples. The expression of Ep-CAM was utilized as a marker for non-leukemic testicular cells.

After selection of specific markers, FACS was performed with four different protocols: (1)

positive selection of germ cells using a conjugated primary antibody (Ep-CAM), (2) positive selection of germ cells using a secondary antibody (to enhance the signal from primary antibody), (3) negative selection of malignant cells using two markers (CD4 and MHC-I), and finally (4) combining positive and negative selections (Ep-Cam+/CD4- and MHC-I-). In all protocols, as previously described [19], cells displaying spermatogonia characteristics with SSC^{low} and FSC^{high} were considered for sorting.

To evaluate the sorting procedure, cells obtained from each sorting protocol were used for transplantation into healthy rat testes or further labeled for FACS analysis. Cytospin slides were also prepared from these samples for immunohistochemistry. All the healthy rats that received unsorted leukemic testicular cells (as a control) died of terminal leukemia in less than 20 days. Two out of three rats which received cells yielded from protocol 1 and 2 (Ep-CAM positive) died of leukemia 25 and 33 days after injection. In protocol 2, repeating the experiment with higher germ cell concentration and more rapid sorting leads to prolonged procedure cell viability reduction. All the rats which received cells obtained from protocol 3 (CD4 and MHC-I negative) died from leukemia. The most promising results were shown in protocol 4 (combined positive and negative selection) as the rats that received this fraction survived disease-free for up to 120 days after injection. No cells expressing CD4/MHC-I were detected among them in the post-sort flow cytometry.

Immunohistochemistry staining for Oct-4 (a marker for spermatogonia) showed that 96.5% of cells selected by positive selection of Ep-CAM (protocol 1 and 2) were Oct-4 positive. Cells which were sorted according to negative selection of CD4/MHC-I (protocol 3) were 1.8% Oct-4 positive only. All the cells sorted by positive selection of Ep-CAM and deletion of CD4/MHC-I were Oct-4 positive, proving this protocol's enrichment ability.

This study reveals that the efficacy of FACS for purification is entirely dependent on the availability of specific surface markers for spermatogonial or cancer cells. Low specificity for surface markers, aggregation of leukemic and testicular cells, and heterogeneity of leukemic cell population seriously impair the procedure's efficacy. The fact that few SSCs and leukemic cells share specific markers makes their absolute separation highly difficult. Only combined positive and negative selection approaches for spermatogonial and leukemic cells showed promising results, but with very high cell loss due to two sorting cycles. It is crucial as the number of stem cells present in testicular biopsy from prepubertal boys would be already low.

A couple of years later, Hermann and colleagues in 2011 [20] tried using FACS to decontaminate prepubertal rhesus monkey testicular cells from MOLT4 human T-cell leukemia cells both in fresh and cryopreserved models. MOLT4 cells were cultured and labeled with GFP-expressing lentivirus to facilitate definitive determination of tumor origin. CD45 as a marker for MOLT4-GFP cells and CD90 (Thy-1) as a marker for testicular cells were used for flow cytometry analyses and sorting. Sorting was performed by positive selection of CD90 cells and negative selection of CD45 cells. Mixtures of testicular cells and 10% MOLT4 cells were used for sorting into three fractions: CD90+/CD45-, CD90-/CD45-, and CD90-/CD45+ cells.

After sorting, immunocytochemistry for VASA (germ cell marker) was done for unsorted and sorted cells. Both sorted and unsorted cells were used for xenotransplantation into the interstitial space of nude mice testes to evaluate tumor growth.

Nearly all the VASA-positive cells were only in the sorted CD90+/CD45 fraction (putative SSC fraction). However, only 15% of starting VASA-positive cells were recovered after FACS due to high cell loss during the sorting procedure. Examining malignant contamination in sorted CD90+/CD45- fraction and purity check by flow cytometry indicated that it contained 0.1% GFP+ CD90-/CD45+ (putative MOLT4) cells, which was enough to develop GFP+ tumors in the testes of recipient mice. Thus, even a low level of MOLT4-GFP contamination was sufficient to produce tumors in nude mice.

Four additional sorting procedures were done using singlet discrimination (SD) to reduce the

possibility of cell clumping, which may explain the presence of MOLT4+ cells in CD90+/CD45- fraction. After SD sorting, the purity check showed the absence of GFP-positive cells in three out of four replicates. These sorted cells did not develop tumors in nude mice testes.

This study showed in a nonhuman primate model that multistage single discrimination sorting could successfully achieve near-complete elimination of malignant cells from testicular cell biopsy. However, high germ cell loss after sorting is still a limitation.

Hou and colleagues followed their previous work using FACS on rat cells [18] and performed another study utilizing MACS as a method of purification [21]. They tried immuno-magnetic separation of testicular cells from Roser's rat T-cell leukemia cells by negatively selecting malignant cells. Donor testicular cells were obtained from the testes of radiation-induced leukemic piebald variegated (PVG) rats. The immunophenotype of Roser's T-cell leukemia closely resembles that of human ALL. Cells obtained from leukemic rat testis were labeled with anti-CD4, a known specific marker for Roser's leukemia cells. Sorting was done twice for the cell suspension, and the flow-through cells were used for injection into the testes of healthy PVG rats. Some of the cells were used for FACS analysis and immunocytochemistry. For FACS analysis, the CD90 surface marker was used instead of CD4 because the CD4 epitope was already occupied by anti-CD4 coupled to magnetic microbeads.

Results for purity check after MACS showed that reduction in leukemic cells achieved was in the range of 0.07–27% as determined by immunocytochemical analysis (CD4+ cells) and 0–52% according to flow cytometry analysis (CD90+ cells). Thus, the extent of this reduction is highly variable. Results also showed that ability of MACS to reduce CD4+ cells could be increased from 27% to 49% by increasing the antibody concentration from 20 to 35 μL per 106 cells. However, no further improvement could be achieved by further increasing the antibody concentration or the incubation time. On the contrary, cell yield was significantly reduced.

The recipient rats were divided into four groups and a control group that received unsorted cell injection. The testicular cells derived from sorting were split into four groups according to the antibody concentration and incubation time used for labeling. The injection was done into the testes of the recipient rats. In each experiment, 1.5 million cells were injected into each testis. All the rats in the control group died within 14 days of terminal leukemia, while all rats that received MACS sorted cells also died a few days later.

In conclusion, this study demonstrated that, although MACS is a clinically available method in the hematology field, it is not an efficient approach to eliminate malignant cell-contaminated testicular cells. Variations in cell size and the immunogenic characteristics of the leukemic cells may explain this approach's failure.

Geens and colleagues in 2007 published a study on mice [22]. The study aimed to evaluate the efficacy of the combination of MACS and FACS in decontaminating the testicular cell suspension from malignant cells. In this study, cells from B6CBAF/Juco mice testes were contaminated with 5% EL-4 cells (mouse T-lymphoma cells). Half of the contaminated cells were frozen-thawed before the experiment, and the rest was kept fresh to compare the effect of sorting in both conditions. Cell markers used for sorting were CD49f (α6 integrin), an SSC enrichment marker in mice, and H-2Kb, a known MHC-1 molecule expressed on T-lymphoma cells but not on germ cells.

Initially, MACS was done using CD49f-PE-conjugated antibody followed by anti-PE microbeads to enrich the spermatogonia population. FACS was performed on MACS-sorted cells, using the anti-H-2Kb-FITC monoclonal antibody. FACS procedure combined both positive selection of CD49f cells and negative selection of H-2Kb cells. Viability of cells was tested by live/dead fluorescence staining after digestion of testes, after labeling with antibodies, after MACS, and after FACS. To evaluate sorting purity, cells were tested by flow cytometry and cultured for tumor colony formation in every step. In vivo

evaluation of decontaminated cells was done after final sorting by transplantation via the efferent duct into W/Wv sterile mice's testes and followed up for tumor formation. Furthermore, to validate this evaluation technique's sensitivity, 44 W/Wv mice were transplanted with serial numbers of EL-4 cells, and tumor formation was checked after 120 days or natural death.

This study showed significant enrichment of CD49f-positive cells (spermatogonia) from an average of 3.94% in the unsorted population to 40.46% after MACS and 76.55% after FACS. On the other hand, the H-2Kb-positive cells decreased from 10.35% in the unsorted population to 3.54% after MACS and then 0.39% after FACS. Cell culture wells showed 100% formation of tumor colonies before sorting. This decreased to 50% of wells after MACS, but only 1 out of 32 wells after FACS (3.1%). Eleven mice were injected with fresh cell suspension, and nine were injected with frozen-thawed cells. Only 1 out of the 20 mice (5%) showed an abdominal tumor after transplantation.

The viability of the cells did not decrease significantly during the process. The viability of the cells after digestion was 70.5%. This percentage was retained after labeling and MACS (69.7%) but decreased to 60.5% after FACS. Also, no significant difference was observed when comparing the fresh and frozen-thawed samples for sorting.

The results of this study contradict the results of previous experiments [17]. The research shows that there was still 0.4% H-2Kb+ cells in suspension after MACS and FACS in murine setup and that these cells can develop colonies in vitro and develop tumors in vivo.

Hou and colleagues in 2007 [23] studied xenograft of testicular tissue from rats with terminal Roser's T-cell leukemia into the back of nude mice as a way of detecting malignant contamination in testicular tissue samples. Healthy PVG rats acted as controls. For transplantation, nude mice were divided into three groups; the first group was injected with four fresh testicular grafts. The second group was injected with four frozen-thawed grafts, and the third group was injected with eight cryopreserved grafts. Other nude mice were divided into three groups and injected intraperitoneally by either 20, 200, or 6000 cells/mouse. Mice with transplanted grafts and mice injected with leukemia cells were monitored for signs of leukemia and local tumor formation.

Xenograft results showed that local tumors developed 11–13 days after transplantation in all the mice, with 1–2-day delay in mice receiving frozen-thawed grafts. A larger number of testicular grafts did not increase leukemia incidence or influence the length of survival of leukemic mice; also, there was no difference between fresh and frozen grafts.

In the injected mice, 2 and 3 out of 4 mice injected with 6000 cells and 200 cells, respectively, developed terminal leukemia within 31–33 days. The remaining group survived for 3 months of surveillance period without any signs of disease.

This study revealed that xenograft of testicular tissue from rats to nude mice provides a promising technique toward developing a practical malignancy detection method. Also, leukemia development in mice injected with the low number of cells (200 cells) confirms the high risk of leukemia transmission.

In 2008 Fujita and colleagues conducted a study to evaluate the effect of leukemia cell contamination on testicular xenografts [24]. They designed a rat model that receives human Jurkat T-cell leukemia. Jurkat cells were injected in the numbers of 100, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, or 1×10^8. Cells were injected subcutaneously into nude mice, and tumor development was observed 8 weeks later. In the other part of the experiment, 200,000 Jurkat cells were injected into SCID mice's testes (as the adult model) and C57/BL6 mice (as the prepubertal model). Five days after injection, the donor mice were killed, and their testes were fragmented to 6–8 mm^3 pieces and used for grafting into castrated nude mice. The recipients were evaluated for tumor growth after 8 weeks, and then the grafts were dissected and examined histologically and with RT-PCR.

The results of the Jurkat cell injection into nude mice showed that the mice which were

injected with less than 1×10^5 cells did not develop tumors. However, 20% of mice injected with 1×10^6 cells, 80% of mice injected with 1×10^7 cells, and 100% of mice injected with 10^8 cells developed tumors.

When evaluating the transplanted mice, tumor formation was detected in 25% of the total grafts. Testicular tissue could not be seen by histological examination in any of the grafts. Also, the RT-PCR for germ cell (DAZL and protamine 2), Sertoli cell (MIS), and Leydig cell (LHR) markers did not detect any remaining testicular cells. This confirmed that leukemic cells replaced all the testicular cells in the grafts.

This study concludes that testicular tissue's xenografts may eliminate the potential risk of reintroducing leukemia to cancer survivors. However, leukemic cells that have infiltrated the testicular tissue may impair spermatogenesis in xenografts. Results also demonstrated that xenograft was not a reliable method to detect human cancer cell contamination, as only 25% of recipient mice developed tumors.

Nanoparticle-targeted drug delivery has been recently under extensive clinical research to improve cancer therapy and decrease drug-related toxicity [25]. Shabani and colleagues in 2018 [26] performed a study using a novel method for removing leukemia cells from neonatal mouse testicular cells using folic acid-conjugated poly(lactic-co-glycolic acid) nanoparticles (PLGA NPs) as drug delivery vehicles for the cisplatin chemo agent. They used folic acid-conjugated poly(lactic-co-glycolic acid) nanoparticles as drug delivery vehicles for the cisplatin chemo agent. The therapeutic effect of cisplatin is limited due to its low water solubility, high affinity to binding plasma proteins, and degradability [27, 28]. Therefore, using NP-targeted delivery for cisplatin could be promising.

Neonatal mice SSCs were mixed with 5% EL-4 ALL cells in the same culture. The SSC-EL-4 cell culture was divided into four groups: the control group with medium only, group 2 treated with blank NPs only (NPs without cisplatin), group 3 treated with effective cisplatin, and group 4 treated with cisplatin-loaded folic acid-conjugated PLGA NPs (NPs-cisplatin). The toxicity of NPs and cisplatin on EL-4 cells and SSCs were determined separately using MTT assay. Also, in situ cell death detection was performed for EL-4 cells with cisplatin and cisplatin-loaded PLGA NPs using In-Situ Cell Death Detection Kit. Surface markers were identified before as α6 integrin for SSC and H-2Kb for EL-4 cells.

After 48-h culture, the cells in all four groups were incubated with FITC-conjugated anti-H-2Kb (a marker of leukemia cells) and PE-conjugated anti-CD49f (a marker for SSCs) and analyzed by flow cytometry to evaluate the cancer cell elimination effect in vitro. Flow cytometry analysis showed no significant differences in the percentage of CD49f+ and H-2Kb+ cells between control and blank NPs groups. However, in the NPs-cisplatin group, the percentage of leukemia cells was lower (0.8 ± 0.14 vs. 1.90 ± 0.17), and the percentage of SSCs was higher (59.64 ± 2.5 vs. 39.43 ± 6.33) in the cisplatin group. There was also no significant difference in cell viability in control and blank NP groups, which confirmed the safety of NPs by itself.

For in vivo evaluation of the decontamination process, the cell mixture from each of the four groups was injected into the efferent duct of recipient nude sterile mice to evaluate tumor production. No tumor formation was observed 8 weeks after injection of NP-cisplatin-treated SSC-leukemia co-cultured cells. However, SSC colonization and initiation of in vivo spermatogenesis were noticed in many seminiferous tubules of recipient mice.

This study showed that the cisplatin-loaded PLGA NPs significantly affect the depletion of leukemia cells in SSCs culture. However, this could not eliminate the malignant cells from the mixture.

Tian and colleagues, in a new study in 2019 [29], compared different purification methods for testicular cells in mice. They experimented using a human B-cell ALL cell line to create a leukemia mouse model of nude GFP mice. The markers for B-ALL cells and mice SSC were determined via literature review and validated to confirm their

specificity. Validation was done using immunofluorescence staining and flow cytometry. They ended up using markers CD20 and CD38 for B-ALL cells and CD90 and CD49f for SSCs. Mouse leukemia model was created by injecting 10^2–10^8 B-ALL cells into the caudal vein. The onset of leukemia in each group of mice was recorded. It was found that with the increase of injected B-ALL cell number, the beginning time of leukemia and survival time of mice gradually decreased. Testicular biopsies were taken every 7 days, and after the onset of cachexia, mice were sacrificed, and their testes were cut and digested to extract SSCs. They tested three methods on these extracted cells: density gradient centrifugation, MACS, and FACS.

Percoll density gradient liquid was prepared in the order of 20%, 30%, 35%, 40%, 45%, 50%, and 60%. The separation tube was centrifuged for 10 min at 4 °C, 500 g. The cells from the 35–45% gradient were collected as putative SSCs.

For the MACS, cells were resuspended with magnetic microbeads attached to antibodies against CD20, CD48, CD90, and CD49f. For the FACS, antibodies against CD20, CD38, CD90, and CD49f were added to the cell suspension. After incubation, FITC-labeled secondary antibody was added.

SSCs that were isolated and purified by each of the three methods were transplanted into the seminiferous tubules of 4-week-old nude mice and C57BL/6 mice after being treated with busulfan. After transplantation, extensive GFP expression in recipient mouse testis suggested that the GFP-positive SSCs proliferated and differentiated in the recipients' seminiferous tubules and started spermatogenesis. GFP+ spermatozoa were first detected in the recipient mouse testis as early as 32 days after transplantation, with an average time of 47.6 ± 16.4 days posttransplantation. GFP+ spermatozoa that were obtained from recipient testes were used for fertilization and led to GFP+ progeny. As for leukemia incidence in transplant recipients, it was found that all the nude mice who received density gradient centrifugation-purified SSCs developed leukemia symptoms after transplantation, with the earliest detection time of 11 days.

Moreover, no GFP+ spermatozoa were detected in these nude mice's testes, suggesting that the spermatogenic function of testicular tissue invaded by B-ALL cells was impacted. However, none of the nude mice receiving SSCs purified by MACS and FACS developed leukemia symptoms. No B-ALL cells were detected in the blood and testis tissues of recipient nude mice by flow cytometry. This study suggests using MACS or FACS for purifying SSCs from leukemic testicular tissue samples by using a combination of multiple sorting markers.

Human Studies

The same methods, mainly FACS, used in animal settings, have also been applied to human tissue and human malignant cell lines (Table 2).

Fujita et al. in 2006 [30] experimented on human germ cells using the same method they used in their previous study [17]: FACS for two surface markers for malignant cells. Human testicular cells were obtained from a patient undergoing surgery for blunt trauma to the testis. In this study, multiple human malignant cell lines were tested: Jurkat T-cell leukemia, MOLT4 T-cell leukemia, HL60 promyelocytic leukemia, KU812 chronic myeloid leukemia, Raji Burkitt lymphoma, and DHL8 B-cell diffuse large non-Hodgkin's lymphoma. These cell lines represent the most common pediatric hematologic malignancies. Hematologic and nonsolid malignancies have more tendency to infiltrate the testis [11].

After obtaining the human sample, the tissue was digested to obtain testicular cell suspension. A panel of malignant and germ cell markers was first tested using immunohistochemistry to determine the specific surface markers for each malignant cell line. They selected the markers HLA-ABC, CD45, and α6 integrin (CD49f) to be used. Spermatogonial cells were negative for CD34 and HLA-ABC, but leukocytes present between seminiferous tubules and malignant cells were positive for CD45 and HLA-ABC.

Table 2 Summary and comparison of human studies

Study	Year	Purging method	Malignant cells	Source of testicular cells	Quantity/ percentage of malignant cells	Malignant cells markers	Germ cells markers	Detection method
Fujita et al.	2006	FACS	Human T-cell leukemia (Jurkat, MOLT4), human promyelocytic leukemia (HL60), human CML (KU812, K562), human lymphoma (U-937, Raji, DHL8)	Human germ cells	Not determined	CD45, HLA-ABC	α6 integrin (CD49f)	Flow cytometry
Geens et al.	2007	FACS	Human B-cell leukemia (SB)	Adult human testicular cells	0.05%, 5%	HLA class I	α6 integrin (CD49f)	PCR
Geens et al.	2011	Selective matrix adhesion	Human SB ALL	Adult human testicular cells	1%, 5%, 10%	HLA-ABC		Flow cytometry, PCR (DNA, B-cell receptor)
Dovey et al.	2013	FACS	Human leukemia (MOLT4, TF-1a)	Adult human testicular tissue	10%	CD49e, HLA-ABC, CD45	Ep-CAM	Xenotransplantation (interstitial injection or intratubular transplantation)
Sadri-Ardekani et al.	2014	Selective culture system	Human B-cell ALL	Adult and prepubertal human testicular cells	0.04%, 0.4%, 4%, 40%	CD1, -2, -3, -5, 10, -19, -20		MRD PCR (patient-specific real-time quantitative PCR)

The isolated germ cells and the malignant cells were plotted in a similar area on the forward scatter (FSC) and the side scatter (SSC) histograms in flow cytometry. In the FSC^{high} and SSC^{low} area, which presumably indicates enriched SSC population [19], all the leukemia cells and lymphoma cells were stained for anti-HLA-ABC and anti-CD45 antibodies. Around 1.45% of the K562 cells (human myelogenous leukemia) were found in the HLA-ABC- and CD45 fraction. This was attributed to MHC-I (HLA-ABC) low expression, reported in other leukemia cell lines [31]. To achieve complete removal of these cells, treatment with IFNγ [32] was applied to induce HLA-ABC expression on K562 cells before FACS. With the combination of two surface markers and gating of FSC^{high} and SSC^{low} cell population, it was possible to remove all malignant cells from the germ cell fraction.

Real-time PCR for HIWI, DAZL, VASA, and NANOG as germ cell markers and STELLAR and OCT4 as stem cell markers confirmed germ cells' presence, including SSCs, in the sorted fraction. Staining with α6 integrin showed 18.3% positive cells in the unsorted germ cells versus 76.7% in the sorted fraction. This indicated that sorting not only could purge malignant cells but can also lead to enrichment of the stem cell population.

This study shows a good promise for the purification of human testicular tissues from malignant cells. However, this depends entirely on identifying specific surface markers for each malignant cell line knowing how human cancers have various immunophenotyping.

As part of their experiment in 2007 [22] on combining MACS and FACS, Geens and colleagues performed FACS on human testicular cells. MACS was not applied in the human experiment due to the limited amount of testicular tissue.

Cells were obtained from patients undergoing vasectomy reversal and had normal spermatogenesis. The malignant cells were SB cells from a patient with B-cell ALL, the most typical leukemia in children. The testicular cells were contaminated by 5% of SB cells in five patients and 0.05% in six patients. The mixed cell suspension was frozen and thawed before the experiment.

FACS was done by negatively selecting HLA class I-positive cells, which is a marker expressed on SB cells but not on germ cells. PCR was done after FACS to evaluate the efficiency of purging of SB cells. After FACS, results showed that 10 of 11 patient cell suspensions still contained SB cells (0.58%) as proven by flow cytometry and PCR with no significant difference between the 5% and 0.05% initial contamination.

Dovey and colleagues in 2013 [33] tested the same approach for human testicular cells, validating the use of FACS and xenotransplantation. They used human testicular cells and MOLT4 T-cell acute lymphoblastic leukemia cell line. First, they validated the markers CD49e and HLA-ABC for MOLT4 cells and Ep-CAM marker for spermatogonial stem cells.

Testicular cells were contaminated with 10% MOLT4 cells, and then the mixed cell suspension was used for the sorting process. Sorting yielded two main fractions: (1) $Ep\text{-}CAM^{-}/CD49e^{+}/HLA\text{-}ABC^{+}$, which was considered putative MOLT4, and (2) $Ep\text{-}CAM^{+}/CD49e^{-}/HLA\text{-}ABC^{-}$ which was deemed to be putative spermatogonia. Both two fractions were used for xenotransplantation. Sorted and unsorted cells were transplanted either into the interstitial space (between seminiferous tubules) or via the efferent duct into the seminiferous tubules of nude mice (intratubular). The interstitial injection is used as a tumor bioassay, while the intratubular injection is used to test the germ cells' colonization activity.

The unsorted cell population formed tumors in 62% and 41% by interstitial and intratubular injections, respectively, in the recipient testes. On the contrary, the $Ep\text{-}CAM^{-}/CD49e^{+}/HLA\text{-}ABC^{+}$ fraction produced tumors in 55% and 23% of testes when injected interstitially and intratubularly, respectively. In the testes injected with the $Ep\text{-}CAM^{+}/CD49e^{-}/HLA\text{-}ABC^{-}$ fraction, tumor formation was never found, in the case of either interstitial or intratubular injection.

This study was repeated using a different leukemic cell line, TF-1a, with another leukemic cell marker. CD45 was used instead to demonstrate that the multiparameter FACS strategy could be applied to remove different malignancies.

According to the recent findings in the literature, all the studies that used FACS methods before SSC transplantation concluded that it has a potential clinical application. However, careful identification of reliable specific cell markers to differentiate malignant cells from SSCs should be established initially. Also, the issue of high germ cell loss during the sorting procedures needs to be addressed.

Geens et al. in 2011 [34] conducted a study using a selective matrix adhesion technique to enrich germ cells and deplete cancer cells in a human setting. Human testicular cells from patients undergoing a vasectomy reversal procedure were obtained via testis biopsy. After enzymatic digestion, testicular cells were mixed with 1%, 5%, or 10% of human peripheral blood B-lymphoblast cells from a leukemic patient (CCRF-SB cell line).

The mixed cell suspensions were seeded at a concentration of two million per cm^2 in a medium containing 20% fetal bovine serum (FBS) on uncoated culture dishes for 4 days. On Day 4, they performed a stepwise selection process. The attached cells were harvested from culture and labeled with rat anti-CD49f-R-PE antibody, a marker for germ cells, and anti-PE MACS microbeads. MACS was then performed to enrich CD49f+ cells. After that, the sorted cells were used in a matrix-based selection. The cells were plated on collagen-I-coated dishes in a medium containing 15% FBS and incubated for 4 h. Unbound cells were harvested and plated in a concentration of 0.5–1 million/cm^2. On laminin-coated dishes for 45 min, laminin-bound cells were harvested and used for flow cytometry analyses and DNA extraction for PCR. All the cell incubations and cultures were performed at 32.5 °C.

For flow cytometry analyses, cells were incubated with anti-CD49f-R-PE and anti-HLA-ABC FITC as markers for germ cells and CCRF-SB cells, respectively. The percentage of CD49f+/HLA-ABC- cells was around 6% in unsorted, uncontaminated cells, but this was enriched to 87.8% after MACS/matrix-based selection. The HLA-ABC+/CD49f- cells were still present at the end of cell selection in a range from 0.9% to 4.6%. PCR for B-cell receptors confirmed the presence of leukemia cells in all samples after final selection. This strategy showed the ability to enrich germ cells significantly, but it was not efficient in depleting malignant cell contamination.

Sadri-Ardekani et al. in 2014 [35] tried the long-term co-culture of malignant cells and spermatogonial cells in a culture system that they established previously for human adult and prepubertal spermatogonial cells [36, 37]. Due to their relatively low number, SSCs are expected to be propagated in culture before using them to restore fertility.

Testicular cells were obtained from one orchiectomy from an adult male with prostate cancer and two prepubertal boys from an experimental banking program for fertility preservation. Acute lymphoblastic leukemia (ALL) cells were obtained by bone marrow aspiration from three patients with B-cell ALL. The percentage of lymphoblast cells was determined using multiple markers for flow cytometry.

Before starting the experiment, testicular cells were grown in vitro for 3–5 weeks to increase their number. ALL cells were added after that to the culture. They used multiple serial concentrations of ALL cells to the culture (0.04%, 0.4%, 4%, and 40%). Culture conditions were kept the same as culturing testicular cells alone. The co-culture of cell mixture went on for up to 52 days. The ability of ALL cells to survive in these culture conditions was evaluated by culturing ALL cells alone in parallel to the co-culture as controls. Cell viability was evaluated using the live/dead flow cytometry analysis. With each passage, the surplus co-cultured cells were used for DNA and RNA isolation. Patient-specific real-time quantitative PCR analysis for minimal residual disease (MRD-PCR) [38] was used to detect ALL cells in each passage using isolated DNA. The sensitivity of MRD-PCR was initially validated to detect a single ALL cell in 10,000–100,000 testicular cells.

All the leukemia cells died after 14 days in the control culture. At the first passage (14–16 days of culture), leukemia cells were not detected in 0.04%, 0.4%, and 4% initial concentration groups of two out of three patients in the co-culture. At

the second passage (20–26 days of culture), all leukemia cells were undetectable by PCR in all groups. As for spermatogonial cells, they kept growing and proliferating and maintained viability throughout the culture period.

This study confirmed that the current culture system for propagating SSCs could also efficiently remove malignant leukemia cells obtained from bone marrow biopsy. However, future studies are required on different types of malignancies to test the ability to eliminate them.

Conclusion and Final Remarks

The body of work trying to achieve fertility preservation for those at risk of infertility has dramatically increased over the past few decades. The prospects of using SSCs for novel methods of in vitro spermatogenesis or SSC transplantation are gaining more interest from the male fertility research community. However, there are still some obstacles to achieving SSC transplantation in a clinical setting, most notably the risk of malignant cell contamination. In this chapter, we tried to spotlight the most significant attempts by researchers to find a reliable method of purifying SSCs, both in animals and humans. Some studies were successful, and some were not. This controversy in different results from different groups leads us to think that more work and more studies need to be done to reach this goal of a standardized protocol for handling testicular tissue samples taken from prepubertal cancer patients.

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Transplant Therapies for Male Infertility

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Introduction

Chemotherapy and radiation treatments for cancer or other conditions, such as autoimmune diseases and myeloablative conditioning prior to bone marrow transplantation, can cause permanent infertility. Cancer survivors report that fertility status has an important impact on their quality of life [1]. Therefore, the American Society for Clinical Oncology [2], the American Society for Reproductive Medicine [3], and the International Society for Fertility Preservation [4] recommend that all patients be counseled about the reproductive risks associated with treatment of their primary disease as well as options to preserve fertility. Adult patients have the options to cryopreserve eggs, sperm, or embryos prior to treatment that can be used in the future to achieve pregnancy using established assisted reproductive technologies [5–7]. Those options are not available to all adult patients (e.g., women who cannot undergo ovarian stimulation) or to prepubertal patients who are not yet making mature eggs or sperm. This is an important human health concern because most children will survive their cancer and still have their entire reproductive life in front of them [8]. Studies show that adult survivors of childhood cancers desire to have children [9–13]. For those reasons, centers around the world are actively cryopreserving gonadal tissues for patients in anticipation that those tissues can be matured in the future to produce eggs or sperm and offspring [14–34].

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Gonadal tissue cryopreservation has been considered experimental and typically is performed at academic institutions with appropriate regulatory approval for human subject's research. The earliest documented cases of ovarian cortical tissue cryopreservation appear to be in the mid- to late 1990s in young adult women who could not undergo ovarian stimulation for oocyte or embryo cryopreservation [35–37]. The first reports of autologous transplantations of those tissues back into the patient survivors were in 2004 in Belgium [35] and 2005 in Israel [37]. Now more than 130 live births have been reported after orthotopic transplantation of frozen and thawed ovarian cortical tissues [38]. Those live birth outcomes prompted the practice committee of the American Society for Reproductive Medicine (ASRM) to recommend that ovarian tissue banking is an acceptable fertility preservation technique and should no longer be considered experimental [39]. The guidance did not distinguish between adult patient and prepubertal patients although there is only one published report of a live birth from cryopreserved peripubertal ovarian tissue (14 years old) [40] and one from cryopreserved prepubertal ovarian tissue (9 years old) [41]. Most patients who cryopreserved ovarian cortical tissues during childhood (prepuberty) are still young, and it will take many years to accumulate live birth outcomes for childhood cancer survivors. The ASRM acknowledged the limited data from childhood cancer survivors but argued that ovarian tissue cryopreservation is the only fertility preservation option available to prepubertal girls. Removing the experimental label from ovarian cortical tissue freezing could have important implications for access to care because several states in the United States have recently passed laws mandating insurance coverage for *standard* fertility preservation techniques [42].

In contrast to ovarian tissues, there are no documented live births from frozen and thawed immature testicular tissues, and testicular tissue freezing for prepubertal patients is still considered experimental [39]. Our Fertility Preservation Program in Pittsburgh (https://fertilitypreservationpittsburgh.org/) and its coordinated centers have cryopreserved testicular tissues for 371 patients since 2011 [29, 43] (STUDY19020220, STUDY19070264) with diagnoses including leukemia/lymphoma, CNS cancers (e.g., glioblastomas), sarcomas, nonmalignant diseases requiring bone marrow transplantation (e.g., sickle cell disease, β-thalassemia), and gender dysphoria (Fig. 1). Immature testicular tissues have been cryopreserved and stored for more than 1000 patients worldwide, based on published reports [30], and the actual number of cases is certainly much higher. Therefore, the research and medical communities are obligated to responsibly developing next-generation reproductive technologies that can be used in the future to mature those tissues and produce fertilization competent sperm. This chapter will briefly describe spermatogonial stem cells (SSCs) and spermatogenic lineage development, review research progress developing SSC-based therapies, and discuss the potential for application of those therapies in the human fertility clinic in the near future, as well as implications for access to advanced reproductive health care.

Spermatogonial Stem Cells and Spermatogenic Lineage Development

Spermatogonial stem cells are the adult tissue stem cells in the testes that balance self-renewing and differentiating divisions to maintain the SSC pool and support continuous sperm production throughout the postpubertal life of men [44–47]. In humans, spermatogonial stem cell activity is thought to reside in the populations of A_{dark} and A_{pale} spermatogonia that are located on the basement membrane of seminiferous tubules (Fig. 2a, b) and are present from the time of birth through adulthood [48, 49]. Undifferentiated A_{dark} and A_{pale} spermatogonia may undergo 1–2 transit-amplifying mitotic divisions before giving rise to differentiating type B spermatogonia, which divide once to produce primary spermatocytes that lift off the basement membrane and enter the adluminal compartment of the seminiferous tubules [47, 50]. Two subsequent meiotic divisions give rise to secondary spermatocytes and haploid round spermatids, which undergo spermiogenesis to produce termi-

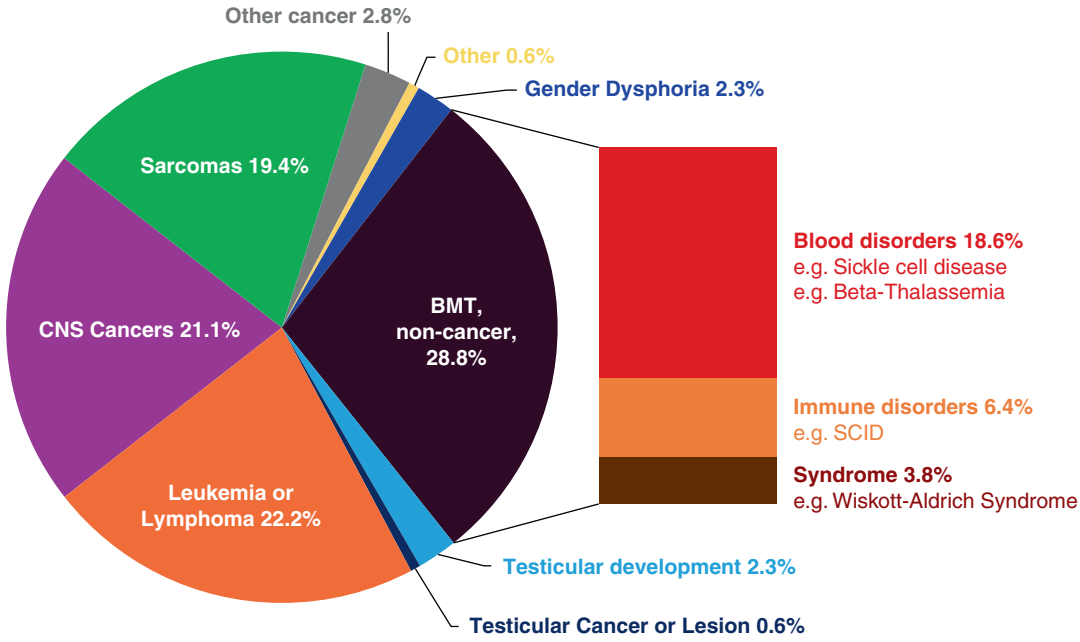

Fig. 1 Diagnoses for testicular tissue cryopreservation. The Fertility Preservation Program in the UPMC Magee-Womens Hospital has cryopreserved testicular tissues for 371 patients from January 2011 through March 2021. Indications for testicular tissue cryopreservation and percent of total cases are indicated in the pie chart

nally differentiated sperm (Fig. 2b) [50]. Spermatogenesis occurs in the seminiferous tubules of the testes that are connected at both ends to a common collecting reservoir, the rete testis (Fig. 2a). Since spermatogenesis is a stem cell-based process and occurs in a plumbed system of tubules, reservoirs, and ducts that can be easily accessed for infusion of therapeutics, it is particularly amenable to stem cell transplant therapies.

For male patients, there are several stem cell-based therapies in the research pipeline that may be used in the future to produce sperm from immature testicular tissues [51]. Those technologies include spermatogonial stem cell (SSC) transplantation [52–58], de novo testicular morphogenesis [59, 60], testicular tissue grafting/xenografting [61–67], and testicular tissue organ culture [68–71]. One day it may even be possible to produce transplantable germline stem cells or sperm from adult somatic cells (e.g., skin or blood cells) in a process called in vitro gametogenesis (IVG). For IVG, somatic cells are reprogrammed into induced pluripotent stem cells (iPSCs) that are differentiated into primordial germ cell-like cells (PGCLCs) that can be transplanted for in vivo differentiation or differentiated to sperm in vitro [72–74]. The path to the clinic for in vitro germ cells or in vitro gametogenesis techniques is long because those techniques have not been independently replicated in any species except mouse. In contrast, autologous SSC transplantation and testicular tissue grafting are mature technologies that have been replicated in numerous animal species and may be ready for translation to the human fertility clinic today. The next two sections describe the historical development of those two technologies and the state of readiness for translation to the human fertility clinic.

Spermatogonial Stem Cell Transplantation

History

SSC transplantation was first described over 25 years ago in mice by Brinster and colleagues [75, 76] who demonstrated that donor SSCs

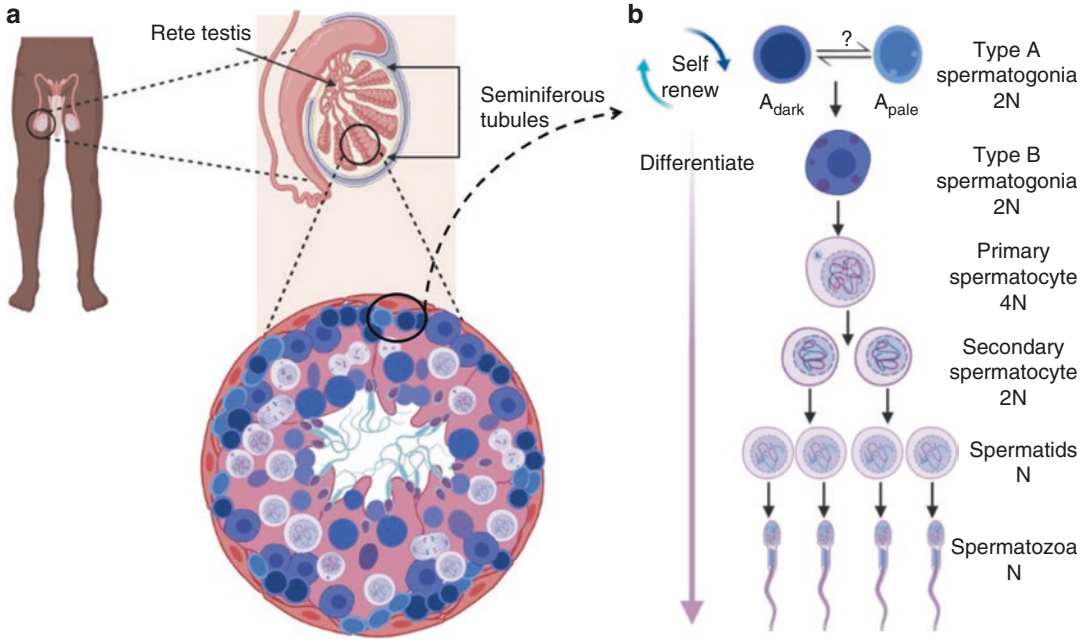

Fig. 2 Anatomy of the testis and spermatogenic lineage development. Spermatogenesis occurs in the seminiferous tubules of the testis that are each connected to the rete testis space, a structure that can be accessed for infusion of stem cells and other therapeutics (**a**). Undifferentiated stem and progenitor spermatogonia (type A_{dark} and A_{pale}) and differentiating type B spermatogonia are located on the basement membrane of the seminiferous tubules. Type B spermatogonia give rise to primary spermatocytes that lift off of the basement membrane and enter the adluminal compartment of the testis. Two subsequent meiotic divisions give rise to secondary spermatocytes and haploid round spermatids. Spermiogenesis produced terminally differentiated sperm (**b**)

could regenerate spermatogenesis and produce donor-derived offspring after transplantation into the testes of mouse recipients that were rendered infertile by chemotherapy treatment. SSC transplantation is a robust technology that has now been replicated in rats, pigs, goats, bulls, sheep, dogs, and monkeys with donor-derived embryos or offspring produced in mice, rats, goats, sheep, and monkeys [52, 54, 56–58, 77–83]. SSCs from donors of all ages, newborn to adult, are competent to regenerate spermatogenesis [54, 84], and SSCs can be cryopreserved and retain spermatogenic function upon thawing and transplantation [58, 85, 86]. Wu and colleagues reported that mouse SSCs were competent to regenerate spermatogenesis and produce offspring after 14 years of cryostorage [87]. Thus, it appears feasible that a testicular tissue biopsy (containing SSCs) could be obtained from a prepubertal boy prior to gonadotoxic therapy, frozen, thawed at a later date, and transplanted back into his testes to regenerate spermatogenesis.

Radford and colleagues reported the first SSC transplantation in human patients in 1999 [88] and in 2003 [89]. Briefly, testicular cell suspensions (including SSCs) were cryopreserved for a total of 12 patients with Hodgkin's disease. Seven of those patients returned to have their frozen and thawed testis cells transplanted back into their testes. The outcomes of those transplants were not reported, but the study provides insights into the motivation of men who were willing to undergo an early-stage experimental procedure for the possibility of having a biologically related child. Homologous species SSC transplantation had only been performed in mice and rats when Radford and colleagues reported the first autologous human SSC transplantations in 1999. The technique has now been replicated in numerous mammalian species, demonstrating safety and feasibility that supports application in the human clinic.

Methodology

In mice, SSC transplantation is a surgical procedure. Testes are accessed through a mid-abdominal incision and oriented under a dissecting microscope to visualize the efferent ducts that connect the rete testis space to the head of the epididymis. A pulled glass capillary pipet is passed along the efferent ducts until the tip of the pipet emerges into the rete testis space, which can be visualized on the surface of the mouse testis [90]. By infusing a cell suspension or other therapeutic into the rete testis space, it is possible to fill all seminiferous tubules of the testis at the same time.

The rete testis space in monkeys and humans is located in the center of the testis, and therefore, it cannot be targeted by visual inspection of the surface of the testis. However, the rete testis space is echo-dense and can be visualized by ultrasound. Schlatt and colleagues pioneered the method of ultrasound-guided rete testis injection into dissected bovine, monkey, and human testes as well as in vivo injection into cynomolgus monkey testes in 1999 [91]. This method has now been used to infuse testis cell suspensions to the seminiferous tubules of several large animal species with regeneration of spermatogenesis and in some cases embryos or offspring [56–58, 79–82]. Unlike the approach used for SSC transplantation in rodents, ultrasound-guided rete testis injection in larger mammals does not require surgery. A hypodermic needle is simply inserted through the base of the scrotum and through the testicular parenchyma until the needle emerges into the rete testis space. The injection needle and the rete testis space are both echo-dense and visible on ultrasound (Fig. 3a–c). Infusion into the rete testis space fills all seminiferous tubules at the same time because all seminiferous tubules are connected to the rete testis (Fig. 3c). In our experience and others, about 250–500 μL of fluid or cell suspension can be injected into the seminiferous tubules of prepubertal rhesus macaques, and 500–1000 μL can be injected into the seminiferous tubules of adult rhesus macaques [58, 83, 92]. It is important not to overfill the tubules because this can impede blood flow and cause ischemia to the testis. We believe the ultrasound-guided rete testis injection can also be applied in humans because the rete testis can be easily visualized by ultrasound inspection of human testes (Fig. 3d, e).

Other Considerations

The tissue biopsies from young patients are usually small and may not contain enough SSCs to produce robust spermatogenesis after transplantation. Thus, it may be necessary to expand SSC numbers in culture before transplantation. SSC culture has been firmly established in rodents [93–98], including development of conditions that do not require supporting feeder cells [99, 100], which may be an important consideration for clinical application. SSC culture has been extended to rats, hamsters, and rabbits [95, 101, 102], but extension to larger animal species has been a challenge, perhaps due to species-specific differences in factors that regulate SSCs [103–105]. Many laboratories have described protocols for human SSCs culture [14, 15, 106–121], but definitive evidence of long-term SSC expansion in higher primates is lacking, and no methods have been independently replicated among laboratories [114, 122, 123]. In the absence of a robust method to expand human SSCs in culture, the best recourse may be to transplant the cells to their native environment in the seminiferous tubules of the testis with the proper structural support and niche factors that support human SSC proliferation, self-renewal, and differentiation.

In mice, the efficiency of SSC engraftment and regeneration of spermatogenesis are better in 5–8-day-old mouse pups than in adult recipients [54]. Mice do not have a prolonged prepubertal period like humans. The spermatogonial stem cells or prospermatogonia migrate to the basement membrane of seminiferous tubules within a few days after birth and initiate spermatogenesis [124–126]. Therefore, testis development in a teenage boy may be similar to a 5–8-day-old mouse pup where the testis is growing under the influence of gonadotropic hor-

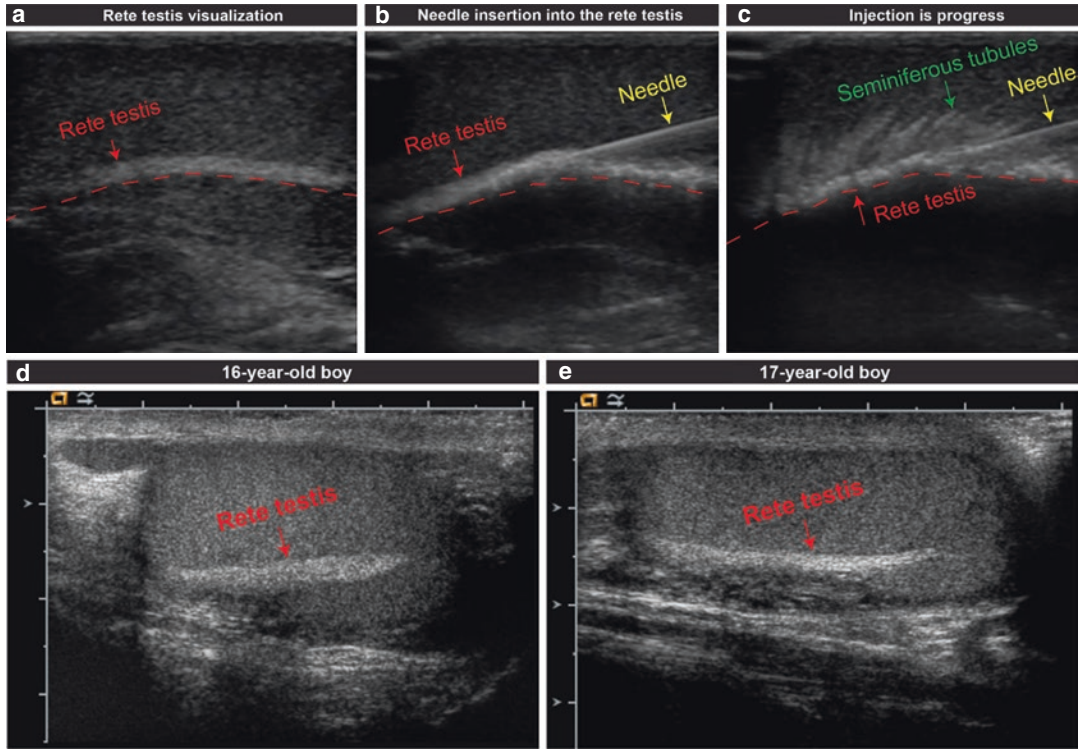

Fig. 3 Spermatogonial stem cell transplantation by ultrasound-guided rete testis injection. All seminiferous tubules of the testis are connected to the rete testis space, which is echo-dense and visible by ultrasound. Images of rhesus macaque testes are shown in (**a**–**c**). A 25-gauge, 1.5 in. hypodermic needle (also visible on ultrasound) is inserted through the base of the scrotum and testicular parenchyma until the needle emerges into the rete testis space (**b**). Microbubbles are added to the donor testis cell suspension to allow tracking of injection progress. Infusion fills the rete testis space and then simultaneously fills all seminiferous tubules (**c**). The anatomy of human testes is similar to rhesus macaques. The ultrasound imaging clearly identifies the rete testis space in the testes of 16-years old and 17-years old patients, suggesting that the same ultrasound-guided rete testis injection approach should work in human patients (**d**, **e**). (Portions of this picture are reprinted with permission from Hermann et al., *Cell Stem Cell* 2012)

mones and there is a burst of Sertoli cell proliferation [127, 128], which is likely accompanied by an expansion of SSC niches. The rete testes of teenage boys should be accessible for SSC transplantation (Fig. 3d, e).

Testicular Tissue Grafting

History

Testicular tissue grafting and xenografting are established technologies in which pieces of immature testicular tissues, containing seminiferous tubules with SSCs, are grafted ectopically under the skin. The objective of this technique is not to regenerate normal spermatogenesis in the recipient seminiferous tubules. The objective is to promote the maturation of the grafted immature testicular tissues pieces and produce sperm that can be recovered for fertilization by intracytoplasmic sperm injection. Immature testicular tissues from mice, pigs, goats, rabbits, hamsters, dogs, cats, horses, cattle, and monkeys have been grafted under the back skin of immunodeficient nude mice and matured to produce sperm [66, 129]. Graft-derived sperm were competent to fertilize oocytes in mice, pigs, goats, and monkeys [62, 65, 130] with production of offspring in mice, pigs, and monkeys [61, 67, 130].

Therefore, it is theoretically possible to graft immature testicular tissue from a childhood cancer survivor into an animal host to produce sperm that can be used to achieve pregnancy with established assisted reproductive technologies. This approach may be particularly applicable in patients with leukemia or testicular cancer for whom it may be unsafe to transplant their tissues back into their own bodies or for transgender females who do not want to experience male puberty that would be required to mature testicular tissues. However, the possibility that viruses or other xenobiotics could be transmitted from the animal host to the patient needs to be carefully considered [131–133].

Homologous species immature testicular tissue grafting was pioneered in mice with the production of complete spermatogenesis and offspring [61–63]. Several groups have reported homologous and/or autologous testicular tissue grafting in nonhuman primates to establish safety and feasibility that may support translation to the human clinic [66, 134–136]. Luetjens and colleagues investigated graft success from immature tissues versus adult testicular tissues transplanted ectopically under the back skin of hemi-castrated monkeys (i.e., normal hormonal milieu). Adult tissues degenerated while immature tissues survived with spermatogenesis arrested at the level of spermatogonia. A second experiment compared graft location at ectopic (back skin) versus orthotopic (scrotum) sites in young, castrated recipients and whether cryopreservation affected graft outcomes. In that experiment, none of the cryopreserved grafts survived. Ectopic fresh grafts survived with spermatogenesis arrested in meiosis, while orthotopic fresh grafts developed complete spermatogenesis. In that experiment, cryopreserved grafts were only transplanted under the back skin, not in the scrotum, so it is not clear whether it was the cryopreservation, the ectopic graft site, or both that contributed to graft demise [135]. This question was answered in part by Jahnukainen and colleagues who transplanted cryopreserved prepubertal and pubertal testicular tissues to the orthotopic location in the scrotum. Similar to the results with adult tissues, pubertal grafts that already contained sperm at the time of grafting could not be recovered 5 months later. Prepubertal, cryopreserved grafts transplanted to the scrotum of castrated autologous recipients could be recovered. The graft recovery rate was low (5%), and complete spermatogenesis was observed in only 13% and 17% of seminiferous tubules in the two surviving grafts. Both studies transplanted small pieces (~0.5–1 mm^3) of testicular tissue to the graft site (4–6 pieces per graft site). Sperm function was not tested by fertilization or with production of offspring in either study [135, 137].

Methodology and Outcomes

We recently repeated those experiments with slight modifications in a rhesus macaque model of cancer survivorship. Prepubertal animals with immature testicular tissues were hemi-castrated. The immature testicular tissue was cut into small pieces that were somewhat larger than previous studies (9–20 μm^3, Fig. 4a) and cryopreserved by controlled slow rate freezing in a medium containing 5% DMSO and 5% serum, as previously described [21]. Five to 7 months after hemi-castration, the remaining testis was removed and cut into small pieces (9–20 μm^3), some of which were designated for fresh tissue grafting. Immediately after removal of the second testis, fresh and previously cryopreserved tissues from the same animal were autologously grafted under the back skin (three sites fresh, three sites cryopreserved) or under the scrotal skin (one side fresh, one side cryopreserved) by individually suturing four pieces of testicular tissue to the subcutaneous aspect of a skin flap (Fig. 4b). This experimental design was repeated in five individual animals for a total of 40 graft sites (30 under the back skin and ten under the scrotal skin). Testicular tissues were recovered from 39 of the 40 graft sites; one graft was lost when the recipient animal opened the incision after surgery. Testosterone levels rose to the normal range for peripubertal rhesus macaques within 6–8 months after grafting. Testosterone could

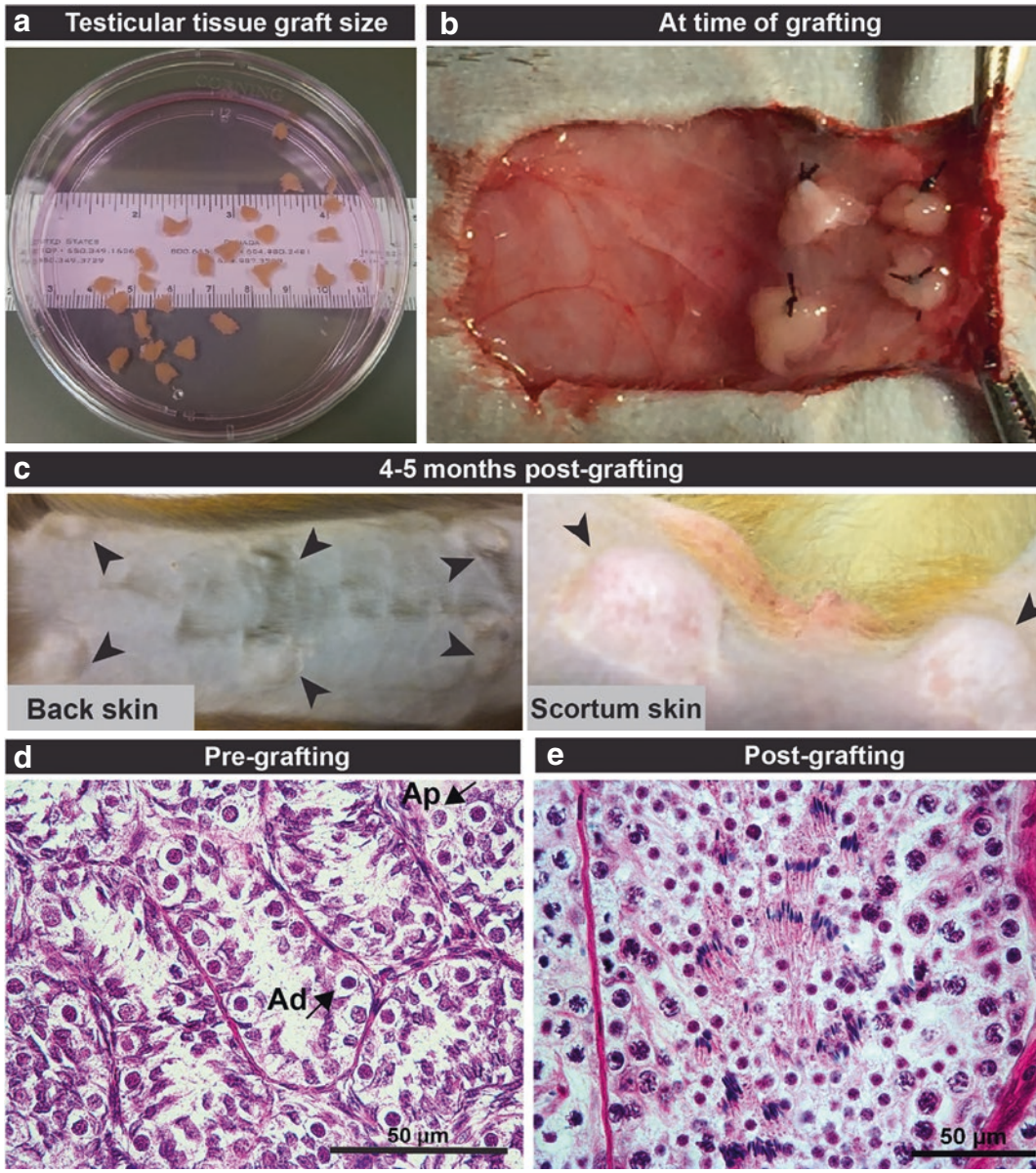

Fig. 4 Testicular tissue grafting. Testicular tissues are collected by wedge resection biopsy. In the fertility preservation laboratory, biopsied tissues are cut into small pieces measuring 2–5 mm in diameter (estimated 9–20 mm^3) and cryopreserved by controlled slow rate freezing (**a**). After thawing, testicular tissue pieces are individually sutured to the underside of a skin flap (**b**). Grafted tissues grew continuously under the back skin or scrotal skin for 8–12 months after grafting (**c**). Testicular tissues were immature at the time of grafting, containing only undifferentiated A_{dark} and A_{pale} spermatogonia in the seminiferous tubules (**d**). When grafts were collected 8–12 months after transplantation, 70% of tubules contained complete spermatogenesis with fertilization competent sperm (**e**). (Images reprinted with permission from Fayomi et al., *SCIENCE* 2019)

only be from grafted tissues because recipient animals were castrated. Grafts grew continuously throughout the duration of the experiment (8–12 months) and were not impacted by graft location (Fig. 4c), cryopreservation, or addition of Matrigel to the graft site. Testicular tissues that were immature at the time of grafting (Fig. 4d) exhibited complete spermatogenesis

with spermatids or sperm in >70% of seminiferous tubules at the time of recovery (Fig. 4e). Sperm were recovered by manual dissection or enzymatic digestion and used to fertilize rhesus oocytes by ICSI in collaboration with the assisted reproductive technology core at the Oregon National Primate Research Center. A healthy graft-derived baby (Grady) was born on April 18, 2018 [66]. We speculated about factors that may explain the improved graft recovery and extent of spermatogenesis. First, the concentration of DMSO cryoprotectant in our study (5%, 0.7 M) was lower than previous studies (10%, 1.4 M). Second, testicular tissue pieces were larger in our study (9–20 μm^3) than previous studies (0.5–1 mm^3), which may increase the local concentration of autocrine or paracrine factors. Third, the larger pieces allowed us to individually suture each piece of tissue to the capillary-rich underside of the skin flap rather than depositing a slurry of small pieces into a subcutaneous pocket.

Other Consideration

Similar to SSC transplantation, testicular tissue grafting and xenografting are established technologies that have been replicated in numerous mammalian species, including production of fertilization competent sperm and offspring in nonhuman primates [66, 67, 129]. In most species, cryopreserved grafts retained potential to regenerate complete spermatogenesis, an important consideration for adult survivors of childhood cancers. Immature testicular tissue grafting will not regenerate normal spermatogenesis in the endogenous tetses or natural fertility but can produce fertilization competent sperm that can be used to achieve pregnancy by intracytoplasmic sperm injection. In almost every report of immature testicular tissue grafting or xenografting, recipient animals were castrated, in theory to eliminate negative feedback from the endogenous testes on the hypothalamus and pituitary. Of course, our patient survivors will not be castrated, so it will be important to demonstrate in future studies that graft development can occur in patients with intact testes.

Concluding Remarks

Testicular tissues have already been cryopreserved for over 1000 patients worldwide [30], and some of those patients may be ready to use those tissues for reproduction. Spermatogonial stem cell transplantation and testicular tissue grafting are mature technologies that have been replicated in numerous labs and across numerous mammalian species over the past two decades. Translation to nonhuman primates provided critical safety and feasibility data that may justify translation to the human fertility clinic. Specifically, the demonstrations that cryopreserved, prepubertal testicular cells or tissues could produce spermatogenesis highlight the potential application in adolescent or adult survivors of childhood cancers or bone marrow transplantation for benign diseases. Autologous transplantation approaches may not be appropriate for leukemia or testicular cancer patients where there is a risk of reintroducing malignant cells to a patient survivor. For those patients methods to screen and/or remove malignant cells may be required [138]. Alternatively, it may be possible to mature testicular tissues ex vivo [69, 139]. However, the majority (>60%) of our patients who cryopreserved testicular tissues had solid tumors (sarcomas, neuroblastomas) that do not metastasize to the testes or nonmalignant diseases (e.g., sickle cell disease, β-thalassemia) (Fig. 1). Those patients may be ideal candidates for first autologous testicular cell or tissue transplantation trials.

There are no human live births from frozen/thawed immature testicular tissues or cells, and therefore, testicular tissue cryopreservation remains experimental in the United States. In contrast, ASRM recommended that the experimental label could be removed from ovarian tissue cryopreservation [39] based on reports of over 130 births after transplantation of frozen and thawed ovarian tissues [38]. This

helps reduce a significant barrier in access to fertility preservation care because it opens the door for some patients to get insurance coverage. It is important to note however that ovarian tissues have been cryopreserved for both prepubertal and adult patients and most documented births are from women who were already adult at the time of ovarian tissue cryopreservation [35]. Immature testicular tissue cryopreservation has been used almost exclusively in prepubertal patients. That means it could be years before the first males return to use their cryopreserved testicular tissues. How many more years and how many births will be required to remove the experimental label from testicular tissue cryopreservation? Furthermore, if a man produces sperm and/or offspring after autologous transplantation of spermatogonial stem cells, how will we know whether sperm were from transplanted or endogenous cells? That question can be addressed in part with the testicular tissue grafting option because those tissues can be removed and dissected to release sperm that are unequivocally from the frozen and thawed immature testicular tissues. Live births from those tissues will still be many years away because most of those patients are still young. The Danish experience may be instructive. In 1990, the Danish Minister of Health concluded that there were no restrictions in freezing ovarian tissues or testicular tissues if only autologous transplantation was considered. This ruling placed gonadal tissue cryopreservation in the context of normal medical practice. Perhaps this perspective along with published reports indicating few adverse outcomes associated with testicular tissue biopsies and cryopreservation could be adequate to justify removing the experimental label from testicular tissue cryopreservation [19, 22, 29, 30], one author's opinion.

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In Vitro Spermatogenesis

Christine Wyns and Marc Kanbar

Abbreviations

AG	Agarose gel
AL	Air-liquid
DTM	Decellularized testicular matrix
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epithelial growth factor
EiS	Elongating spermatid
ES	Elongated spermatid
FGF	Fibroblast growth factor
FSH	Follicle-stimulating hormone
GC	Germ cell
GDNF	Glial-derived neurotrophic factor
hCG	Human chorionic gonadotropin
ICSI	Intracytoplasmic sperm injection
IGF1	Insulin-like growth factor 1
IGFBP2	Insulin-like growth factor protein 2
IHC	Immunohistochemistry
ITT	Immature testicular tissue
KS	Klinefelter syndrome
LC	Leydig cell
LIF	Leukaemia inhibitory factor
MCS	Methyl cellulose
MIP2	Macrophage inflammatory protein 2
mTESE	Microsurgical testicular sperm extraction
NGS	Next-generation sequencing
NOA	Non-obstructive azoospermia
OA	Obstructive azoospermia
PDMS	Polydimethylsiloxane
PGT-A	Pre-implantation genetic testing-aneuploidy
PS	Primary spermatocyte
PTMC	Peritubular myoid cell
RA	Retinoic acid
ROSI	Round spermatid Injection
RS	Round spermatid
SC	Sertoli cell
SDF1	Stromal cell-derived factor 1
SS	Secondary spermatocyte
SSC	Spermatogonial stem cell
ST	Seminiferous tubules
TCS	Testicular cell suspension
TEC	Testicular endothelial cells
TESE	Testicular sperm extraction

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Introduction

While the relationship between the product of the male organ and a pregnancy dates back to 5000 years ago, it was only around 150 years ago

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that concrete understanding of the spermatogenic process came to light with the work of Enrico Sertoli [1] (for review see Geyer).

While spermatogenesis might appear as a simple science, more than a century of research pointed to the intricacy of this process that leads to the production of billions of sperms over an individual's life span. The recent spike in male infertility with decreasing semen quality over time [2], as well as concerns on quality of life including the reproductive capacity after cancer cure [3], heightened research interests in spermatogenesis.

In vivo, the spermatogenic process occurs within an organized tissue and organ architecture and is orchestrated by a multitude of complex signalling pathways [4], many of which still need to be unravelled before the full process can be reproduced in vitro.

With the development of techniques for cells and tissues culture ex vivo in the late nineteenth century, Goldschmidt was the first to report in vitro spermatogenesis (IVS) using sperm follicles (containing spermatogonia and spermatocytes) of the moth cultured in haemolymph hanging drops [5]. This work, among others that followed in rabbits, mice and rats [6, 7], initiated the study of in vitro gametogenesis.

Major findings and achievements in the field were undoubtedly reached by the Steinbergers and their colleagues between the 1960s and 1990s. Their studies had an important impact on the determination of appropriate culture conditions including, i.e. culture media, pH, temperature, oxygen (O_2) and carbon dioxide (CO_2) for the survival, maturation and meiosis initiation of mammalian testicular tissue in vitro [8–10] (for review see Staub [11]). Despite the fact that the late pachytene stage was the most advanced stage achieved in their organotypic culture model, it was widely used and adapted by scientist for the in vitro study of the first steps of spermatogenesis.

Two main breakthroughs helped position IVS as a potential fertility restoration strategy. The first was the development of cryopreservation protocols that allow the survival of human testicular tissue [12–18] and cell suspensions [16–20], and the second was the achievement of oocyte fertilization after intracytoplasmic sperm injection (ICSI) [21].

Tesarik et al. were the first to take advantage of these achievements and reported in 1999 a total of four live births obtained after ICSI with elongated spermatids (ES) developed in vitro from testicular cell suspensions (TCS) containing primary spermatocytes (PS) and round spermatids (RS) cultured in vitro for 48 h [22]. However, the extreme speed at which meiosis occurred in their study and the absence of further achievements with the technique raise many questions on its real potential.

While the organotypic culture technique seemed unable to complete meiosis in vitro in the early times, several studies using dissociated human germ cells (GCs) in mono- [23] and co-culture with either Sertoli [24] or Vero cells [24–26] reported in vitro GCs meiosis. However, these techniques were either not compatible with human clinical practice (Vero cells) or could not reproduce meiosis starting from the level of the spermatogonial stem cell (SSC) itself.

The true breakthrough for IVS came from the team of Ogawa in Japan when they succeeded, by using the agarose gel (AG) culture method, a modified organotypic culture technique, with a medium containing knockout serum replacement (KSR), to achieve complete spermatogenesis in vitro from neonatal fresh and cryopreserved mice testicular tissue fragments, up to the birth of healthy and fertile progeny from both fresh [27] and frozen samples [28].

Lessons from these first experiments, together with the increased knowledge of the testicular microenvironment regulation in mammals and humans [29, 30] as well as advances in the biotechnology field (3D cell culture, microfluidics, 3D printing) led to the development of a wider variety of IVS culture systems in the past decade.

While so far no live birth from in vitro-derived GCs has been achieved in any other species than the mice, some milestones were reached in other mammals and humans. Reports in higher-order mammals described the completion of meiosis in rat, bull, rhesus monkey, Guangxi Bama mini-pig and domestic pig [31–37]. With regard to human

IVS, the development of post-meiotic cells as the most advanced achievement was reported in only eight papers, two from immature testicular tissue (ITT) [38, 39] and six from adult testicular tissue [32, 40–44].

It is also worthy to note that many researchers failed to achieve complete meiosis in both mammals and humans [11, 45–48] and that culture conditions most probably need to be species- and even strain-specific as it was recently shown that human SSCs displayed limited proliferation in vitro under mouse SSC culture conditions [49] and that testicular tissue of mice from different strains cultured under the same in vitro condition led to different results (blockage at the spermatocyte stage vs. complete spermatogenesis) [50]. It may also be reasonably assumed that the degree of sexual maturation of the testicular tissue to be cultured, i.e. prepubertal or peripubertal will likely influence the IVS protocol.

In this chapter we will try and cover the different culture systems that fall under IVS and discuss the differences between those that use isolated/mixed cell population and those that use whole testicular tissue fragments with their respective advantages and disadvantages. Special attention will be given to culture systems that could be potentially used in a clinical setting for human fertility restoration.

Spermatogenesis: Its Initiation, Regulation and Completion

The production of spermatozoa in vivo is conditioned by the testis anatomy and several hormone-depending and immune mechanisms [51, 52]. While the epididymis is responsible for the final maturation of the spermatozoa, i.e. acquisition of surface proteins, the three major stages for the generation of these ‘gene-transmitting’ haploid cells take place within the testes. The first is the proliferative stage, also termed spermatocytogenesis, where spermatogonia in the basal compartment of the seminiferous epithelium undergo a series of mitoses in order to give rise to primary spermatocytes. The second is the meiotic stage where preleptotene and leptotene spermatocytes that cross a tight and highly selective Sertoli cell (SC) juncture—called the blood-testis barrier (BTB)—give rise in the tubular adluminal compartment to haploid GCs known as spermatids. The third and last stage is the differentiation stage, also termed spermiogenesis, where the spermatozoa gain its final shape and function before being released in the seminiferous tubule (ST) lumen [53].

While the importance of endocrine effectors, e.g. FSH, LH and testosterone, on both the maturation of the testis at puberty and the initiation and maintenance of a normal spermatogenesis after puberty is well established [54] (for review see Ramaswamy and Weinbauer [54]), much remains to be elucidated regarding the exact role of the different paracrine factors, developmental pathways and GC interactions within the somatic microenvironment regulating spermatogenesis [55, 56].

This section will briefly review the current knowledge on the SSC, its niche and the somatic microenvironment that need to be reproduced to ensure a successful human IVS.

The SSC and Its Microenvironment

Among the diploid spermatogonial population, the SSCs are a pool of undifferentiated cells located on the basement membrane of the STs which are able to both self-renew and differentiate [57, 58]. This peculiar capacity makes SSCs key effectors in various fertility restoration strategies used as isolated cell populations (SSC transplantation), whether within a testicular tissue fragment (tissue autografting or tissue/organotypic culture) or within a reconstructed microenvironment (3D culture/organoid formation from TCS) [59] (for review see de Michele et al. [59]). However, there are currently no phenotypic markers that can accurately identify SSCs [60] although recent advances in single cell transcriptome sequencing look promising in achieving this cell selection [61, 62].

The SSCs niche is a very specific site in the seminiferous epithelium (along the basement membrane, between SCs, behind the BTB and in

close proximity to blood vessels) where SSCs can be found [63]. Within the niche, the fate of undifferentiated SSCs (i.e. self-renewal and differentiation) is mediated by a multitude of paracrine factors secreted by the somatic cells' microenvironment (SCs, Leydig cells, myoid cells, macrophages and endothelial cells). Glial-derived neurotrophic factor (GDNF), fibroblast growth factor (FGF2), leukaemia inhibitory factor (LIF), epithelial growth factor (EGF), stem cell factor (SCF), vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF1) were already described mainly in rodents [29, 64, 65] (for review see Oatley and Brinster [29]).

With regard to humans, besides GDNF and FGF2, insulin-like growth factor protein 2 (IGFBP2), stromal cell-derived factor 1 (SDF1) and macrophage inflammatory protein 2 (MIP2), all secreted by the testicular endothelial cells (TECs), have been recently identified as important regulators of human SSC survival and renewal in vitro. Indeed, when TCS from ITT of prepubertal boys containing SSCs were cultured in vitro with GDNF and FGF2 alone or endothelial cells (ECs) (from human-induced pluripotent stem cells) alone, cultures with GDNF and FGF2 alone died after 2 weeks, while those cultured with ECs survived and formed numerous SSC colonies [66].

Figure 1 represents the testicular somatic microenvironment needed to achieve the completion of in vivo spermatogenesis which was also suggested to be essential for IVS [30].

The SC is the cornerstone of the somatic microenvironment that plays an indispensable role in the progression of GCs towards differentiation by secreting essential factors such as retinoic acid (RA) to initiate meiosis [55] but also by forming the BTB, a dynamic structure that maintains the immunological barrier between the basal and adluminal compartments. The BTB is almost exclusively formed by specialized SC junctions (i.e. tight, adherence, gap junctions and desmo-

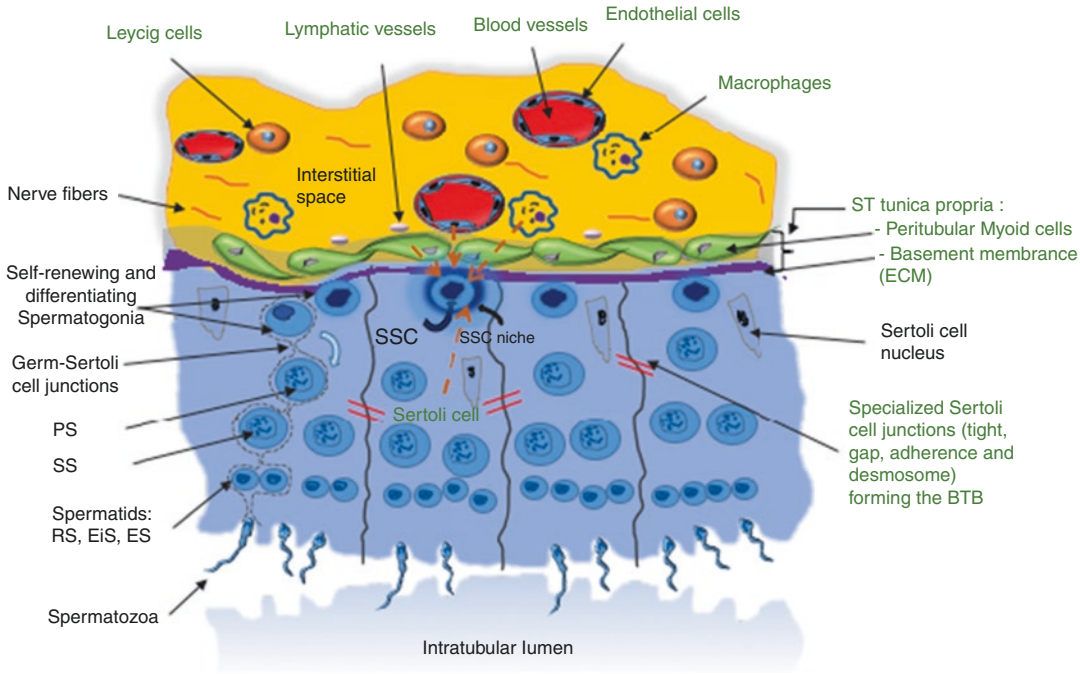

Fig. 1 The testicular microenvironment. Dotted orange arrows indicate cells that directly regulate the SSC within its niche (Sertoli cells, endothelial cells, myoid cells, macrophages). Important cellular and structural components of the somatic microenvironment are written in green. Red strikes indicate the specialized Sertoli cell junction part of the BTB that separates the seminiferous epithelium into a basal and an adluminal compartment. *PS* primary spermatocyte, *SS* secondary spermatocyte, *RS* round spermatids, *EiS* elongating spermatids, *ES* elongated spermatids, *BTB* blood-testis barrier, *ST* seminiferous tubule, *ECM* extracellular matrix

some) that interact with various collaborators (i.e. adhesion protein complexes, steroids, etc.) and allow the passage of the preleptotene spermatocytes towards the adluminal compartment [67] (for review see Cheng and Mruk). Mice that lose important proteins (Claudin 11 and connexin 43) of the BTB become infertile with their GCs losing the ability to progress beyond meiosis [68], and in humans, an abnormal pattern of BTB proteins was hypothesized to be the cause of infertility in males suffering of early maturation arrest and Sertoli cell-only syndrome [69–71].

The Leydig cell (LC) secretes testosterone a well-known factor promoting GC survival [72] that acts through androgen receptors located on SCs and contributes to meiosis as well as spermiogenesis [73].

While macrophages contribute to the microenvironment depending on their location within the interstitial space [74], their exact role in humans is still to be fully understood [61]. Further important structural components, like the ST basement membrane and peritubular myoid cells (PTMCs), also play key roles. Extracellular matrix (ECM) proteins are the main components of the basement membrane. In vivo they are in close contact with spermatogonia and SCs [75] and regulate important biochemical and structural events within the testicular microenvironment [76]. PTMCs do not only provide mechanical support through their contractile nature, but they have been shown to have secretory properties that make them effectors in the paracrine regulation of spermatogenesis [77].

As disruption of the testicular microenvironment proved to be deleterious to in vivo spermatogenesis [78, 79] (for review see Stukenborg et al. [79]), preserving its functional integrity in vitro is key to achieve IVS.

Candidates for In Vitro Spermatogenesis as a Fertility Restoration Strategy

The main advantage of IVS for fertility restoration in humans is that it might offer adequate in vitro conditions for SSC self-renewal and differentiation when there is a contraindication to their transplantation. In fact, for some patients (Table 1), IVS could be the only hope to father progeny with their own genetic material.

Table 1 Potential candidates for in vitro spermatogenesis using a cryopreserved immature/mature testicular tissue fragment containing SSCs

IVS from immature testicular tissue for:	IVS from mature testicular tissue for:
1. Prepubertal patients cured from cancer until safe technology to eliminate malignant cells from testicular samples becomes available	1. Patients with a known dysfunctional somatic compartment (e.g. NOA with maturation arrest, cryptorchidism)
2. Patients suffering from cryptorchidism	2. Patients with Klinefelter syndrome
3. Patients that underwent orchidectomy for DSD	3. Transgender male to female under hormone therapy that precludes development of tissue or cell transplants
4. Klinefelter patients	

DSD disorders of sex development, *NOA* non-obstructive azoospermia

Prepubertal boys are among the most important candidates for IVS as part of a fertility preservation strategy [80] as 35–41.7% of boys [81, 82] that elect to undergo a testicular tissue biopsy suffer from haematological malignancies where the risk of having a contaminated cryopreserved testicular tissue is elevated. Strategies like re-transplantation of thawed testicular tissue pieces or isolated SSCs carry a high risk of cancer cell reintroduction in the recipient [80, 83]. Despite efforts spent for the removal of malignant cells prior to autotransplantation of SSCs [84] (for review see Del Vento et al. [84]), IVS still remains the preferred strategy for fertility restoration in cancer patients that suffered from haematological or metastasizing diseases.

Other candidates labelled as high risk for infertility in adulthood (i.e. Klinefelter syndrome and cryptorchidism) are being included as part of pilot studies in fertility preservation programmes [85, 86].

Klinefelter syndrome (KS) occurs with an incidence of 1/450 to 1/600 boys and is characterized by an extra X chromosome and an important loss of germ cells starting already in utero [87]. Around 90% of these patients have fertility problems in adulthood, and spermatozoa can be

found in around 50–60% of azoospermic KS patients after testicular sperm extraction (TESE)/microsurgical TESE (mTESE) with a cumulative live birth rate of 50% with assisted reproductive technologies [88, 89]. However, after failed recovery of spermatozoa on mTESE, spermatogonia can still be retrieved in 21% of adults, 31.5% of peripubertal boys, and in three (out of four cases) prepubertal patients [85].

While there is some evidence that KS patients have a damaged testicular environment that hampers the spermatogenic process [90, 91], it was shown that XXY spermatogonia are able to lose the extra X chromosome when propagated in vitro [92], which could then presumably be used for IVS. However, no attempt to achieve IVS of KS SSCs has been reported yet, and studies in this regard are therefore needed. As the exact timing of complete GC loss is unknown [85] and because of the absence of a difference in sperm recovery rates between adolescent and adult KS patients [89], recommending ITT banking aiming at IVS can only be offered as part of research programmes [93].

Cryptorchidism, part of the disorders of sexual development (DSD), affects around 1–4% of newborn males at term and up to 30% in case of prematurity [94]. It is a frequent cause of non-obstructive azoospermia (NOA) reaching up to 89% in cases of untreated bilateral cryptorchidism [95]. When orchidopexy is performed in a timely manner, this risk drops to 46% [95]. When TESE/mTESE is performed, sperm retrieval rates vary between 60% and 74% in patients with a history of orchidopexy for unilateral or bilateral cryptorchidism [96, 97]. As evolutive impairment of the testicular tissue morphology was observed with age in a cryptorchid mice model [98] and because SC and LC dysfunction was described in cryptorchid boys [99, 100], cryopreservation of the ITT at the time of orchidopexy could be considered for fertility preservation involving IVS, especially in those identified at high risk of infertility [86].

Other rare diseases within the DSD spectrum like gonadal dysgenesis (46 XY gonadal dysgenesis and 45 X/46 XY mixed gonadal dysgenesis) and 17βHSD-3 deficiency that could lead to orchidectomy at a prepubertal age [71] (for review see Giudice et al. [71]) might also be candidates for ITT cryopreservation aimed at IVS. However, it should be noted that for patients with gonadal dysgenesis, the chances of finding GCs are highest before the age of 2 years and become close to zero afterwards due to the early progressive testicular hyalinization and atrophy observed in these cases [101].

Meiotic arrest accounts for around 4–30% of all NOA cases that undergo a testicular biopsy. While some cases are reversible (i.e. varicocele, gonadotropin or nutritional deficiency), others due to somatic or genetic anomalies are not [102]. The blockage occurs most frequently at the meiotic metaphase level [103], and a damaged testicular microenvironment has been described as the main cause of meiotic arrests [104]. Therefore, in the absence of GCs for round spermatid injection (ROSI) or ICSI on mTESE/TESE, IVS could be an option to overcome the unfavourable conditions for in vivo SSC differentiation in these patients.

In Vitro Spermatogenesis Strategies

As mentioned before, IVS can be achieved by culture of complete testicular tissue fragments known as ‘organ/organotypic culture’ or by the culture of isolated TCS (mechanically, enzymatically or both). The advantage of the organotypic culture technique is providing the SSCs with their physiologic 3D microenvironment and keeping intact cell cohesion and cell adhesion with the ECM, preserving thereby all interactions.

Although it seems simpler to favour the organotypic culture technique for IVS, this cannot always be considered. Indeed, as mentioned in the previous section, many patients that could benefit from IVS have a damaged underlying somatic testicular microenvironment that cannot support spermatogenesis in vivo and presumably also not in in vitro conditions. In this regard, experiments using presumed normal donor GCs and recipient tissue with presumed normal somatic cells were enlightening. The organotypic culture of testicular tissue explants (containing

normal donor SSCs injected into the recipient's functional testicular microenvironment) led to spermatogenesis recovery (from the donor GCs) and the birth of healthy offspring [105].

While this approach cannot be considered in a clinical setting, it points to the interest of developing an artificial testicular model that mimics the in vivo SSC microenvironment and structure. Such model will be useful to complete the IVS spectrum for fertility restoration in patients with underlying niche damage (see section on 3D cell culture systems).

While there seems to be a general consensus on temperature and gas culture conditions for human IVS (33–35 °C and 5% CO_2) [106], tremendous heterogeneity exists with regard to culture media (volume, composition, timing of medium change, single versus sequential) (for review of culture media components, see Richer et al. [107]) [107] and culture systems (i.e. organ vs. TCS, static vs. dynamic, 2D vs. 3D, hanging drop/air-liquid interface, etc.). Figure 2 summarizes available literature on the subject specifying culture conditions and systems that could become compliant with clinical practice in the future. Techniques will further be reviewed with a special focus on studies performed with human tissue.

SSC Culture Within an Intact Microenvironment

Air-Liquid Interface Organotypic Culture Systems

Air-liquid (AL) interface culture systems were the first to be applied on human testicular tissue by the Steinbergers [108]. Their system was a modification of Trowell's [109] and consisted of a stainless-steel wire mesh grid contained within a small Petri dish (60 mm) that was covered with a thin sheet of agar. Cultured human testicular tissue explants, under rat testicular tissue

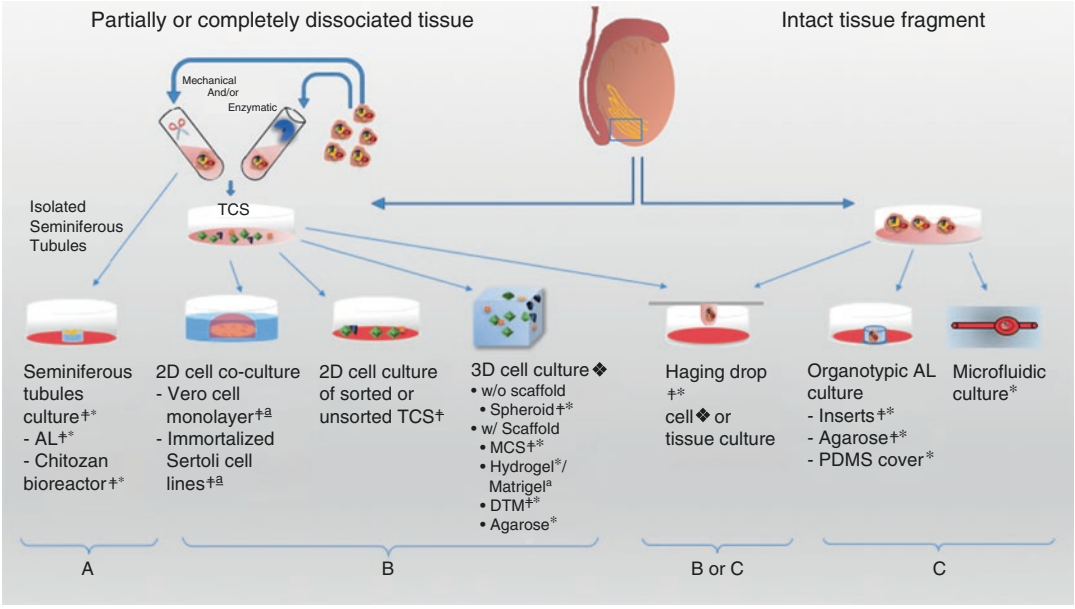

Fig. 2 Currently available techniques for IVS described in the literature using either a preserved, partially and completely dissociated testicular SSC niche. *AL* air-liquid, *MCS* methyl cellulose, *DTM* decellularized testicular matrix, *PDMS* polydimethylsiloxane, *ST* seminiferous tubules, *TCS* testicular cell suspensions, *w/* with, *w/o* without. *: Techniques that can be used for IVS in a clinical setting. a: Techniques that cannot be used in a clinical setting. Techniques that have already been applied to the culture of human testicular cells/tissue. Strategies where reconstruction of the testicular environment is possible, leading to organoids. (A) SSCs within a partially dissociated original testicular microenvironment. (B) SSCs dissociated from the original testicular microenvironment. (C) SSCs within their original testicular microenvironment

culture conditions, showed that, similar to the rat, type A spermatogonia and somatic cells remained viable for 4 weeks and that GCs could differentiate in vitro from the preleptotene spermatocyte stage up to the late pachytene stage during a 3-week culture using H3-thymidine tagging of GC nuclei and radioautography [8, 9, 108]. Nonetheless, the pachytene stage was the most advanced stage at which GCs could differentiate in all of their culture experiments [110–112].

In the early 2000s, the organ culture technique consisted of a testicular tissue fragment that was placed on a polyester insert (with a bottom formed of micropores), deposited in a microwell plate, and surrounded by culture media. Three short-term culture experiments used this modified organotypic culture technique to compare the efficacy of different freezing and thawing protocols on the prepubertal testicular tissue integrity and spermatogonial survival [13, 113, 114].

In 2006, Roulet et al. modified the AL technique by culturing 3 mm^3 testicular tissue fragments from fertile but elderly patients undergoing orchiectomy for prostate cancer on polyester inserts deposited in a 6-well plate containing culture media. After 16 days of culture, they denoted some differentiation of preleptotene spermatocytes into pachytene spermatocytes using bromo-2′-deoxyuridine (BrdU) labelling. While occasional newly formed spermatids could be observed using transmission electron microscopy, these cells could have been present before culture. The authors also observed considerable loss of GCs regardless of the addition of gonadotropins (200 ng/mL of FSH and 1 IU/mL of hCG) [40].

It is only recently that this system was applied to ITT aimed at developing mature sperm in vitro for future fertility restoration with cryopreserved ITT in a fertility preservation programme. Frozen-thawed 1 mm^3 ITT fragments from three patients were cultured in two different culture media supplemented with either testosterone or hCG showing a preserved tissue integrity up to 139 days with SC maturation, testosterone release and a good proliferation rate of spermatogonia although their numbers decreased over time [115]. However, no differentiation was observed which was attributed to an incomplete establishment of the BTB, i.e. adluminal location of connexin 43 [48].

Based on experiments in mice [27] and peripubertal physiologic levels of blood-supplied hormones, culture media were adapted in the same culture system [115]. Haploid GCs were obtained and documented by both immunohistochemistry (IHC) and chromogenic in situ hybridization after organotypic culture of ITT from five prepubertal boys aged between 2 and 12 years when FSH (5 IU/L) and serum-free, xeno-free KSR at 10% were added regardless of other media enrichment [38].

However, the scant numbers of haploid germ cells, the failure to achieve spermiogenesis and the loss of the SSCs pool over the culture period indicated that we still need to define the optimal conditions for IVS. While the addition of KSR is what led to the generation of haploid GCs, its presence in the culture media is a non-neglectable restricting factor to the research process as its components are undisclosed by the supplier [38].

Following these studies, Medrano et al. cultured testicular tissue (1–2 mm^3 fragments) from four prepubertal boys using the AG culture system used by Sato and colleagues [27]. In their study they tested two different temperatures (37 or 34 °C) and compared four different culture media containing either KSR 10% or FBS 10% with or without the addition of gonadotrophins (FSH and LH both at 5 IU/L). A temperature of 34 °C with KSR and gonadotrophins in the media provided optimal support for spermatogonia and SC survival, but no post-meiotic cells were obtained [47]. Differences in culture systems and media composition could have accounted for these discrepancies.

Microfluidic Organotypic Culture Systems

The aim of microfluidic technology is to challenge the classical static culture methods. Its dynamic nature makes it closer to the in vivo conditions where cells in a 3D complex environment

draw their nutrients and oxygen in a diffusion-limited manner from the neighbouring blood vessels [116].

While most applications of microfluidics are for cell culture, organotypic culture in a microfluidic device was only attempted in 2016 [117]. In their study, the authors developed a microfluidic gas permeable chip using polydimethylsiloxane (PDMS), a gas permeable biopolymer frequently used in biomedical research and cell culture. In their first device which consisted of two PDMS layers, both containing channels and a tissue chamber, separated by a porous membrane and connected to a closed circuit between a medium reservoir and an aspiration pump, complete spermatogenesis from murine neonatal testicular tissue was achieved and maintained over a period of 6 months. Healthy pups were born by both ICSI and ROSI from GCs obtained after 41 and 185 days in culture.

This was the first study to report ongoing spermatogenesis for 6 months in an in vitro culture system. More importantly, the technique proved to be significantly superior to its classical AG gel counterpart in terms of spermatogenesis efficiency. Two subsequent studies to improve the overall system design and user-friendliness followed and found the same results with regard to spermatogenesis efficiency [118, 119].

Microfluidic systems were so far not applied to human testicular tissue.

Hanging Drop Tissue Culture Systems

The hanging drop culture method maintains the tissue in growth media hanging on an inverted glass slide and was originally described by Harrison [120]. The first application was in a murine model to study a signal implicated in testis growth [121]. According to the authors, the presence of a small fragment of tissue in a small volume of media allows the concentration of different locally produced factors that might be crucial for the function of complex tissues [121].

In humans, the system was used with the objective to analyse the impact of drugs on the germ-somatic niche interactions in culture of adult testicular tissue fragments [122]. Even though their culture system was able to support GC proliferation from normal testicular tissue up to 14 days, their numbers dropped significantly compared to Day 0 [122], which suggests that the system was not able to support a sustained spermatogenic process in vitro if cultures were to be prolonged.

SSC Culture Within a Partially or Completely Dissociated Microenvironment

Seminiferous Tubule Culture Systems (Partially Dissociated)

Studies using ST tubule cultures were highlighted by Parvinen et al. because of the possibility of identifying by transillumination the stage of the spermatogenic cycle at which the culture started [123]. This was interesting because using this method GC development could be followed without using radioactivity [11] (for review see Staub).

The first report on human isolated ST culture was from Seidl and Holstein in the 1990s who cultured STs of nine elderly patients undergoing orchidectomy for testicular carcinoma [124]. Cultures were done on freshly isolated STs and frozen-thawed STs in 96-well microtiter plates using Dulbecco's Modified Eagle Medium supplemented with various nutrients with or without foetal calf serum (FCS). The authors demonstrated in their study that STs' distal ends needed to be closed to maintain cell survival. After 5–20 days of culture, Ap and Ad spermatogonia were the only GCs to survive, and the number of mitotic cells was higher in the group where FCS was added to the culture media.

Tesarik and colleagues studied partially dissociated testicular tissue from 16 men with OA in culture for 48 h, with or without the addition of FSH (25 IU/L) in drops of a gamete-100 culture medium at 30 °C. They demonstrated progression of meiosis especially in the group supplemented with FSH regardless of a direct contact with SCs or not [125]. However, analyses in their study were based on morphology using mainly a manual counting technique. The same culture system and media supplemented with FSH

(25 IU/L) and testosterone (1μmol/L) were applied to testicular tissue from nine men suffering from meiotic arrest at the PS stage (n = 5) or RS stage (n = 4). Normal RS and abnormal elongating spermatids were observed in the PS meiotic arrest group (two out of five patients) and abnormal elongating spermatids in the RS meiotic arrest group (four out of four) after 48 h in culture. Morphological analyses and fluorescence in situ hybridization (FISH) were used to confirm the cell ploidy, and pregnancies were obtained after ROSI/ICSI in both groups [22].

More recently, isolated STs (fresh and frozen/thawed) from adult patients undergoing orchidectomy as part of a gender change procedure were cultured for 60 days using serum-free culture conditions in a chitosan hydrogel bioreactor made of squid chitosan, a biocompatible material. Five to 20 spermatozoa were observed per bioreactor for fresh (on Day 55) and frozen/thawed (on Day 34) samples. Using FISH for detection of aneuploidy on Day 60 (using probes for chromosomes 13, 18, 21, X and Y), the authors found 2% to 3.8% of cells that were haploid [32]. However, besides the low efficiency, the authors could not prove that spermatozoa were developed from SSCs and not spermatogonia already committed to differentiation. Moreover, chitosan has been reported to modulate immune functions if clinical application was considered [126] (for review see Ahmadi et al. [126]).

2D Cell Culture Systems

Isolation and enrichment of the different cell types that constitute the testis was a pioneering step that allowed the study of individual cell functions and, in the case of SSCs, their propagation to increase their numbers in vitro [127].

Even though these systems were important to study cell behaviour and interactions under various stimuli (growth factors, cytokines, hormones), there is more and more evidence that cells in a 2D environment have an altered bioactivity compared to their in vivo counterparts and can therefore bring to misleading results [128, 129].

Moreover, with regard to IVS, a significant disadvantage of this model is that GCs lose their 3D architecture and become more difficult to recognize outside their regular spatial distribution [130]. In humans several studies attempted to reproduce spermatogenesis in a 2D environment; most were in co-culture with Vero cells or contained Vero cell-conditioned media [24–26]. Blockage at the human RS stage could be reversed in vitro to obtain ES and even fertilization-competent spermatozoa with development of a blastocyst-stage embryo [25]. The addition of Vero cell-conditioned medium to a mixture of different types of spermatogonia co-cultured with SCs, supplemented with FSH and testosterone, induced differentiation of human primary spermatocytes from NOA men into RS at a rate of 3–7% and from RS into normal elongating and elongated spermatids at a rate of 5–32% [24]. Co-culture of isolated primary spermatocytes with Vero cells generated chromosomally normal RS [26]. However, even though these studies suggested that Vero cell secreted factors are especially effective in promoting differentiation beyond the end-meiotic stage and spermiogenesis, these cells, due to their animal origin are not compatible with a clinical-grade IVS protocol.

In 2012, Riboldi used TCS from men with NOA and obstructive azoospermia (OA) cultured with integrin-α6-positive cells, presumed to be SSCs, in a media enriched with GDNF before transferring them on a SC (virally transfected by red fluorescent protein) and collagen monolayer [131]. While the presence of haploid cells was demonstrated by IHC and FISH, all (except one patient in NOA group that showed haploidy on FISH only) were obtained in patients with OA that usually have a normal spermatogenesis and who are thus not candidates for IVS.

3D Cell Culture Systems

While cells in the organotypic culture are found within their original 3D environment, 3D cell culture systems aim to grow isolated cells into cell aggregates that would mimic their native configuration. When compared to its 2D counterpart, cells grown in a 3D system are more similar

to the in vivo in terms of cell to cell and cell to ECM interactions and thus to the microenvironment [129]. Due to the ability of the cells to reorganize and mimic both the architecture and function of the in vivo organ in 3D culture, such systems were also assimilated to organoids [132].

The two main strategies described for 3D testicular cell culture depend on whether a scaffold is used or not. Scaffold-free techniques usually rely on the auto-assembly properties of cells, thus generating spheroids [129, 133].

As for scaffold-based techniques, the advancements in tissue engineering strategies [107] made it possible to design more complex 3D matrices that mimic the in vivo support. While scaffolds can be either natural or synthetic, solid or liquid, this section will mainly stress on those that are compatible with human clinical application (see Table 2). With regard to rodents and other non-human mammals, 3D culture systems were able to support the achievement of spermatogenesis with post-meiotic GC formation in prepubertal mice [106, 134–136], prepubertal rats [137] and prepubertal monkeys [35] (for review see Alves-Lopes et al. [138] and Richer et al. [107]).

With respect to humans, Table 3 shows studies that cultured either mature or immature testicular cells in 3D systems.

Regarding studies using mature testicular tissue, Lee et al. cultured adult TCS from 18 patients suffering of NOA in a collagen-Matrigel® ECM preparation together with foetal bovine serum (FBS) and an enriched culture media. After 12 days only, haploid cells represented 11–37% of the cultured cell population [139]. However, as the authors stated that few spermatids were present before the culture start, it is difficult to judge whether differentiation eventually occurred in vitro or not.

Table 2 Scaffolds currently described for 3D testicular cell culture

Potentially suitable for clinical application	Potentially unsuitable for clinical application
Human DTM	Matrigel®
Alginate	Collagen-Matrigel®
Human ECM-derived hydrogels	
Collagen	
Soft agar	

DTM decellularized testicular matrix

In 2016, with the aim to develop the first human testicular organoid, Baert et al. cultured (4-week duration) testicular cells from seven adult patients (including one peripubertal boy of 15 years old), using both a scaffold-based (human decellularized testicular matrix containing ECM components) and a scaffold-free (on top of agarose layer) approach in the apical compartment of hanging Transwell® inserts. Their culture medium were enriched with 10% KSR, 1% Glutamax® and gonadotrophins (FSH and hCG both at 5 IU/L). While they obtained spheroid-shaped organoids and showed GC proliferation and a functional somatic microenvironment using both adult and peripubertal tissue, there was no reorganization into a testis-specific cell arrangement, and no GC differentiation was observed on whether a scaffold-free or scaffold-based approach was used [133].

Pendergraft et al. aimed at developing a testicular organoid for the study of spermatogenesis and gonadotoxicity in vitro. Isolated human SSCs (from three brain dead adults) were cultured with immortalized adult SCs and LCs for 48 h in hanging drops and then in microwells for 23 days using a medium supplemented with 1μg/mL solubilized human ECM, 2μM retinoic acid, 2.5 IU FSH and 100 ng/mL stem cell factor. Despite the absence of a testis-specific architecture, 0.2% of the cells were post-meiotic GCs at the end of the culture period [42]. While their study was the first to report the use of solubilized ECM in a human culture system, in the absence of a control group (without addition of solubilized ECM), it is difficult to judge whether the achievement of meiosis in vitro was due to the addition of the solubilized ECM or to the production of the native ECM itself.

A significant milestone for IVS using a 3D cell culture system was achieved in 2018 [43] with the development of RS showing normal chromosomes, similar global gene profiles and DNA methylation (compared to their in situ counterparts) that were able to fertilize mice oocytes and develop into eight cell embryos after

Table 3 Summary of studies using human testicular cell suspensions for 3D culture

	3D culture aimed at IVS	3D culture aimed at organoid formation	
		Scaffold-based	Scaffold-free
Immature TCS	*Abo Foul et al. 2018*: sperm-like cells after 15 weeks based on morphology and IF [39]	*Baert et al. 2017*[a]*:* germ cell proliferation; no differentiation [133]	*Baert et al. 2017*[a]*:* germ cell proliferation, no differentiation [133]
			Sakib et al. 2019[b]: presence of GCs in the organoid after 5 days [140]
Mature TCS	*Lee et al. 2007:* haploid cells obtained after 12 days based on IHC and FACS [139]	*Baert et al. 2017:* germ cell proliferation; no differentiation [133]	*Baert et al. 2017:* germ cell proliferation; no differentiation [133]
	Abo Foul et al. 2019: sperm-like cells after 105 days based on PCR and IF assays [44]		*Pendergraft et al. 2017:* development of post-meiotic cells at Day 23 based on digital PCR and IF [42]
	Sun et al. 2018[c]*:* post-meiotic cells at Day 20 that could fertilize mice oocyte [43]		

IF immunofluorescence, *IHC* immunohistochemistry, *FACS* fluorescence-activated cell sorting, *PCR* polymerase chain reaction

[a]One patient was peripubertal aged 15 years old

[b]No GC proliferation or differentiation assays were done

[c]Only study that used a functional assay for the in vitro derived gamete

culturing GRP125+ spermatogonia (putative SSCs) from 60 patients suffering from OA in a Matrigel® hydrogel onto an inactivated SC feeder layer [43]. However, the use of a Matrigel® matrix is a truly limiting factor for a clinical application.

More recently, TCS (six samples) from five adult patients with a so-called diagnosis of Sertoli cell-only syndrome (as per the authors) were cultured in a 3D methyl cellulose (MCS) synthetic matrix and enriched medium containing KSR (25%), GDNF, FGF and ILF. The authors claimed that de novo post-meiotic cells (based on IHC and RT-PCR) were developed in three donor TCS after 6–7 weeks [44]. However, five out of six analysed samples already had one positive post-meiotic marker (out of two analysed) at the beginning of the culture, meaning that these testicular fragments contained spermatogenic activity with cells undergoing and achieving meiosis.

Concerning studies involving immature testicular tissue, the group of Huleihel reported the development of sperm-like cells based on both morphology and immunofluorescence staining in one out of eight ITT samples from chemotherapy-treated prepubertal boys after culture in a 3D MCS system [39]. However, despite their interesting result, the use of chemotherapy-exposed testicular tissue (six out seven patients underwent the testicular biopsy less than a year after receiving gonadotoxic treatment) limits the conclusions especially regarding culture conditions as the treatment could have modified both the germ and somatic cell functions.

The presence of ECM components in the 3D culture system allowed the in vitro development of post-meiotic cells when adult human testicular tissue was cultured. As achievement of meiosis in 3D cell culture systems did not require the presence of a testis-specific architecture, the question of how organoids could be of advantage for IVS aimed at fertility restoration remains open. However, despite the many challenges, improving the 3D/organoid culture systems is crucial as these models hold the only potential to achieve IVS for patients that have SSCs as the only germ cell within a damaged in vivo microenvironment.

Future Perspectives, Safety and Ethical Considerations

Several questions arise regarding the future of human IVS as a clinically applicable fertility restoration strategy. A better understanding of the SSCs niche and the testicular somatic microenvironment in vivo is primordial to faithfully repro-

duce the process in vitro. In this regard, studies using single-cell transcriptomics to measure the expressions levels of the myriad of genes found in the testes cell population could be an important contributor to this understanding [141] (for review see Gille et al. [141]).

Improving culture conditions to achieve human spermiogenesis and increasing spermatogenesis efficiency are other important challenges. For instance, oxidative stress from direct air contact is known to modify the properties of cells in culture [142]. In this respect, strategies using a reactive oxygen species (ROS) scavenger [143] or a gas permeable cover on top of the tissue fragment to avoid direct air contact [144] were both shown to increase spermatogenesis efficiency in mice.

Microfluidic systems could also prove to be beneficial in their ability to both maintain spermatogenesis for longer periods in vitro and increase IVS efficiency.

Eventually, the most crucial challenge is the safety of in vitro-derived spermatozoa as in vitro conditions cannot be compared to the in vivo environment. Proving the genetic integrity and epigenetic stability of in vitro-derived spermatozoa is mandatory before clinical pilot research programmes could be implemented (Fig. 3). The advances in single sperm cell [145] and embryo [146] genome profiling, through multi-parameter next-generation sequencing (NGS), appear to be promising for this matter.

More importantly, patients need to be counselled regarding the potential safety of ROSI as reassuring data from of 90 babies followed over 2 years showed that there was no difference between the physical and cognitive development in ROSI-born babies when compared to the natural-born ones [147]. Although reassuring, more large-scale studies are needed in this regard.

Additionally, similar to the challenges found in fertility programmes for prepubertal boys [148–151], implementing IVS for fertility restoration raises new challenges in clinical practice and specific ethical issues that should be listed and addressed.

Apart from IVS using human SSCs, it is probable that the potential utility of non-germinal or genetically modified SSCs will also come into consideration in the future. Even though

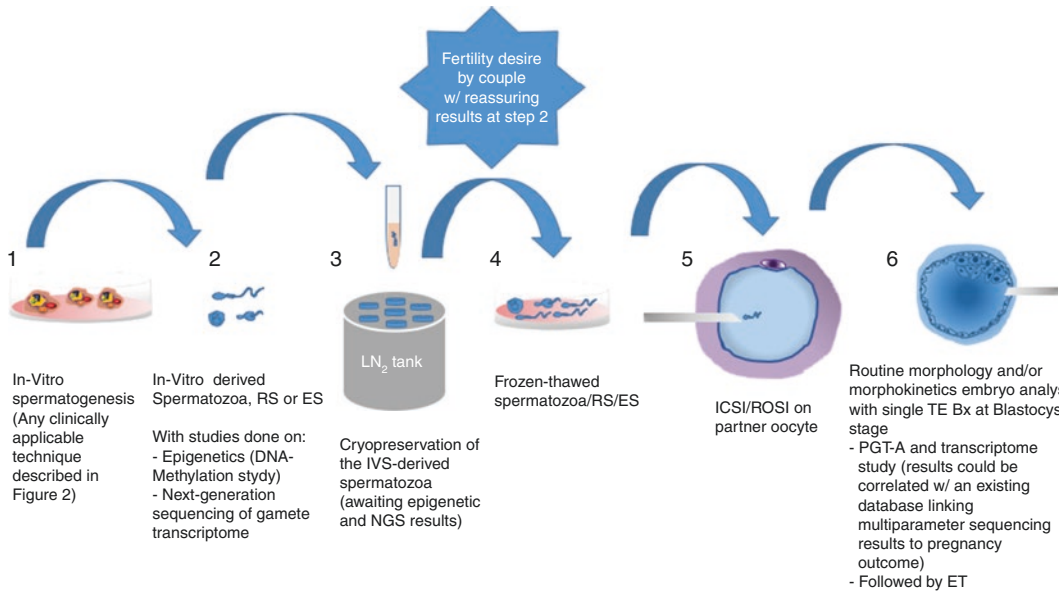

Fig. 3 Schematic process of using IVS-derived gamete for couples participating in pilot studies. *RS* round spermatids, *ES* elongating and elongated spermatids, *w/* with, LN_2 liquid nitrogen, *ICSI* intracytoplasmic sperm injection, *ROSI* round spermatid injection, *TE* trophectoderm, *Bx* biopsy, *PGT-A* pre-implantation genetic testing-aneuploidy, *ET* embryo transfer

results seem to be encouraging in mice for both strategies [152, 153] (for review see Fang et al. [153] and Mulder et al. [152]), the many uncertainties, risks and ethical dilemmas that come with the current knowledge of these techniques should keep their use confined to an experimental set-up and preclude them from being offered as therapeutic procedures to patients for now [154].

Conclusion

Nine years have elapsed since complete spermatogenesis was reproduced in mice, and still no research group could reproduce it completely and efficiently in humans. However, with continuing proof that significant differences exist between different species, even at the level of the SSC niche regulation [155], more studies using both mature and immature human testicular tissue are urgently needed to hasten the pace of IVS as a fertility restoration strategy especially for prepubertal boys who banked their testicular tissue prior to gonadotoxic treatments.

The appropriate culture technique to apply is highly dependent on whether the patient's testicular microenvironment is impaired or not. Strategies that privilege specific cell to cell contacts to faithfully reproduce the testicular microenvironment—a requirement for a normal spermatogenesis in vivo—are most likely the most promising. Improving cell and tissue perfusion in dynamic culture systems seems also valuable to improve spermatogenesis efficiency. Most importantly, all scientists performing experiments aimed at IVS for fertility restoration should consider switching to serum-free, xeno-free media as well as ECM that could become clinical grade to meet the requirements for clinical application. Disclosure of culture media will further help to enhance knowledge.

In addition to improving in vitro culture technology, a full understanding of SSC regulation through the combination of molecular, genomic, proteomic [61] and microanatomical approaches is warranted. Moreover, correlating the gene expressions to SSC fate during in vitro culture under different conditions might allow to determine the necessary signals needed at each time point to reproduce and sustain spermatogenesis in vitro.

Clinical Case 1

An 8-year-old boy was diagnosed with ALL in 2005; his treatment required a bone marrow transplantation following total body irradiation. His risk of infertility after treatment was evaluated at 90%. A testis biopsy was taken the same day the central line for chemotherapy was inserted. Small testicular tissue fragments were removed from the left testicle and frozen in the institution's reproductive tissue and cell bank. The presence of spermatogonia was confirmed by IHC. Twenty percent of the sample was given to research after parent consent and 80% for fertility preservation. In 2019, the patient returned to the clinic with his girlfriend, with a desire to have a child together in the next couple of years. His last sperm analysis done in 2013 showed azoospermia 5 years after treatment completion in 2008, and a repeat sperm sample done 1 week before the visit in 2019 still showed azoospermia. Testosterone measurements showed a markedly decreased free testosterone concentration.

Practical Clinical Tip

1. Update the patient/couple about the state of the art of fertility restoration strategies—including IVS—with cryostored prepubertal testicular tissue.
2. Discuss sperm donor programmes or adoption as current alternatives, and propose liaison psychologic to support the patient in the decisional process.
3. Refer the patient to an endocrinologist for follow-up/treatment of his low testosterone level.

Clinical Case 2

A 55-year-old man with NOA (testicular volume 5 mL left and 6 mL right) following a severe orchitis in childhood, after a negative micro-TESE, arrives to your consultation asking for options for his fertility restoration. His Johnsen score was 3, and the pathology report confirmed the presence of MAGEA4-positive cells as a marker of spermatogonia and spermatocytes.

He wonders if there are any options for fertility restoration from his own genetic material.

Practical Clinical Tip

1. Inform the patient that human spermatogenesis from adult testicular tissue suffering from NOA has not entered clinical practice and that in vitro-derived haploid cells should be further analysed for their genetic integrity and epigenetic stability before entering pilot clinical trials.
2. Recommend sperm donor programme.
3. Contact the patient only once IRB-approved clinical studies are in place.

Take Home Messages

1. Human IVS is not ready for clinical application. Until IVS protocols for fertility restoration are validated and standardized, the key approach remains the adequate counselling about current limitations and possible future applications.
2. ROSI/ICSI will be the only way for fertility restoration through IVS.
3. Ensuring the genetic integrity and epigenetic stability of in vitro-derived spermatozoa is of paramount importance as a validation step of the procedure.
4. IVS can be a promising tool to increase knowledge on human spermatogenesis as well as for in vitro drug testing for various testicular impairments or to evaluate drug-induced testis damage.
5. Spermatogenesis reproduced from non-germ line cells should only be restricted to research purposes

Key Readings

1. Sato et al. [27]
2. De Michele et al. [38, 48, 115]
3. Medrano et al. [47]
4. Komeya et al. [117, 144]
5. Sun et al. [43]
6. Pendergraft et al. [42]
7. Perrard et al. [32]
8. Baert et al. [133]
9. Abofoul-Azab et al. [39, 44]
10. Alves-Lopes et al. [138]
11. Richer et al. [107]

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Part III

Fertility Preservation Considerations

Psychological Aspects of Fertility Preservation

Verena Ehrbar and Sibil Tschudin

Introduction

Case 1

Sarah had just turned 30 when she got the diagnosis of breast cancer. A few weeks before, she had felt a suspect lump in her right breast, and not much later, she got a definitive diagnosis. It all happened very quickly, but luckily, Sarah was referred to a specialist in reproductive medicine only a few days after diagnosis and before the start of her cancer treatment. The specialist explained her options for fertility preservation before the planned chemotherapy. Sarah had been in a relationship with her boyfriend Jack for 3 years. They were talking about having children—one day. Sarah explained: "Of course, my first thought was survival. But next was my fertility. I want to have children one day. But never in a thousand years did I think cancer would be part of this decision making."

Being confronted with a life-threatening diagnosis such as cancer is obviously a major stressor, and young patients in particular face an emotionally challenging time. But is survival always the only priority? Of course it is. However, with today's advancements in medicine, a cancer diagnosis is no longer necessarily a death sentence. The survival rate of young cancer patients is rising and nowadays exceeds 80% [1]. For a meaningful and satisfactory life after cancer, some issues need to be considered early, that is, at the time of diagnosis. For instance, providing patients with information about fertility preservation (FP) and giving them the opportunity to choose whether they want to undergo FP are not just a medical concern but also of very high psychological importance.

How do young adult cancer patients in their reproductive years cope with the distressing prospect of fertility impairment? What are the factors influencing the psychological impact of fertility issues? How should healthcare professionals (HCPs) inform, support, and counsel patients about fertility?

After reading this chapter, you will understand the high psychological impact impairment of fertility has on young cancer patients. You will also recognize that it is important to inform patients of their options, because well-informed patients experience a more satisfactory decision-making process. You will have learned that decisional conflict and regret are to be expected up to a certain point but may decrease with information provision and additional support tools. You will understand that even if counseling with a repro-

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ductive specialist is the gold standard, online decision aids are seen more and more as an irreplaceable complement to counseling. By the end of this chapter, you will have an overview of what is known regarding the psychological aspects of FP and where more research is still needed to improve patient care and support. With the clinical examples and practical tips for clinical routines, this chapter should help you understand and integrate the psychological perspective of fertility issues into the care of young cancer patients.

The Psychological Dimension of Fertility and FP

The effect of impaired fertility on cancer patients is undeniable, influencing quality of life and increasing the risk of mental health issues [2]. Many young cancer patients may feel unprepared to make a decision on whether to opt for FP. They might consider it an additional burden to think about their future fertility and family planning, while also facing a life-threatening disease. Most affected cancer patients, however, perceive being able to choose to preserve their fertility as a source of hope and a reason to think about a life after cancer. Nonetheless, being confronted with the decision-making about FP is a challenging situation for all patients, as well as their families and HCPs.

Fertility Concerns

The potential risk of infertility is one of the top five concerns of cancer patients [3, 4]. The majority of cancer patients want to preserve their fertility, and many wish to experience a pregnancy with their biological child [5]. Fertility concerns are even important enough to influence cancer treatment decisions: a recent study reported that 30% of cancer patients would opt for a less optimal cancer treatment to reduce the negative effect on fertility [6].

Fertility concerns vary depending on the age or gender of the patients as well as stage of cancer treatment and parity [7]. Patients without children seem to be more concerned about fertility (76% for both men and women) compared to those with children (31% for women, 26% for men) prior to a cancer diagnosis [8, 9]. However, a cancer diagnosis does not change the desire for a pregnancy [10]. What all patients have in common is that due to the impairment of fertility, they experience a disruption in family planning that also affects the partners of cancer patients. And as the desire for a child is very individual, it is not surprising that not only patients who are in a relationship showed infertility-related distress but also those who were single at the time of diagnosis [11].

> → *Key reading: Keep in mind that fertility concerns have a high impact on patients, independent of relationship status, birth history, or gender. Fertility concerns are important enough to influence cancer treatment decisions.*

Psychological Distress

Being confronted with potential infertility is a distressing adverse effect of cancer therapy [8]. There are numerous studies showing an increase in psychological distress when patients did not have the opportunity to discuss reproductive concerns with a specialist [12–15]. Infertility-related distress usually arises at the time of diagnosis and often lasts into survivorship. Both patients and cancer survivors have retrospectively reported a range of negative emotions at the time of diagnosis, such as loss of control and feeling scared, frightened, overwhelmed, depressed, and frustrated [14, 16]. Increased rates of depression, anxiety, and trauma have been reported within the cancer population at the time of diagnosis and when accessing FP treatment [17]. Cancer survivors, who have an unfulfilled wish to have children prior to their cancer diagnosis and who therefore have heightened reproductive concerns, have shown an increased risk of depression and poorer mental health [14, 18]. It has to be noted that depression is generally the most common mental health disorder in cancer survivors, however, and is not specifically linked to fertility. Indeed, distress experienced by cancer patients is

not only caused by the potential loss of fertility but is closely associated with the diagnosis itself. Nevertheless, offering FP options to young cancer patients leads to an improvement in quality of life [5, 19]. This emphasizes the importance of taking fertility concerns seriously.

→ Key reading: Infertility distress may occur at the time of diagnosis as well as during survivorship care with a variety of psychological consequences. Ask patients also during follow-up consultations about any concerns regarding fertility.

Information Provision

Case 2

Heather was 35 years old and had just started her new job in childcare. In her first week of work, she started feeling sick. Only after seeing several doctors, the gynecologic oncologist, she was finally referred to, found what was wrong. Heather was confronted with the diagnosis of a FIGO stage IIIb cervical carcinoma.

The FP options were limited for Heather, as the relevant organs would be greatly affected by the planned cancer treatment. Furthermore, given the recommended oncological treatment, the specialist did not expect that Heather would be able to carry out a future pregnancy. From an oncological perspective, the need to start treatment was the first priority. In the multidisciplinary discussion of the case, however, the specialist in reproductive medicine pointed out the importance of nevertheless offering counseling to Heather.

Guidelines

Knowing now the common fertility concerns and the psychological consequences that can be expected, the question is how to address this issue adequately with patients. First, patients need to receive information about FP. The reality is that not all cancer patients are informed about the potential impairment of their fertility due to their cancer treatment and that there are FP options. Yet, according to international guidelines and recommendations, all cancer patients who are in their reproductive years need to be informed about the available options to preserve fertility before the start of cancer treatment by a specialist in reproductive medicine [4, 20]. This information should be provided to patients regardless of their current partnership situation and independent of previous birth history, given that fertility concerns are very individual and can affect all cancer patients. Referral to a specialist in reproductive medicine should be initiated soon after diagnosis, to guarantee the possibility of starting FP before the onset of cancer treatment. In counseling, patients must be informed about the existing options but also about financial aspects and specific regulations of their country of residence regarding FP procedures. The costs and follow-up costs of FP are not always covered by health insurance, depending on the country as well as the planned procedure. Furthermore, there are differences within countries about the legislation regarding cryopreservation of oocytes and ovarian tissue.

As we saw in the case of Heather, it is important to inform all young patients in their reproductive years, regardless of their prognosis. Even if there are no FP options to recommend, it is the patient's right to be informed about any potential impairment. With this information, the patient has the chance to process the information properly and, depending on the situation, to feel the attendant emotions (e.g., grief about the loss of fertility, the inability to have children).

Logan and Anazodo [17] summarized 33 published guidelines or recommendations regarding FP available as of 2019. These documents were developed by 19 different organizations from 12 countries. This global perspective provides insight into the differences regarding the underlying healthcare systems, the availability of FP, and the provision of information. This may have an impact on the importance that FP receives and highlights the different use of FP across countries and institutions. Furthermore, these guidelines vary in content according to age, gender, and

treatment factors. However, almost all mention the need for referral to counseling as a crucial part of the FP process.

→ Key reading: Guidelines recommend that all young cancer patients be informed about FP options before the start of cancer treatment.

Barriers to Information Provision

Despite the existence of these guidelines and recommendations, not all young cancer patients benefit from a referral to a specialist in reproductive medicine. In fact, even though an increase in referrals to FP counseling has been observed in recent years, it is still only a small percentage of patients worldwide who benefit [21].

When it comes to a fertility discussion, it is often the patients themselves who initiate it, rather than the oncologist or other HCPs. What keeps professionals from bringing up the topic of fertility in young cancer patients? Various barriers have been reported in the recent literature. First, not all institutions and cancer centers have developed a pathway specifically for FP, and not all of them are able to offer FP onsite. Although FP counseling should be provided to any patient, independent of where he or she is being treated, this is unfortunately not the case today. Complicated and long referral paths lead to prioritizing the start of cancer treatment over giving the patient the opportunity to see a specialist in reproductive medicine. Second, there is evidence that patient characteristics, such as age or family situation, influence whether a patient receives the offer of fertility counseling. Studies have shown that a woman who already has children at the time of diagnosis is less likely to be asked about her family planning after a cancer diagnosis [13]. Third, cancer characteristics also act as a barrier to referral for FP counseling. Patients with some diagnoses and prognoses are referred less often. Finally, there are considerable knowledge deficits in professionals, which have been seen to influence the referral rate. Due to their lack of knowledge, they hesitate to address FP and consequently do not refer patients to specialists in reproductive medicine. Many HCPs are concerned about distressing patients and hesitate to start a fertility-related discussion, especially with patients who have a poor prognosis or with those who they assume are not concerned about parenthood, because of their relationship status, sexual orientation, or age. However, knowledge in HCPs is of the utmost importance. A recent study reported that without special training regarding FP, only 6.7% of participating physicians indicated having initiated a discussion about FP in cancer patients. After receiving the appropriate training, the number of physicians who mentioned FP increased significantly to 46% [22]. Therefore, education for HCPs is critical to providing all cancer patients with information on FP.

→ Key reading: Information provision is essential. In the healthcare system, there are still barriers to all patients receiving information about FP. Currently, too many care providers show knowledge deficits. Specific education for professionals and clear guidelines for FP are crucial.

Knowledge Deficits in Patients

Case 2

(continued) *Heather confirmed that the discussion with the specialist in reproductive medicine was of great importance to her. She said that there was a time after receiving the diagnosis when she was more worried and threatened by the likelihood of not being able to have a child of her own than of the cancer itself. To give up this dream and to have this chance taken away from her was very hard to accept. But being adequately informed helped her cope with the situation. She also saw a psychologist regularly during and after her treatment where her fertility was every now and then a topic of discussion.*

When there is no referral by an HCP, patients obviously also show a lack of knowledge. Consequently, there are still many patients who are unaware of the potential impairment of fertil-

ity as well as about possibilities of FP. Nevertheless, it is known that patients wish to be informed about FP options [10] before the start of cancer treatment, even though they express concerns about their emotional reaction (e.g., that they will feel overwhelmed) when receiving such information. Patients still report feeling inadequately informed about their FP options and wish to be more involved in the decision-making process. It may be that some patients do not have an urgent wish for a child at the time of diagnosis or that they may not have a high chance of a healthy pregnancy in the future. But even these patients still report that they want to be informed about possible options and are not satisfied when HCPs automatically assume that they have no desire to discuss FP options. As we have seen in the case of Heather, it is often more about understanding than really doing everything.

Patients should understand the impact of their cancer diagnosis and its treatment on fertility. They need to know if there are options available to make an adequate and informed decision about their future fertility. It is overall beneficial to patients when they are referred to a specialist in reproductive medicine. After seeing a specialist, they have demonstrated greater knowledge, at least with regard to the FP technique they opted for, and better psychological health [23, 24].

Specialists in reproductive medicine, but also any other HCP, should keep in mind that every patient shows a different level of health literacy. For patients with low health literacy, it might be difficult to understand the detailed medical information given to them. HCPs who provide information about fertility should therefore pay particular attention to the health literacy of their patients when counseling to ensure that they understand the information they receive. Several predictors have been identified that are likely to improve knowledge after counseling, such as higher education, additional contact with a specialist in reproductive medicine, discussing FP options with someone else, and using educational material such as specific websites and/or decision aids [25]. The good news is that most of these predictors are modifiable. They should be used in clinical practice to meet the needs of cancer patients. Therefore, offering follow-up counseling or providing additional education material may be efficient ways to improve clinical care.

> → *Key reading: Currently, too many patients show knowledge deficits. Patients need to understand the information received. Counseling has to be adapted to patients' health literacy. Feeling informed increases psychological health in cancer patients.*

Decision-Making

Whereas 40 years ago it was generally a physician who paternalistically made a decision for the patient, nowadays there has been a shift toward shared decision-making. In shared decision-making, patients and their medical providers are actively involved in the decision-making process, but in the end, the patient has to make the choice. In the specific situation of FP, patients typically have only a few days in which to think about future fertility and make decisions regarding FP before the start of cancer treatment. Furthermore, it has to be taken into account that in a critical situation such as receiving a cancer diagnosis, an individual's capacity to process information becomes limited. For some patients, life issues apart from overcoming cancer recede to the background when they are confronted with such a diagnosis. Furthermore, most patients are unfamiliar with the medical and practical aspects of FP and therefore may feel unprepared to make a major decision about their future fertility and family planning. Young age or a recent start of a romantic relationship may further complicate decision-making. Especially for female patients, it has been shown that FP decision-making is perceived as one of the most challenging decisions to make.

To decide whether to undergo a FP procedure—and if yes, which option to choose—requires being adequately informed and involves reflecting on personal values regarding family planning as well as on how to realize one's goals. For many young cancer patients, it might be the first time that they think about reproductive plans in a serious manner. Nonetheless, confronting

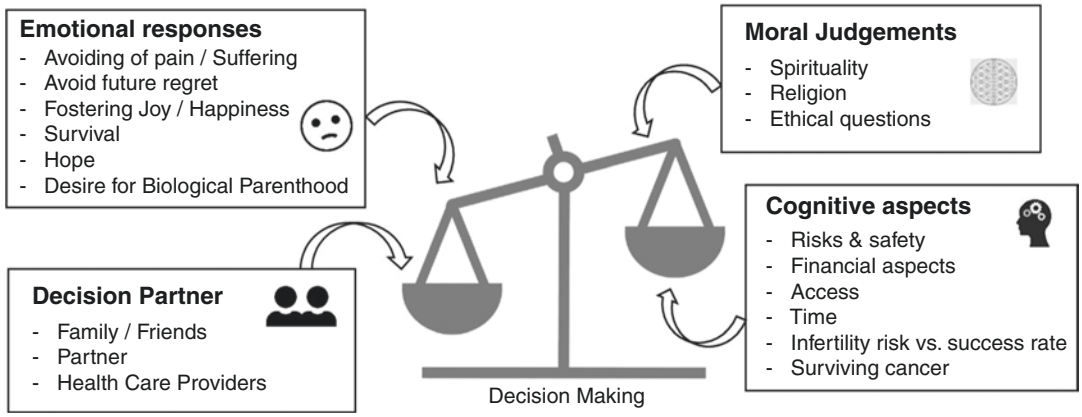

Fig. 1 Decision-making: an act of balancing out. (Adapted according to Hershberger et al. [26])

this subject is necessary to reach clarity. In addition to promoting value clarity and reducing uncertainty when counseling patients about FP, HCPs must provide information and support the patients' weighing up of pros and cons. In their qualitative study, Hershberger et al. [26] aimed at understanding young women's reasons for accepting or declining FP and categorized the identified reasons into four different dimensions (see Fig. 1). All participants described the cognitive appraisals as an influencing factor in decision-making. Moreover, emotional factors and moral judgment have a significant impact. However, decision partners seemed to be the dimension, which was crucial regarding the final decision. With all these contributing factors in mind, it comes as no surprise that many patients experience decisional conflict.

→ *Key reading: Shared decision-making regarding FP is crucial but also challenging.*

Decisional Conflict

Definition

Decisional conflict is defined as a state of uncertainty about a course of action to take [27] *"when choice among competing options involves risk, loss, regret, or challenge to personal life values"* [28] *(p. 61).*

Decisional conflict can be assessed with the decisional conflict scale (DCS), a validated self-report questionnaire developed by O'Connor [29]. There are four versions of the DCS: the traditional DCS consists of 16 items, which are divided among five subscales: feeling informed, value clarity, feeling supported, uncertainty, and effective decision. Lower scores on the DCS indicate that the patient perceives his or her choice to be well informed and based on his or her own values. Higher scores indicate more uncertainty about the choice. Decisional conflict is not a static construct and may vary over time and with various influences. Complex decisions are most likely accompanied by a certain amount of uncertainty, and being highly involved in the decision-making process may temporarily increase decisional conflict. Many studies have shown that the majority of patients report considerable decisional conflict when it comes to making FP choices [30–32]. Factors that increase decisional conflict in young cancer patients regarding fertility concerns are uncertainty about the impact of cancer treatment on future fertility, safety concerns, time constraints, and financial considerations. Lack of knowledge was also reported to lead to an increase in decisional conflict. Consequently, if patients feel well informed and have a sense of their own personal values, it is more likely that they can make an informed choice. Giving patients the opportunity to ask questions in counseling and offering them follow-up visits with a specialist in reproductive

medicine were shown to lower decisional conflict in these patients.

→ *Key reading: Decisional conflict in cancer patients has to be expected to a certain degree. It may be lowered with adequate and individualized information provision about fertility and FP throughout the course of cancer care.*

Decisional Regret

Definition

Decisional regret is defined as "remorse or distress over a decision" [33].

The validated Decisional Regret Scale (DRS) is the official instrument to assess decisional regret and is interpreted in the same manner as the DCS with a standardized scale of 0–100. The DRS is a five-item self-report questionnaire where lower scores indicate low decisional regret and therefore lower remorse or distress after making a decision [33]. There is no official cutoff for high decisional regret. Patients who have to decide about FP are at high risk of decisional regret because of the need to make a decision within a short time frame. Often, these decisions are rushed and made at a time of high vulnerability. Decisional conflict and decisional regret are associated with each other, and individuals who report decisional conflict at the time of diagnosis are significantly more likely to have subsequent decisional regret.

In the specific context of FP, there are various factors leading to a decrease in decisional regret, mostly linked to the factors that also lower decisional conflict. These include having had counseling by a specialist in reproductive medicine (compared with no counseling or counseling only by an oncologist) and therefore feeling appropriately informed. In addition, patients who had counseling showed more satisfaction with their decision, regardless of their choice about FP. Especially in young patients, it was reported that just having a choice regarding FP helped keep decisional regret low [34]. Consider the examples of Sarah and Heather. They both stated that the most important thing was not having the choice taken away by the cancer.

→ *Key reading: Decisional regret is linked to decisional conflict. They are driven by similar factors.*

Decision Aids

Case 1

(continued) For Sarah, the discussion with the specialist was helpful but also overwhelming. It was very difficult for her to concentrate and to understand the medical aspects of FP, as she was being confronted with many new medical topics around cancer diagnosis and fertility. Jack accompanied her, as she had been advised to bring someone along to the counseling. Sarah felt it was important for Jack to be there, as they were about to receive information about options for future family planning, and she was relieved he came. The specialist in reproductive medicine also suggested they use an online DA to supplement the information received in counseling and help them in their decision making. They used the DA at home in the evening right after the counseling. Sarah also sent the link to her mother, with whom she usually discussed everything.

Patients wish to actively participate in decisions about FP, but they need support in this decision-making process to keep decisional conflict and regret low. Even if a specialist in reproductive medicine or another HCP counsels the patient appropriately, there is no guarantee that the patient will be able to process this information properly. More information and support tools might be necessary to complement orally provided information. Informational material should be tailored to the patient's individual situation, that is, age, diagnosis, and life situation. The quality of information and the support

offered to patients positively correlate with both the experience of counseling and the decision-making process in general. However, the availability of helpful information is still low. This is where DAs come in. They have been known and used in other healthcare areas, and a Cochrane review recognized evidence-based DAs as the gold standard in facilitating decision-making [35]. Especially when there is more than one reasonable choice as well as when personal values influence the weighting of risks and benefits of competing choices, DAs are seen as a helpful source of support. The Cochrane review with 115 studies about DAs provides high-quality evidence that DAs compared to usual care were able to increase patients' knowledge, facilitate faster decision-making, decrease decisional conflict and regret, and include patients more actively in the decision-making process [35]. DAs consist of educational materials and/or tools designed to support people as they make a specific decision. They help patients understand potential risks and benefits of different options as well as engage actively in the decision-making process by considering their own personal values, using the technique of value clarification exercises [35]. These exercises consist of rationally evaluating pros and cons but also allow users to estimate their gut feelings for or against the respective option (see example shown in Fig. 2). Evidence-based DAs are developed according to the international patient DA standards [36]. DAs are very helpful instruments, especially when the time for decision-making is limited, which is the case in decision-making about FP. Particularly in the cancer context, DAs have shown to be successful in encouraging patients to involve themselves in the decision-making process [37].

Only a few DAs exist today in the context of FP and cancer, but the number is growing (see Table 1). Those available so far cover different cancer types and languages, with English and breast cancer being the predominant language and cancer type. The development of DAs in more languages or for other cancer diagnoses is underway. We next provide an overview of current DAs and their effects (see also Table 1).

An Australian research group developed and evaluated the first DA booklet for breast cancer patients [32]. The use of the DA led to higher fertility-related knowledge, lower decisional conflict and regret, and higher satisfaction with the decision. Since the development of their first DA, they have updated and adapted the content. It is publicly available and the booklet can be downloaded as a pdf via their website.[1] A research group in the Netherlands developed an online version based on the Australian work. They translated the content into Dutch and provided the information as well as the value clarification exercises online. They were able to show higher knowledge in breast cancer patients using the DA compared to usual care [38]. However, since completion of the study, this online DA is unfortunately no longer available. Soon after, a Swiss research group developed an online DA in German for women with any type of cancer and evaluated it within a randomized controlled trial. Not all results have been published yet, but so far, they have been able to show lower decisional conflict in a group who used the DA compared to a group who had only counseling with a specialist in reproductive medicine. Furthermore, satisfaction with the DA was good, and the time to make the decision was reduced [39]. The online DA in German is publicly available[2] and has just recently been translated into French.

Some but not all DAs are web-based. There are many reasons why a web-based DA may be preferable to offline versions. As the medical techniques are evolving and FP legislation is being adapted regularly, an online version allows the content to be immediately updated to the current state of the art. Moreover, a web-based DA is available to patients anytime and anyplace and allows for a more interactive process. Additional helpful resources such as other webpages can be linked and updated if needed. This implies, of course, that someone is regularly in charge of this task. In any case, having a DA available online means it can be updated quickly and offer infor-

[1] https://www.bcna.org.au/resource/booklet-fertility-related-choices/.

[2] www.fertionco.ch.

The pros and cons of egg freezing:

Use the side columns to mark personal importance to you using one to five stars (*****)

(*****)	PROs	CONs	(*****)
	This needs only a slight delay in my cancer treatment	This may involve the use of high dose hormones which may affect my cancer	
	This may increase my chances of having children in the future	There is no gurantee that I will have a baby (success depends on the number and quality of eggs collected)	
	I can think about my fertility later	This can be costly	
	Children born from frozen eggs will be genetically related to me	I am not comfortable with having to discard frozen eggs	
	I can fertilise my eggs with a future partner's sperm	This is a minor surgical procedure requiring sedation	
	I will be able to look back after cancer treatment and know that i gave it a go		
	This is a sort of 'insurance' against possible future infertility		

At this point time, are you learning towards egg freezing or not? (Mark the position on the line that is closest to how you feel)

I am leaning towards egg freezing		I am not sure yet		I am NOT leaning towards egg freezing

Fig. 2 Example of a value clarification exercise. (Retrieved from the Australian DA [32], published by the Breast Cancer Network Australia (BCNA). https://www.bcna.org.au/resource/booklet-fertility-related-choices/)

mation to a wide range of patients across the globe.

The available DAs were all developed specifically for female cancer patients. This may be because the choice for women may be seen as more challenging, as they have more complex options than men do. There are a few information platforms, but the number of resources available is still very low for men [44–46].

It is important to emphasize that additional support tools such as DAs should never replace counseling with a specialist in reproductive med-

Table 1 Overview of decision aids for female cancer patients

Authors	Country	Language	Cancer type	Format	Data about effectiveness	Implementation
Peate et al. [32]	Australia	English	Breast cancer	Booklet	Increases knowledge, satisfaction with decision; decreases decisional conflict, decisional regret	Booklet available for download online (pdf)
Garvelink et al. [38]	Netherlands	Dutch	Breast cancer	Online	Increases knowledge	No longer available
Ehrbar et al. [39]	Switzerland/ Germany	German/ French	Any cancer type	Online	Increases knowledge for the chosen option; decreases decisional conflict, time to make a decision; high satisfaction with the decision aid; more data expected (e.g., decisional regret)	Publicly available: FertiOnco
Gonçalves et al. [40]	Portugal	Portuguese	Breast cancer	Booklet	Under development; no final data yet available; study protocol available	Unknown
Speller et al. [41]	Canada	French	Breast cancer	Online	Under development; no final data published yet; development and alpha testing available	Publicly available: BEFORE
Jones et al. [42]	United Kingdom	English	Any cancer type	Booklet/ online	Under development; no data published yet; study protocol available	Available online: Cancer, Fertility & Me
Woodard et al. [43]	United States	English	Any cancer type	Online	Under development; no data published yet, study protocol available	Unknown

icine. Individual counseling is of the utmost importance and necessary for informing patients about the FP possibilities that apply to their situation. However, DAs provide additional support and allow patients to read about the information they were given verbally in counseling, show this information to their partner or family, and reflect on their personal values when rating the pros and cons of the FP options within the value clarification exercises.

→ Key reading: DAs are a helpful additional source of support for patients deciding whether to opt for FP. There are a limited number of evaluated DAs, and most are in English. The online format fits best with the needs of young cancer patients. More DAs should be developed and evaluated systematically.

Case 1

(continued) Sarah eventually decided to cryopreserve oocytes before the start of chemotherapy. With this, she and Jack hope to preserve their fertility options for a time after cancer, when they may decide to start a family. She said that one of the most important aspects was to still have the choice later and that the cancer did not take this choice away.

Support from HCPs

Infertility is an additional source of distress within the already overwhelming situation of having received a cancer diagnosis. About a third of all cancer patients develop a clinically significant mental disorder (mostly affective disorders). The consequences of fertility-related psychological distress highlight the ultimate need for additional psychological support (i.e., psychooncological support) for cancer patients [17]. The timing of information provision and support offers seems to be crucial, with the most appropriate timing being as soon as possible after the diagnosis. Feeling supported is a known key factor in higher choice satisfaction, and the importance of support from HCPs is unquestioned. Overall, the literature shows that interdisciplinary support from collaboratively working groups of HCPs is beneficial [47]. But how and when should HCPs offer support regarding fertility issues?

The Role of the Oncologist

Oncologists play a major role from the beginning to the end of cancer-related care, from diagnosis to follow-up. As the experts in the treatment of cancer, they generally become the primary care provider, earning the trust of their patients. It is important for oncologists to be aware of this position and to refer patients to other HCPs for additionally needed support. Their role in fertility care is to initiate the referral to a specialist in reproductive medicine. Given the relationship oncologists have with their patients, they have an impact on decisions about FP.

The Role of the Specialist in Reproductive Medicine

Within existing guidelines, the term "fertility counseling" usually refers to a discussion with a specialist in reproductive medicine covering information about the medical aspects of FP. Therefore, fertility counseling can be understood as information provision and decision-making support. It has a significant effect on the emotional health of cancer patients and may be very important to patients especially in the early phases of diagnosis and treatment. The specialist in reproductive medicine may also play a significant role in aftercare when the patient wants more information about his or her fertility status or wants to continue with family planning after successful cancer treatment. Already having a contact person is very helpful to patients, making it easier for them to receive the information and support they seek.

The Role of the Psychologist

Medically trained HCPs counsel and support patients regarding the medical aspects of fertility, but this does not replace the often-necessary psychological support given by a clinician with mental health training. Infertility has many possible consequences, potentially affecting identity, body image, sexuality, and relationships. These topics can be addressed with a psychologist and explored more deeply regarding their impact on the personal values of the patient. Psychological support within fertility counseling is mentioned in only a few guidelines and recommendations. In future versions of guidelines, it may be important not just to mention the need for psychological support but also to promote the assessment of psychological distress, in terms of a clinical cut-off point for referral [17]. Psychologists might also offer support during cancer treatment and into survivorship.

→ *Key reading: Providing support is essential and a multidisciplinary approach is most appropriate. Different disciplines of HCPs are important at different stages of treatment.*

Beyond the Acute Phase (Oncofertility Aftercare)

Cancer patients welcome the possibility of FP, and having their eggs or sperm "in the bank" may allow them to concentrate on other things during cancer treatment. But what about after the successful end of cancer treatment? Does having done FP mean that there is no longer a need for support or counseling regarding fertility? Not at all. Patients have made clear that they feel neglected regarding follow-up care for the physical and psychological consequences of the cancer treatment [48]. This underlines the importance of improvements and further developments regarding aftercare. About 90% of cancer patients described 5 years post-diagnosis at least one emotional concern. This means that aftercare should cover not only the medical and physical needs but also the emotional well-being of patients. Furthermore, not all patients have the opportunity to do FP before the start of cancer treatment. This means that there are a significant number of patients who could profit from the in some cases arising window of opportunity for FP after completion of cancer treatment.

Patients should be followed within oncofertility care from the time of diagnosis into survivorship. So far, reproductive health issues have often been inadequately considered in posttreatment survivorship care. However, it is known that fertility concerns outlive the termination of cancer treatment, and a comprehensive multidisciplinary approach for oncofertility care is needed. There are no official survivorship care plans regarding fertility issues for patients who have successfully completed their cancer treatment. Whereas official guidelines underline that all patients should be informed directly at the time of diagnosis about FP options, they are inconsistent with regard to recommendations for when follow-up consultations should take place. However, reproductive concerns affect areas such as sexual health, body image, and relationships. Persistent sadness, grief, anxiety, depression, panic, and social isolation may remain for up to 20 years post-diagnosis. These consequences are sufficient to highlight the importance of offering appropriate psychological care during the whole cancer journey.

The lack of consistency among guidelines discussed earlier in this chapter may increase the difficulties HCPs have in providing optimal care regarding fertility counseling after cancer treatment. Furthermore, it remains unclear where patients get information from and to whom they turn for guidance, answers, or general support regarding post-cancer fertility. Thus oncofertility care that covers medical and psychological needs should be an integral part of cancer care from diagnosis through survivorship.

Macklon and Fauser [49] stated that all centers should offer oncofertility services that begin at diagnosis and continue until the patients had the opportunity to approach their desire for a family. They suggested a concrete model for a complete oncofertility service (Fig. 3). As cancer survivors need reproductive care in many ways, various topics should be covered in the follow-up

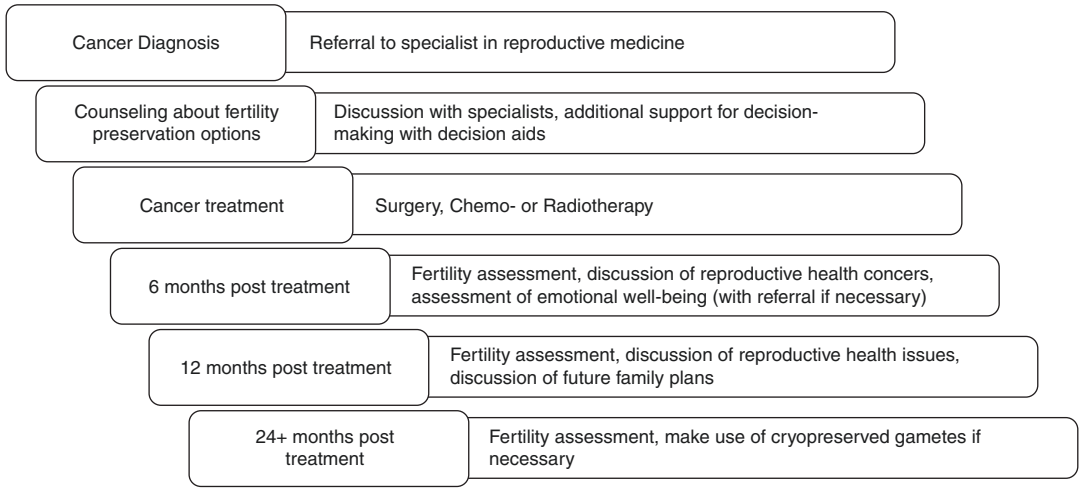

Fig. 3 Comprehensive oncofertility care: a suggestion. (Adapted according to Macklon and Fauser [49])

counseling, such as sexual health, sexual dysfunction, or hormone insufficiency. Ideally, these oncofertility units should be multidisciplinary, including specialists in reproductive medicine, endocrinology, cardiology, and psychology. Patients who have been asked what should be included in oncofertility care have mentioned information provision, age-appropriate communication, financial support, and psychological support [9]. The aims of a model of care regarding comprehensive oncofertility care from the beginning of the cancer journey until survivorship should be (a) to facilitate management of care for HCPs in terms of clarifying their responsibilities and (b) to ensure that patients know whom to talk to about fertility concerns. However, more research is needed to develop and implement a model of care that covers the needs of HCPs as well as patients.

→ *Key reading: Fertility is also an issue in aftercare, and cancer survivors have concerns not only about their fertility but also about sexual health in general. Therefore, interdisciplinary support should be provided to survivors during follow-up care.*

Further Research/Outlook

Much is known about the psychological aspects of FP in cancer patients, but there are still some aspects that need to be covered in-depth in future research as well as implemented in current clinical routine. Psychological support has been shown to be beneficial for cancer patients at the time of diagnosis as well as into survivorship. As the medical advances in FP are just beginning, there is little long-term data on the psychological aspects of cancer and fertility issues. Future research should explore how cancer patients can avoid negative psychological consequences in the long term.

It has to be noted that FP for male and female cancer patients is very different at least from the medical point of view. It is true that the options vary more for female FP than for male but also that the procedure might be simpler for male than for female cancer patients. However, from a psychological perspective, research has shown that men as well as women show a similar need for support during this challenging time of their life [34]. Male cancer patients have reported feeling neglected when it comes to appropriate fertility counseling and support. In fact, there is more research on female cancer patients and less knowledge about the experiences of male cancer patients. More research for male cancer patients needs to be done with the aim of adequately meeting their needs regarding oncofertility support at cancer diagnosis and into survivorship.

→ *Key reading: More long-term data on the psychological well-being of patients receiving*

oncofertility care as well as more emphasis on male cancer patients is needed.

Clinical Implications

In this chapter, we have described both achievements and weaknesses in the current health system approach to oncofertility care. The latter need to be addressed and discussed in each clinic and center that treats cancer patients as well as survivors. Next we summarize the difficulties that need to be overcome in the future.

The lack of referral of newly diagnosed cancer patients to fertility counseling with a specialist in reproductive medicine clearly needs to be addressed. The number of patients who receive counseling should increase. Internal institutional policy matters a lot. A helpful approach might be to develop standardized pathways to support a multidisciplinary setting that provides the best supportive care for cancer patients and survivors. Additional training for HCPs is needed. HCPs that are more knowledgeable will be better prepared to open the discussion about fertility with patients.

Interestingly, reproductive concerns may persist independent of evident physical difficulties in reproductive ability. It would therefore be beneficial for HCPs to explore not only the reproductive function of patients but also their concerns surrounding fertility potential and the impact on quality of life. Depending on patients' concerns, a referral to a specialist might be needed. Clinicians should be aware that some patient groups (e.g., those who have no children before diagnosis [11]) are in greater need of psychological support. Such patients may profit from additional support from a psychologist; HCPs can accelerate this process with a timely referral.

DAs are seen as the gold standard when it comes to additional support for decision-making. Some evaluated DAs already exist and have been implemented in clinical practice, whereas others are still being evaluated or lack the resources needed for clinical implementation. DAs have been shown to be a helpful source of support, and more DAs in various languages and targeting different cancer types are needed. A greater body of evidence is needed to support the implementation of DAs in clinical practice.

A very important aspect of clinical routine is to ensure consistent support. A cancer diagnosis and a potential impairment of fertility may seem most threatening at the beginning, but the psychological impact further down the road when a patient might already be in recovery should not be neglected. Therefore, every HCP who sees cancer patients should emphasize the psychological well-being of the patients with a few questions about their well-being and quality of life. If a patient affirms having fertility concerns, a referral to an appropriate specialist should be guaranteed.

→ *Key reading: Optimal cancer care requires HCPs to have a high awareness of fertility concerns in patients and survivors, to promote continuity in care, and to have an attentive eye on the psychological well-being of their patients.*

Conclusion

Fertility is an important aspect of quality of life and identity in young cancer patients and cancer survivors. A considerable number of these patients have reported a desire for parenthood. Infertility can be one of the most difficult long-term psychological consequences of cancer diagnosis and its treatment. It is associated with a higher risk of depression, anxiety, grief, low self-esteem, poor sexual well-being, and challenges to gender identity. Fertility counseling with a specialist in reproductive medicine before the start of cancer treatment can reduce fertility-related distress, lower decisional regret, and increase psychological well-being.

The possibility to undergo FP procedures is dependent on cancer type, oncological treatment protocol, time to start of oncological treatment, and likely prognosis, as well as access to services. Decisions regarding FP may be further complicated by the distress associated with a new cancer diagnosis, financial barriers, and psychological components. This highlights the complexity of this decision-making and emphasizes the need for comprehensive decision-making support.

The situation regarding provision of support, however, is not always adequate. Fertility counseling with a specialist in reproductive medicine is the primary need, but in many cases, support from a psychologist might be necessary and beneficial. Furthermore, patients and caretakers alike should consider evidence-based DAs as a helpful complement.

Fertility concerns outlive the termination of cancer treatment, and counseling should be offered beyond the acute phase. As part of a comprehensive care plan, fertility concerns should be addressed during follow-up consultations. A multidisciplinary approach is seen as beneficial to best meet the patients' needs with regard to fertility distress.

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Ethical Considerations of Fertility Preservation

Heidi Mertes and Guido Pennings

Introduction

As has become apparent throughout the previous chapters in this book, the possibilities for the preservation of fertility by both women and men are constantly expanding. In principle we should welcome this development as an infertility diagnosis is linked to a substantial decline in well-being. Yet, several of these current and potential interventions come with societal concerns and difficult ethical dilemmas in which it is not always straightforward which option optimally balances the ethical duties of respecting the patient's autonomy and refraining from harming patients. In this chapter, we will first consider the desire for genetic parenthood as this is the main focus of fertility preservation and then discuss some ethical concerns linked to particular interventions in woman and men.

The Desire for Genetic Parenthood

The ethical basis of fertility preservation is the concept of reproductive liberty and the knowledge that many people value and desire genetic parenthood. Fertility preservation is performed because one wants to enable people to have genetically related children. However, there is much debate about the importance of genetic parenthood [1–3]. Multiple studies and the general behavior of people show that most people prefer to have a genetically related child [4]. However, that fact in itself does not tell us what society should do to accommodate that preference. It is not because people want something that others, or society at large, should act to give it to them. Would-be parents may be willing to go to great lengths to obtain this goal. Others, such as healthcare professionals and society, may evaluate the goal differently and draw the line at a different point. There has been strong criticism of medically assisted reproduction in general because it confirms and reinforces a too strong emphasis on the genetic link in the parent-child relationship [5]. In the specific context of fertility preservation, Asch [6] and McLeod [7] encourage us to think more critically about some convictions that are very widespread and that we take for granted, namely, that parenthood is an essential component of a person's life, that parenthood makes people happier, and that families based on genetic relatedness are better than other forms of family building [6, 7]. They argue that if in fact these presuppositions turn out to be false, we need not invest our resources in fertility preservation measures but rather counsel patients about the fact that infertility will not necessarily have an impact on their overall quality of life or on their future

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happiness and that other options exist besides genetic parenthood.

An important element when judging the value of a goal is the existence of alternatives. In the case of genetic parenthood, the alternatives are the use of donor gametes or donor embryos and adoption. Adoption in most countries is very expensive, highly selective (or discriminating), and therefore very difficult to obtain. A more realistic alternative is gamete donation. Some people will argue that this is not a real alternative. It is clear that some motives underlying parenthood can only be fulfilled through genetic parenthood. There is for instance the wish to become immortal, to transmit part of myself into a future generation, to spread my genes, etc. The divergence of views on parenthood can be illustrated by postmortem reproduction. Many people find it difficult to accept postmortem reproduction because the person will never know that he/she became a parent and because he/she will never be able to do what parents normally do. Other people consider parenthood as a relationship that is characterized by a focus by the parents on the well-being of the child, unconditional love between parent and child, and the intention of the parents to raise the child to become an autonomous person [8]. This definition of parenthood implies that nongenetic forms of parenthood should be considered as worthwhile alternatives to genetic parenthood: a person can do all these things without having a genetic link with the child.

Although genetic and nongenetic parenthood can be considered as equivalent alternatives, the principle of respect for autonomy demands that if a given patient prefers genetic parenthood over nongenetic parenthood, priority should be given to this preference unless there are good reasons to argue otherwise. Two considerations that carry sufficient moral weight to curb autonomy in some circumstances are the welfare of the future child and the allocation of scarce resources in healthcare. The latter refers to justice and is mainly focused on the question of reimbursement. In many countries, people only have access to medically assisted reproduction if they have the necessary financial means. Other countries consider infertility treatment as part of basic healthcare and provide public funding to increase access. However, all countries (with the possible exception of Israel) limit the contribution of society on the basis of costs and effectiveness. That is the main justification for limiting reimbursement of IVF above a certain age of the woman. It is not cost-effective (good use of scarce money) to spend thousands of euros when the chances of success are very low. A similar justification could be offered to limit reimbursement for fertility preservation if evidence shows that only a low percentage of the patients return to use their cryostored material and even fewer finally end up with a child. So even when reproducing is considered important, it does not override all other considerations. The second reason to limit people's use of medically assisted reproduction is the general well-being of the future child. People who are at high risk of transmitting a genetic disease to their child undergo multiple cycles of IVF combined with PGD in order to avoid transmission of the disease. However, even when there are risks for the welfare and health of the child, some people still prefer genetic parenthood over an unaffected child. An example that led to a fierce debate some years ago is men with microdeletions on the Y chromosome who use ICSI and thus transmit their infertility to their sons [9]. Likewise, some people undergoing IVF and PGD may ask transfer of affected embryos if they have no non-affected embryos rather than shift to donor gametes. Some of these embryos tested positive for serious diseases such as hereditary breast and ovarian cancer [10]. The welfare of the child is also a concern when new techniques are introduced in clinical practice. The manipulation of gametes and gonadal tissue may have implications for the health of the patient from which the material is collected (complications after testis biopsy) and for the health of the future child in case of experimental techniques such as in vitro spermatogenesis [11].

So in summary, although fertility preservation is an intervention that is justified by the wish for a genetically related child, this goal should be balanced against other values such as distributive justice and non-maleficence.

Fertility Preservation in Women and Girls

Several different indications for fertility preservation in women lead to different possible interventions and different ethical considerations. For the purpose of this chapter, we will start by focusing on adult cancer patients and then go on to discuss some particular issues for a couple of interesting other categories of potential beneficiaries of fertility treatment, without claiming to be exhaustive: pediatric patients, Turner patients, and healthy women.

Oncofertility: What Are the Options?

At its introductory phase, fertility preservation was almost synonymous with oncofertility: the preservation of fertility for cancer patients whose reproductive organs need to be removed or who receive pelvic irradiation or treatment with alkylating agents putting them at risk of acute ovarian failure, premature menopause, and pregnancy complications. Both chemotherapy and radiotherapy deplete the number of follicles in the ovaries, and high doses of irradiation cause permanent damage to the uterus. The impact of treatment on fertility varies greatly, with some treatments leading almost certainly to immediate menopause, while others may advance the time of onset of menopause by a couple of years, but do not severely impair the patient's reproductive options [12]. The most common interventions for avoiding or reversing infertility in this group of women include transposition of ovaries (for pelvic irradiation), fertility-sparing surgical options (e.g., radical trachelectomy instead of radical hysterectomy in cervical cancer), oocyte cryopreservation, embryo cryopreservation, ovarian tissue cryopreservation, or concomitant treatment with GnRH analogs (for chemotherapy) [13]. Evidence for the efficacy of the latter intervention to preserve fertility is currently inconclusive in terms of its effect on pregnancy outcomes, although it has been shown to have a positive effect on the recovery of menses and ovulation and appears to lead to better pregnancy outcomes in breast cancer patients [14]. In this chapter, we will focus primarily on embryo, oocyte, and ovarian tissue cryopreservation.

The most established option available to cancer patients is embryo cryopreservation after ovarian hyperstimulation. However, embryo cryopreservation is only available for those women who have the time to undergo an ovarian stimulation cycle and who are in a committed relationship or who are willing to use donor sperm. As illustrated by the Evans v United Kingdom case for the European Court of Human Rights, even when embryos are created within a committed relationship, a man may withdraw consent to use the created embryos to establish a pregnancy, resulting in a situation in which the delay of cancer treatment in order to preserve the patient's fertility turns out not to bring any benefit for the patient [15]. In that respect, oocyte cryopreservation is oftentimes the preferred option, despite the present lack of long-term follow-up of children born from cryopreserved oocytes [16]. Short-term follow-up of the health of children born from cryopreserved oocytes is currently reassuring [17].

Although protocols have been developed that allow the start of ovarian stimulation at any point in the menstrual cycle, ovarian stimulation still requires a 2- to 3-week time investment. Depending on how soon after the cancer diagnosis fertility preservation is presented to the patient, this will result in a postponement of the start of cancer treatment. A way to avoid this postponement is the option of ovarian tissue freezing, which can involve freezing either cortical strips or the whole ovary with the vascular pedicle, and/or the collection of isolated follicles, followed by in vitro maturation and cryopreservation. Advantages of these options are that cancer treatment is minimally delayed and for ovarian tissue that besides preserving the possibility of reproduction, also endocrine function can be restored after transplant (after cancer treatment) of the ovary or cortical strips. A disadvantage of both options is that they are currently considered to be experimental treatments and the main risk for ovarian tissue transplant is the reintroduction of malignant cells, which is a concern

particularly for leukemia, non-Hodgkin's lymphoma, and ovarian cancer. Also, the average life span of a transplant is only 3 years [18]. Concomitant treatment with GnRH analogs is a much offered option, although the current data about its efficiency is, as mentioned above, inconclusive. Although many live births have been reported after ovarian tissue transplant, this option is still considered experimental and therefore should only be offered within a research setting [19].

Oncofertility: What Are the Concerns?

Whether postponement of cancer treatment (in the case of ovarian stimulation), a suboptimal treatment method (in the case of conservative surgery), or the extra physical burden on the patient (in the case of oocyte or ovarian tissue cryopreservation) is warranted will depend on a number of factors, impacting either the utility (and therefore benefit) of the procedure or the risks of the procedure. Starting with the latter, there will often be a correlation between the severity of the cancer, the chances of survival, and the urgency of treatment. If a patient has a low chance of survival and is benefited by an early start of treatment or a radical surgical procedure rather than a conservative one, fertility preservation measures only seem warranted at the explicit and well-informed request of a patient with a very strong desire for parenthood.

Second, the actual risk of becoming infertile after the treatment needs to be taken into consideration. As mentioned, while some treatment options result in immediate sterility, most diminish the ovarian reserve, without completely depleting it right away. This means that many cancer patients may have a sufficient window of opportunity to reproduce after their treatment without the need for fertility preservation measures. In those cases, fertility preservation measures are more likely to cause harm than benefits. However, the eventual outcome of a treatment option is sometimes difficult to predict at the onset of treatment, especially if a mild treatment is started first but is later replaced by a more aggressive one.

Third, the probability that the patient will want to reproduce in the future and will want to do so with her own oocytes needs to be assessed. All too often it is assumed that all young women (ought to) want to reproduce, but this is not necessarily the case. Generally, in the case of male cancer patients, it is justifiable to store a semen sample even when the probability of future benefit is very small due to the minimal invasiveness of the procedure and due to the absence of an impact on the cancer treatment (provided that the option is offered before the onset of treatment). Male patients have "nothing to lose" as it were, and this is a way of keeping all future options open, as reproductive desires can change in the course of a lifetime. Female patients, however, often do have something to lose as postponement of treatment can have an impact on their chances of survival. While clinical oncologists are more likely to focus on survival of the patient and might downplay the importance of fertility preservation, fertility specialists may be tempted to project the despair that they encounter in their infertile patients—who by definition highly value (genetic) parenthood—onto the cancer patient and therefore overestimate (a) the impact of infertility on the patient's future quality of life and (b) the importance of fertility preservation. It is important for fertility specialists to keep in mind that the group of people seeking medical care for infertility is a selected group of people who desire parenthood and suffer due to their infertility. It is estimated, however, that this group represents roughly half of all those who are infertile [20]. While many different factors can contribute to the decision not to seek medical care for infertility such as financial or psychological hurdles, it is fair to assume that a considerable group of infertile people do not experience severe suffering from their infertility, either because they do not have a (strong) desire for parenthood, because they have adopted a beneficial coping style, or because they are open to nongenetic parenthood [21]. Also in cancer patients, this variability is likely to be present. It has, for example, been observed that LGBTQ patients display more

adaptive coping and thus suffer less from treatment-induced infertility than their heterosexual counterparts [22]. In short, while fertility specialists are privileged witnesses of the psychological damage that *can* be caused by infertility, they are also likely biased regarding the proportion of people that will actually encounter this damage. It is important that they are aware of this bias so that they can adjust their intuitions accordingly.

Taking the previous remarks into account, weighing the pros and cons of fertility preservation efforts, and deciding which steps to take should always be a joint effort of a multidisciplinary team and the patient herself resulting in case-by-case decision-making. It is important to notice that in this context, the concept of informed consent is under considerable pressure. Informed consent is based on four criteria: (a) the patient is *informed* about the procedures, risks, and expected outcomes of the medical intervention, (b) she *understands* that information, (c) she *agrees* to the medical procedure *free from outside pressure*, and (d) she is *competent* to make such a decision. In the case of oncofertility decisions, several of these criteria are compromised. First, the patient is likely to be in emotional distress as she just received a cancer diagnosis (impacting on competence). This may cause patients to dismiss fertility preservation interventions which may result in decision regret later on [23]. Second, the patient has to digest a multitude of information regarding both her cancer diagnosis, her treatment options, and her options for fertility preservation. As mentioned, the patient will need to consider many probabilities: chance of survival, chance of impact on fertility, chance of a future desire for parenthood, chance of benefit from the fertility preservation intervention, impact of the fertility preservation intervention on chance of survival, et cetera. This may be such a mental overload that patients filter out everything that is not related to their survival. Finally, outside pressure is quite likely: the risk of the patient losing her reproductive potential does not only affect the patient herself but also—in first instance—her partner and/or parents. Young adults who are not inclined to take up the offer of fertility preservation may, for example, feel like they have to explain and justify this decision to their parents and may be sensitive to emotional requests by the latter to change their minds. Also, phenomena such as the technological imperative ("the technology is available, so I should take advantage of it"), binary thinking (not "I only have a 2% chance of this cryopreserved tissue/gamete ever leading to a healthy live birth" but "I have a chance that this cryopreserved tissue/gamete will lead to a healthy live birth"), and anticipated decision regret ("I will regret not preserving my fertility if I desire to have children later.") may impede free decision-making and lead to a high uptake of fertility preservation interventions even when the potential benefit is very limited, under the motto "at least we tried everything" [24]. The factors limiting informed consent outlined above do not pull the patient's decision decisively in either direction but can cause both an over-uptake and an underutilization of fertility preservation. The most important lesson here is that the medical team supporting the patient in her decision-making should be well aware of all these potential limitations of an informed consent and of the importance of (non-directive) fertility preservation counselling. Even patients who decide to decline fertility preservation report higher levels of psychological well-being than those who were never counselled about their options [25]. This counselling preferably involves a multidisciplinary team, to avoid a one-sided impact of physician authority (of either the oncologist or the fertility specialist) on the decision-making process.

As a final remark, patients and physicians alike may be worried by the risk of passing on cancer susceptibility to the next generation. However, this risk can be limited by making use of PGD and does not seem weighty enough to sideline fertility preservation entirely for these patients [16].

Pediatric Patients

As the harvesting of oocytes is not an option for prepubertal girls, ovarian tissue cryopreservation is oftentimes presented as their only option

of fertility preservation. However, as argued elsewhere, this does not mean that ovarian tissue freezing should become a standard procedure for young patients faced with gonadotoxic treatments [26]. There are additional reasons calling for restraint in this context as data on outcomes in terms of healthy live births from cryopreserved prepubertal ovarian tissue are still lacking for the moment. Also, as children have a much bigger ovarian reserve than adult patients, they are less likely to become sterile immediately after treatment (unless when they undergo very aggressive procedures such as total body irradiation for a stem cell transplant). This means that many girls may have the option of banking egg cells a couple of years after their cancer treatment, when they are not under time pressure or in emotional distress, when they are competent to make their own decisions, and when they have a better grasp on what their desires and expectations regarding the prospect of parenthood are. Offering ovarian tissue preservation to all young cancer patients "just in case" they might lose their fertility is therefore disproportionate and at odds with the principle of non-maleficence. Rather, a strict patient selection should be maintained. Inclusion criteria that have been well validated are the so-called Edinburgh criteria, which succeed in including only patients with a high chance of premature ovarian insufficiency (35% in the selected group vs. 1% in the nonselected group) [27]. A point of attention for the group of girls who are not good candidates for ovarian tissue freezing due to a limited chance of immediate POI but who are at risk of premature menopause is that, ideally, these girls would come to a fertility specialist for an assessment of their ovarian reserve as young adults. However, once cured from cancer, they will not regularly meet specialized physicians anymore. Therefore education of general physicians about the potential impact of different treatment regimens on fertility is very important, so that they can bring up the topic of potentially compromised infertility to childhood cancer survivors at the appropriate time.

Turner Patients

Besides gonadotoxic treatments, also genetic conditions can cause POI. The most prevalent condition (with an incidence of approximately 1/2000) is Turner syndrome (monosomy X), which is characterized by a depletion of the ovarian reserve in childhood or young adulthood. Women with a mosaicism for Turner syndrome (representing about one third of all Turner patients) are most likely to go through puberty, which makes oocyte cryopreservation as young adolescents an option for them. In other patients with a faster depletion of the ovarian pool, ovarian tissue cryopreservation can be considered [28]. Alternatively, cryopreservation of the patient's mother's oocytes is sometimes performed [29]. While the prospect of fertility preservation potentially offers a great benefit in terms of psychological well-being of Turner patients, all options also come with a number of ethical concerns. First, the same concerns regarding experimental invasive treatments in minors as those outlined above apply. Second, pregnancies in Turner patients are known to be high risk pregnancies, with the greatest concerns on the maternal side being the risk of heart failure, aortic dissection, and sudden death, leading to a maternal mortality rate of 1/50 (as compared to 1/10,000 in the general population in developed countries) [30]. Other reported maternal complications include thyroid dysfunction, obesity, diabetes, obstructive nephropathy, hypertension, preeclampsia, and increased rates of miscarriage [31]. Furthermore, increased rates of fetal abnormality, intrauterine growth restriction, low birth weight, and prematurity are risk factors for the well-being of the resulting children. The incidence of some of these risks can be reduced by careful patient selection and intense monitoring of the pregnancy; however, the risks remain elevated. Relying on donor oocytes—either from unknown donors or, for example, from the mother or a sister—is unable to mitigate these problems. Therefore, counselling about these risks, about alternative ways of starting a family, and about the option of childlessness is of critical impor-

tance for all Turner patients and especially for those with cardiac abnormalities. Relying on donor oocytes from a mother, sister, or other family member has the advantage of not requiring an invasive procedure on a minor, avoids the chromosomal abnormalities that may be present in the patient's own oocytes, and establishes a genetic link between the Turner patient and her children. At the same time, psychological counselling will be necessary to explore the extent to which the complicated family relationships that are established in this way might turn out to be problematic in the long run. Also, it has been noted that when a mother cryopreserves oocytes for her daughter, this may put pressure on the Turner patient to use these oocytes, rather than opting for donor oocytes or remain childless [32]. Whether this concern outweighs the potential benefit for those girls who cannot preserve their own fertility but would be good candidates for carrying a pregnancy is up for discussion.

In short, while informing Turner patients and their parents about fertility preservation options and monitoring of ovarian reserve for a timely intervention can be beneficial, in this population, one should be cautious of overselling fertility preservation measures to patients who are bad candidates for carrying an eventual pregnancy to term. The desire to comfort Turner patients by offering hope of genetic parenthood should not eclipse realistic expectations of the utility of the procedure and good clinical practice in terms of not harming the patient and potential future children.

Healthy Women

The possibility to cryopreserve oocytes to be used in IVF treatment later in life has not only enlarged the reproductive options of cancer and Turner patients but also enlarges the reproductive options of healthy women whose personal circumstances (most often the absence of a partner) do not allow them to reproduce in their most fertile years. Especially at its introduction in the clinic, there was quite some controversy over whether the technology of oocyte cryopreservation should be used for "nonmedical purposes." However, this distinction between medical and nonmedical egg freezing (or the so-called social egg freezing) is largely arbitrary [33, 34]. Both aging and cancer treatment cause a depletion of the ovarian reserve, and we typically do not call other age-related health problems (e.g., osteoporosis, bad eyesight, hearing problems) nonmedical problems. The fact that this distinction between medical versus nonmedical/social egg freezing is made in the context of egg freezing indicates that a number of presuppositions and stereotypes are present in this context that warrant critical reflection [35].

First, there is the objection of going against nature. While fertility preservation for cancer patients is considered to support "how nature intended things to be" (i.e., women can reproduce until they are about 40 years old), fertility preservation for age-related fertility decline goes against this natural course of things. As mentioned above, a large part of medicine is focused on remedying age-related diseases, which does not meet the same degree of resistance. So even if one believes that nature is organized in an optimal fashion, there is a consistency problem here.

Second, there is the idea that the need for egg banking in healthy women is avoidable, as it results from a purposeful delay of childbearing. According to this reasoning, by educating women so that they reproduce at a young age and/or by organizing society differently so that motherhood can be combined more easily with professional responsibilities, egg banking for age-related infertility will become superfluous [35]. However, it is inaccurate to state that women choose to delay childbearing in order to advance their careers. The age at which healthy women currently request oocyte cryopreservation indicates that "postponement" of childbearing is seldom planned at a young age and thus that freezing oocytes is rather an emergency intervention than part of a well-designed life plan to "have it all" [36]. Most women (and men) consider the ideal age to begin parenting to be somewhere between the ages of 25 and 35 [37]. However, some postpone childbearing bit by bit by lack of a partner, a demanding job, financial

insecurity, etc. (a phenomenon also known as "perpetual postponing"), and before they know it, their reproductive years have passed [38]. Also, it is inaccurate to say that women have no other option but to delay childbearing due to socioeconomic conditions. When people have their children depends on an interplay between contextual factors and personal values, and neither one will completely override the other. Several studies have found that women find it increasingly important to first complete their education, have financial security, good housing, and a stable relationship before taking on the responsibility of parenthood [37, 39, 40]. These are not selfish concerns that women should throw overboard but considerations that are made in the best interest of their future children. Thus, if we want to support women in exercising their reproductive liberty, they should be supported on several fronts: by educating them about their reproductive life span, by facilitating the optimal circumstances of having children, but also by allowing them access to a technology that can prolong their reproductive life span, within the limits that are already observed for IVF treatment (with or without donor oocytes). As long as women intend to use their oocytes within this age limit, it would be difficult to argue that reproduction with donor oocytes is allowed, but reproduction with their own, previously cryopreserved oocytes is not.

Nevertheless, there are some legitimate ethical concerns linked to AGE banking (egg banking for anticipated gamete exhaustion) and especially to the commercial settings in which the technology is increasingly offered. While there is a benefit to making AGE banking available to those women who have the highest chance of benefiting from it, it would be unethical to "sell" this technology to women who are unlikely to benefit by making them believe it will "stop their biological clock from ticking" [41]. The ideal candidate for AGE banking would be a 34-year-old woman who is not in a (stable) relationship or in a relationship in which parenthood is not (yet) an option and who has a conditional desire for parenthood (i.e., parenthood is desired within certain side constraints such as a dedicated partner, financial stability, etc.). Moreover, this woman would have to be well-informed about the limitations of the possibilities of establishing a pregnancy and reaching a healthy live birth; about the efforts, discomforts, and risks involved; and about the costs associated with obtaining a sufficient number of oocytes, storing them, and using them afterward. Also, she should be free from outside pressure (e.g., from her employer) [42]. In the ideal scenario, AGE banking should be considered as a plan B in case future attempts at natural conception fail: a plan B that has a chance of success but that is no guarantee for success. Women who inquire about AGE banking at a very young age, arguing that they will postpone parenthood to build a successful career first, should be stimulated to consider whether there will be more or less room for raising children 10 or 15 years down the road once they have landed that perfect job they are now aiming for and whether or not they are willing to gamble their odds of being able to establish a family. Finally, women who inquire about AGE banking at a point when they are very close to being infertile, which is currently the majority of candidates, should be counselled properly about their individual chances of success [43]. For many of those women, AGE banking will be a desperate measure with a very small chance of success, rather than a plan B with a reasonable chance of success. It is important to be aware of the fact that many women have reported to be happy about their decision to bank eggs, even if they believe that the chances that they will ever use them are slim [44]. For many women the loss of their fertility may come very suddenly, and egg banking may give them the time to come to terms with that new reality and to take the pressure of new relationships. At the same time, the psychological argument only goes that far. If some years from now it would turn out that very few women come back to use their banked oocytes, psychological support and counselling might be the preferential treatment option for women at the verge of losing their fertility, rather than AGE banking.

Fertility Preservation in Boys and Adolescents

Freezing sperm for adult men is a simple and well-established procedure that raises few ethical issues. The interesting ethical problems regard the interventions on minors (boys and adolescents). The issue of informed consent is tricky both for underage girls and boys. Depending on the minor's age and development, different approaches can be adopted. Although legal consent cannot be obtained, the minor can still be involved in the decision [45]. The moral alternative to legal consent is assent: a lower standard of capacity that does not require the level of understanding and reasoning that is generally assumed in informed consent [46]. The child should be informed about the pros and cons of the procedure at a level appropriate for its age and development. This does not equal shared decision-making: shared decision-making presumes (at least) two competent parties. In this case, the minor should be informed about the different aspects of the procedure but does not decide: he or she only agrees to the proposed treatment. The limits of assent become clear when the minor disagrees.

The general rule is that parents have decision-making authority and act as proxies of the minor. The parents are assumed to provide a substituted judgment, i.e., they should try to make the decision that the patient would have made if he or she were able to make decisions. However, this substituted judgment standard is impossible to apply when there is no information on the person's life plan and values. It only applies if two conditions are fulfilled: there is a competent person with decision-making capacity who lost this capacity (temporarily or permanently), and the proxy knew the person well. The first condition is not fulfilled. How would the parents be able to know whether their child will want to have a child in the future? Is there any reason to assume that the parents know better than anyone else whether this will be the case? Both questions should be answered negatively, and, therefore, the substituted judgment standard should be rejected.

The alternative is the best interest standard. Balancing benefits and risks is highly complex due to several factors. First, there is the lack of scientific and clinical evidence about the efficacy, efficiency, and safety of the intervention. This is a crucial point as it introduces large gray zones, potential bias, and possibly unrealistic expectations. Second, the intervention is performed on a minor, and third, the intervention is frequently discussed in a highly stressful and emotional context (i.e., cancer treatment). All these elements contribute to a setting in which it is not always clear when a way of framing and presenting the cost-benefit ratio crosses the line of what is acceptable. There is some evidence of parents exerting pressure on children and of children feeling pressured by healthcare providers [46]. Cultural norms and expectations about the naturalness of the wish for a genetic child undoubtedly also play a role. At the same time, separating the minor from his or her family and constructing him or her as an autonomous decision-maker are not necessarily good ideas. "Adolescents' decisions tend to reflect the values and morals of their parents and the healthcare system in which they receive care" [21]. This could be interpreted as a good reason to give the parents the right to decide since their values are the same as those of the adolescent due to his or her upbringing in that family. But that also leads to the conclusion that adolescents, even if they seem to decide for themselves, can never be really autonomous since they do not have control over the values they acquire and use. Prying apart the values of parent and child seems impossible and even undesirable. Teaching and passing on values and principles to their children are part of what we consider good parenting.

The experimental nature of the freezing of testicular tissue remains the main ethical problem for fertility preservation in prepubertal boys. The basic idea governing experiments on children is a strict balance between burdens and risk on the one hand and the anticipated benefit for the child on the other hand [47]. Since the potential benefit has not been proven, the technology should only be offered when the risks and burdens for the

child are kept to a minimum. Thus, contrary to the situation for postpubertal patients where fertility preservation should be proposed, testicular tissue cryopreservation in prepubertal boys can but should not be offered [48]. It has been suggested that testicular tissue cryopreservation should not be restricted to the experimental setting [49]. However, this move seems largely premature. Treatments can be categorized in three large types: experimental, innovative, and established treatment [50]. All three steps of the testicular tissue cryopreservation (collection, cryopreservation, and re-transplantation) fail to fulfill the criteria to be considered as innovative treatment: no proof of principle in humans and as a consequence no data on safety, procedural reliability and transparency, and effectiveness. The proposal to allow the application outside the experimental setting intends to augment access. However, problems of access can be solved in other ways than by changing the scientific status of the technique [51].

The benefits of the technology are determined by the importance attributed to genetic parenthood and by the belief in scientific progress [52]. When parents believe that science will advance enough for the cryopreserved material to be usable in the coming decades, they allow their child to undergo the tissue collection [53]. This belief thus increases the potential benefits. Still, it is unclear how this belief should be evaluated: when does belief in scientific progress turn into therapeutic misconception or false hope? The medics working in the field of fertility preservation may not be the right people to evaluate this belief as their personal involvement may result in bias. In addition, other mechanisms may reinforce the tendency to accept the offer such as anticipated decision regret. Knowledge of these factors, in combination with normative rules about good parenthood, should incite caution in the presentation of the benefits of the preservation. Still, one should not downplay the benefits either. The challenge consists in finding the right balance to fit the experimental nature and the potential therapeutic benefit. Testicular tissue cryopreservation can be seen as an intermediate category: an experimental procedure with the prospect of direct personal benefit for the child [54].

The importance of preserving the possibility of having genetically related children is corroborated by studies indicating the importance of fertility in (surviving) cancer patients. However, there are also indications that show that this initial belief is not maintained in later life. One such indicator is the utilization rate of the stored material. While the majority of cancer patients indicate that having genetically related children is important, the utilization rate of frozen sperm in cancer patient populations is very low. In male cancer patients, a utilization rate of merely 8% is reported, and from those 8%, only 49% achieved a pregnancy [55]. While these numbers are expected to increase after longer follow-up, they are likely to remain relatively low. Moreover, the utilization rate regards the use of frozen sperm. When we take into account the need for surgery and possibly IVF combined with ICSI when testicular tissue cryopreservation is concerned, the utilization rate might be even lower. These percentages (and not only the efficiency of the technique) should be taken into account when offering treatment. Regarding the offer, two schools can be distinguished. There are those who believe that preservation measures should be offered to all possible candidates. On the opposite side, there are those who believe that parents should only be approached if the child has a good prognosis and is at high risk of permanent infertility [27]. Given what we said earlier, the latter position seems the most reasonable.

Conclusion

Fertility preservation is a relatively recent subdiscipline of reproductive medicine, aimed at safeguarding a chance to reproduce for those people who are at risk of losing their fertility. Especially in the context of oncology, this has become an important quality of life intervention as the group of young cancer survivors is growing due to a younger average age of cancer onset and higher survival rates. However, also in other contexts where infertility may precede the moment at

which people are ready to reproduce, fertility preservation interventions are increasingly being offered.

While in the light of reproductive liberty this possibility is a positive evolution, it is also important to remain conscious of contraindications to fertility preservation, most notably health risks and issues related to distributive justice. Two situations (that frequently coincide) demand an extra cautious approach: vulnerable populations and experimental treatments. For example, in the case of pediatric cancer patients, who have limited capabilities of weighing the pros and cons of fertility preservation measures themselves and whose options are oftentimes limited to experimental procedures, healthcare professionals should be careful not to impose a pronatalist societal norm onto those patients, pushing them to undergo invasive procedures and risks for an uncertain benefit. At the same time, sometimes other societal norms—such as the norm that women should reproduce at a very specific time in their life, but not too early and not too late—discourage access to fertility preservation measures for well-informed women.

Ideally, a shared decision-making approach is pursued in which fertility preservation candidates or their parents are optimally informed about their different options and their risks, benefits, and uncertainties. For healthcare professionals, it is important to be aware that they are themselves prone to bias due to some deeply rooted societal norms and prejudices regarding (genetic) parenthood, which are likely to be reinforced by stressing the importance of fertility preservation, rather than critically assessed.

Definitions

- Reproductive autonomy/freedom/liberty: the basic right to decide freely whether, how, with whom, and how many times to reproduce. This right is generally considered to be a pro tanto right (meaning that it is valid unless it conflicts with other, more basic rights) and a liberty right (meaning that people should not be hindered in achieving their reproductive goals, but not necessarily that people deserves assistance in reaching them).
- Assent: approval of a medical procedure by a patient who is considered incompetent and thus unable to provide an informed consent, typically children.
- Substituted judgment: the standard of proxy decision-making where the proxy makes the same decision that the incompetent patient would have made if he/she would have been competent.
- Therapeutic misconception: when a person participating in research wrongly believes that he/she receives treatment and attributes therapeutic intent to a research procedure.

Practical Clinical Tips

- The medical team should not assume that all patients have a great interest in preserving their gametes.
- To avoid bias in perception and decision-making by certain disciplines, it is highly recommended that counselling and decision-making involve multidisciplinary teams.
- In case of experimental interventions, the technology should only be offered when the risks and burdens to the child are kept to a minimum.

Take Home Message

The medical professionals should avoid imposing a pronatalist norm on patients and provide nondirective counselling balancing the pros and cons of fertility preservation.

Clinical Cases

We are ethicists and therefore cannot provide clinical cases.

Key Readings

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Legal Aspects of Fertility Preservation

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Introduction

Roughly a third of Americans report a connection to the world of fertility preservation [1]. For many, fertility treatment made starting a family possible. For others, advancements in assisted reproductive technologies ("ARTs") benefitted a family member or a friend. Not only are Americans turning to fertility preservation more frequently when it comes time to start a family, employers now routinely offer subsidized egg preservation as a "perk" to younger employees [2]. Yet, despite this growing popularity, many legal issues associated with fertility preservation remain unsettled—leaving clinicians with limited guidance and patients vulnerable.

To date, US legal doctrines and regulatory bodies have taken a piecemeal approach to regulating fertility preservation and, in doing so, often miss critical issues that warrant intervention. Courts consider individual cases and controversies, which limits their vision to the facts in front of them. While regulatory bodies have a more expansive line of sight, they often regulate in silos. This leaves gaps where typically unrelated areas of law—like federal drug regulation and state family law—intersect. Fertility preservation inhabits exactly such an intersection. To supplement this fragmented regulatory environment, groups like the American Society for Reproductive Medicine ("ASRM") step in. Professional organizations have a more holistic understanding of the challenges posed by ARTs, but self-regulation can only go so far.

Whether in the courtroom, the legislature, or a regulatory environment, the ever-changing scope of the term "ARTs" presents a challenge. The pace of scientific progress makes pinning down the term's definition difficult, yet laws and regulations require precise definitions. ARTs upset our very conceptualizations of life and personhood by moving the reproductive process (at least partially) ex vivo. These scientific advancements force the legal and regulatory worlds to reckon with the "continuum of distinctly identifiable processes involving in vivo (via natural conception or artificial insemination) or in vitro fertilization ("IVF")"—and different entities take different approaches when it comes to defining that continuum [3].

The current regulatory landscape of ARTs highlights this definitional challenge. For example, even within the federal government, different entities don't always follow the same definition. The Fertility Clinic Success Rate and Certification Act of 1992 ("FCSRCA"), overseen by the Department of Health and Human Services

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("HHS"), defines ARTs as follows: "all treatments or procedures which include the handling of human oocytes or embryos including in vitro fertilization, gamete intrafallopian transfer, [and] zygote intrafallopian transfer" [4]. The statute also allows the secretary of HHS to broaden this definition, subject to public notice and comment. Yet, the Centers for Disease Control and Prevention ("CDC")—the agency tasked with collecting reports on the safety of certain fertility clinics—takes a narrower view [5]. For the CDC, the definition of ARTs includes only "fertility treatments in which both eggs and embryos are handled" and expressly excludes treatments involving only sperm (e.g., intrauterine or intracervical insemination) or drugs that stimulate ovulation absent egg retrieval [6]. Disjointed definitions like these represent just one piece of the fertility preservation puzzle—a puzzle that often leaves physicians with limited guidance.

While the legal issues associated with ARTs could fill their own book, this chapter focuses on issues most relevant to fertility preservation. From accessing care in the first place to handling long-frozen embryos, patients and providers face thorny legal and regulatory issues at nearly every stage. These issues include insurance coverage, informed consent requirements, regulation of the cryopreservation process, and legal challenges associated with final disposition of reproductive material. This chapter will proceed in four parts, introducing the legal doctrines relevant at each stage of fertility preservation to give physicians some insight into the legal dimensions of patient care.

Insurance Entities as Regulators in a World of Limited Oversight

In some sense, the fertility preservation process begins before a patient even selects a provider. Insurance entities play a significant gatekeeping function in fertility preservation. Despite growing popularity, the cost of treatment puts fertility care out of reach for many Americans. For patients unable to pay out of pocket, insurance coverage dictates treatment options. The treatments insurance entities will cover vary greatly, and laws that require coverage for certain procedures differ from state to state. Only a handful of states require at least *some* form of ART coverage—but these coverage mandates are often narrow. With a narrow mandate comes additional out-of-pocket costs that can leave fertility preservation prohibitively expensive.

One might assume limited insurance coverage means limited involvement of insurance entities in the regulation of fertility preservation. And yet, insurance entities play an outsized role in regulation. Not only do these entities often dictate *who* can access treatment, but they also define *what* procedures qualify for coverage. Insurance companies may present obstacles to treatment by requiring patients undergo multiple cycles of intrauterine insemination prior to IVF, even against a treating physician's recommendation. But they may also serve an important signaling function for patients by refusing to cover expensive and unproven IVF "add-ons" [7]. In this way, insurance entities both limit access to treatment and "quality control" the procedures available to many patients. While one can easily cast gatekeeping in a negative light, insurance activity offers benefits as well—especially when one considers the fertility space's piecemeal regulatory environment.

The Piecemeal Regulatory Environment of ARTs

Many consider legislatures and federal agencies as the entities best suited to regulate ARTs [8]. Unlike courts, legislatures and administrative agencies have the purview to broadly regulate—therefore, the purview to adapt to changes in the world of medicine more quickly. They produce statutes, administrative rules, and regulations that are prospective and (in theory) better match the cadence of advancements in medical practice. Despite this "ideal" venue, legislatures are beholden to their constituents. This democratic tether imports political and social tensions underpinning issues of reproduction justice and the ever-present question of when human life legally

begins. The polarizing nature of the conversation hinders attempts to pass a comprehensive regulatory scheme for ARTs and likely contributes to fertility preservation's disjointed and inadequate public oversight [8]. Instead of a comprehensive scheme, a piecemeal framework of federal, state, and private actors regulates ARTs broadly and fertility preservation specifically.

Federal Regulation

On the federal level, the Fertility Clinic Success Rate and Certification Act of 1992 ("FCSRCA") directly and purposefully regulates ARTs. The act requires fertility clinics report their pregnancy success rates and certification information of each embryo laboratory the clinic uses to the Centers for Disease Control and Prevention ("CDC"). But while the FCSRCA outlines reporting requirements, the statute provides neither incentives nor penalties for clinics that fail to comply. The FCSRCA lacks any enforcement mechanisms, crippling its regulatory purpose by allowing the estimated 12% of clinics that fail to comply with the act's mandate to keep their doors open [8].

In addition to the FCSRCA, the FDA also plays a role in the federal regulation of ARTs—albeit a small one. In addition to outlining reporting requirements, the FCSRCA tasks the Food and Drug Administration ("FDA") with screening third-party reproductive material for infectious diseases [4]. However, the FCSRCA expressly limits the FDA's regulatory authority over fertility preservation. Thanks to strong lobbying by the fertility industry, "Congress add[ed] a carve out forbidding the agency from 'establish[ing] any standard, regulation, or requirement, which has the effect of exercising supervision or control over the practice of medicine in assisted reproductive technology programs'" [8]. As most fertility preservation does not involve any third-party reproductive material, the act considers fertility preservation a medical procedure. For this reason, most fertility preservation falls outside of the FDA's regulatory purview.

Beyond the restrictions imposed by the FCSRCA, the FDA has minimal regulatory authority over ARTs. Statutes constrain a federal agency's regulatory authority. The FDA can only regulate "articles" whose commercial distribution engages the Commerce Clause and qualify as either *biologics* under Section 351(a) of the Public Health Services Act or as *drugs* or *devices* under the Food, Drug, and Cosmetics Act [9]. Given this limited scope, FDA authority over ARTs varies depending on the specific technology in question—and whether it qualifies as a *biologic* (e.g., semen, oocytes, and embryos), *drug*, or *device*. Certain ARTs do fall within the FDA's regulatory purview, such as "minimally manipulated" human cells, tissues, and cellular and tissue-based products ("HCT/Ps") [10]. But the agency nevertheless stays largely away from the ART world, refusing to do more than discourage research in the mitochondrial transfer, germline embryo editing, and human cloning spaces [11].

State Regulation

To supplement what many consider insufficient federal oversight, some states have enacted their own ART-specific legislation. However, ART-specific legislation varies greatly from state to state. Some focus their legislation on issues related to embryonic stem cell research, insurance coverage for infertility, and surrogacy agreements [12]. A few others go further. Utah, for example, allows provider-conceived children to access the medical records of their sperm provider when they reach majority.

In terms of state legislation, Louisiana likely offers the most "comprehensive" regulation of ART procedures. The state requires providers "possess specialized training and skill in in vitro fertilization in conformity with the standards established by the American Fertility Society or the American College of Obstetricians and Gynecologists" ("ACOG") [13]. Louisiana's legislation also requires that IVF procedures occur in a medical facility meeting ASRM or ACOG standards. While the legislation goes further than other comparable statutes, the state still offers little guidance as to the meaning of "specialized training and skill," instead importing self-regulation by professional organizations.

Though such laws could do more to guide physicians, ART-specific state legislation rests on an established regulatory backbone: state medical boards. State medical boards interpret and enforce state's laws and regulations that govern the practice of medicine and, in doing so, establish the standards of the profession [14]. Regulation occurs through physician licensing, investigating complaints, and tracking all formal actions taken against a practitioner. That said, recent years show a pronounced trend toward more lax disciplinary treatment—with state medical boards disciplining fewer and fewer doctors each year [15].

In the fertility space, specifically, numerous instances exist where state medical boards failed to revoke licenses even after egregious misconduct. For example, one doctor at the Pacific Fertility Center in California saw multiple medical malpractice suits. Claims ranged from false promises, failure to train staff, botched IVF, and performing an abortion without a patient's consent to cover up implantation of the wrong couple's embryos [16]. Yet, the California state medical board failed to act.

Third-Party Regulation

Given limited federal regulation and uncoordinated state efforts, professional groups such as ASRM and ACOG serve a particularly important regulatory function in the fertility space. As evidenced by Louisiana's ART statute, and others like it, states do integrate organizational guidelines into legislation. Therefore, organizational guidelines can have the force of law in some states. That said, many of the same problems that plague federal and state regulatory efforts exist in third-party regulation as well—namely, a lack of enforcement power.

ASRM sets forth industry standards for clinicians, fertility clinics, and sperm banks—however, these standards only apply to clinics that *opt* into the society's Reproductive Laboratory Accreditation Program ("RLAP"). RLAP aims to "make laboratory processes more fail-safe and reduce risk of errors in patient identification, specimen labeling, handling of embryos and gametes and cryo-storage conditions to protect patients" [17]. To achieve this aim, RLAP institutes standards which include ongoing competency of all testing personnel and embryologists, administrative reporting requirements, and facility inspections [17].

While ASRM's guidelines offer a much-needed supplement to federal and state regulation, the same problem exits: limited enforcement power. Fertility clinics are not required to join ASRM, and even those who opt into RLAP "routinely ignore" program requirements [8]. Individual clinicians are not obligated to follow the organization's guidelines, and ASRM does not vet new ART procedures prior to their clinical use. ASRM also lacks the ability to do more than withhold accreditation for those who violate best practices, which does little to deter bad actors. Ultimately, though third parties wield power when it comes to ART regulation—especially in light of limited federal and state regulatory schemes—many agree that a gapping regulatory hole exists in the fertility industry.

Insurance Entities as De Facto Regulators

In addition to professional organizations, another third-party actor wields tremendous influence over the provision of ARTs: insurance entities. Insurance entities serve as gatekeepers—first, by dictating *who* may access treatment and, second, by dictating *what* treatments they will cover. In doing so, insurance entities take on a quasi-regulatory role in the ART space.

Insurance Entities as Gatekeepers: Who Gets Treatment

For patients unable to afford fertility preservation out of pocket—an expense that can total tens of thousands of dollars—insurance coverage dictates who can access treatment [18]. Both public and private insurers often refuse to cover fertility preservation. Only 15 states currently have laws related to covering fertility services, including 1 state Medicaid program (though the program does not cover artificial insemination or IVF) [19]. And even between these 15 state mandates,

coverage varies greatly. Two states—California and Texas—require insurance companies *offer* coverage for infertility treatment, whereas the remaining 13 require insurance companies *cover* infertility treatment [20]. Some state mandates define infertility and those who qualify for fertility preservation broadly, while others limit their mandated coverage to "oncofertility" services.

An outlier, Colorado takes a broad approach when it comes to mandated coverage of fertility preservation. The state requires all individual and group health benefit plans issued or renewed after January 2022 cover diagnoses of infertility, treatment for infertility, and fertility preservation services. The "Colorado Building Families Act" ("the CBFA") features nondiscretionary language and broadly defines infertility to include *all* indicated needs for infertility diagnosis, treatment, and fertility preservation, irrespective of marital status or sexual orientation [21]. The bill covers three completed egg retrievals and unlimited embryo transfers [21]. It also mandates that infertility be treated as any other disease process (i.e., no additional co-pays, coinsurance requirements, or waiting periods) [21]. While increasing the availability of fertility preservation for many Coloradans, the CBFA only applies to health insurance plans regulated by the Division of Insurance ("DOI") in Colorado's Department of Regulatory Agencies [22]. In total, approximately one million Coloradans receive health insurance through DOI regulated plans and may benefit from ARTs they might not otherwise have access to.

In contrast, many states with mandated infertility coverage limit fertility preservation services to patients who receive medical treatment that may jeopardize their fertility. Delaware, for example, only mandates fertility preservation coverage for patients suffering from iatrogenic infertility [23]. The statute defines iatrogenic infertility as "an impairment of fertility due to surgery, radiation, chemotherapy, or other medical treatment" [23]. All group and blanket health plans offered by health insurers, health service corporations, or HMOs in the state must cover "standard fertility preservation" for such patients. Despite these mandates, practitioners in states like Delaware still note the critical role the preauthorization process plays in whether patients actually receive coverage [24].

Whether a state broadly defines those who qualify for fertility preservation or limits services to oncofertility, these mandates only apply to insurance plans regulated by state law. Even in states with inclusive mandates, many states exempt small businesses or religious employers from such legislation. In addition, the Employment Retirement Income Security Act of 1974 exempts self-insured employer plans from state regulation. Instead, federal law regulates these plans [25]. In 2019, 61% of covered workers were enrolled in self-funded plans exempt from state regulation [26]. While progressive state mandates broaden the reach of ARTs, many Americans see no benefit from these efforts. But regardless of variances in coverage, insurers serve a quasi-regulatory function by gatekeeping who can access fertility preservation.

Insurance Entities as Gatekeepers: What Gets Covered

In addition to gatekeeping *who* can access treatment, insurance entities also gatekeep *what* treatment options patients can realistically consider. While legislation differs from state to state, typically, *if* state mandates cover fertility preservation, they often only apply to the preservation of eggs and sperm and do not include "elective preservation," the long-term storage costs of cryopreservation, or "experimental" treatments [27]. In this sense, insurance entities also serve a quasi-regulatory function by limiting clinical practice through coverage decisions.

Many fertility treatments are not considered "medically necessary" by insurance entities and therefore do not receive coverage—but that raises the question: how do we make sense of "medical necessity?" Linda Bergthold sees the term "mainly a placeholder [] in insurance plans," with the national healthcare reform debate prompting a discussion about what "a necessary service actually is and who should decide if it is covered" [28]. Like the term ART itself, both "medical" and "necessity" defy definitional precision—which allows insurers a dangerous level of

freedom in decision-making. Generally, an insurer's calculus for medical necessity includes the following:

1. The scope as determined in the contract
2. The standards of professional practice
3. Patient safety and setting of the intervention
4. Medical service (e.g., service a medical need)
5. Cost-effectiveness of the particular treatment [28]

Given that each of the aforementioned goals intersect with each other in both subjective and variable ways, some suggest that an insurer's calculus "ultimately result[s] in a determination that best suits [their] interests" [29]. As insurance providers do not disclose their process for making individual determinations, patients "implicitly ... struggle to know what [their] plans actually cover and, critically, what they do not" [29]. With the authority to create their own definitions and criteria for medical necessity, insurance entities hold considerable influence over individual patient and provider behavior within the fertility sector [29].

Embedded in the question of medical necessity is the dichotomy of "established" versus "experimental" treatment. Insurance entities favor "established" treatments and regularly deny coverage for those deemed "experimental." Some statutes offer insurance entities guidance in how to distinguish between the two. Colorado, for example, specifically defines "standard fertility preservation" as "procedures and services that are consistent with established medical practices or professional guidelines published by ASRM or ASCO" [21]. In 2012, ASRM shifted its classification of oocyte freezing from experimental to established treatment [30]. In 2018, guidelines issued by the American Society of Clinical Oncology ("ASCO") noted that "[s]perm, oocyte, and embryo cryopreservation are considered standard practice and are widely available" and that "the field of ovarian tissue cryopreservation is advancing quickly and may evolve to become standard therapy in the future" [31]. In the same vein, ASRM's 2019 guidelines declared ovarian cryopreservation nonexperimental [32]. Evolving guidance from professional associations carries weight in how insurance entities classify fertility preservation procedures and make coverage decisions.

In this sense, insurance entities and professional organizations work together to shape clinical practice. The gatekeeping effect created through definitions of "medical necessity" and "established treatment" influences patient and physician behavior. Insurance activity may, therefore, weed out dangerous or cost-ineffective care. But the effect of insurance entities on "quality control" remains less than clear; no studies demonstrate a definitive impact. In the fertility space specifically, patients may supplement their covered care with "elective" procedures and "add-ons," which they pay for out of pocket. This may dilute the effect of coverage decisions on "quality control." While quantifying the effect might be difficult, insurance entities play a significant role in regulation nevertheless by limiting *who* can access *what* fertility procedures.

The Challenges of Informed Consent

Separate from concerns about *who* receives *what* kind of care, *consent* to care introduces its own unique legal questions in fertility preservation. Legally, informed consent requirements obligate physicians to disclose all risks that a reasonable person would find significant in making an informed treatment decision [33]. Even in the best of circumstances, informed consent presents a challenge. The informed consent process surrounding fertility preservation is especially fraught; how can clinicians effectively communicate unknowns about the likelihood of successful pregnancy to patients desperate to start families? How can patients effectively evaluate the risks and benefits of fertility "add-ons" with limited empirical backing?

Despite these challenges, informed consent remains vitally important. Minimal regulations, difficulties proving the elements of medical malpractice, and policy pressures to steer clear of "wrongful life" claims offer patients limited

recourse when things go awry. This lack of recourse hinders recovery, even when gamete banks fail to screen for genetic disorders or neglect to validate information provided by anonymous sperm providers (be it medical information, educational history, or criminal records) [34]. These acute information asymmetries leave patients vulnerable and underscore the importance of the informed consent process.

The Fraught Process of Informed Consent for Fertility Treatment

To satisfy the legal requirements of informed consent, clinicians must discuss the risks and benefits of procedures with their patients. Only after does the law consider patients able to make an "informed" decision about treatment. Courts use two competing touchstones to determine the adequacy of informed consent: (1) whether a similarly situated, competent doctor would have disclosed the risk and (2) whether a reasonable patient would have elected not to undergo the treatment as a result [35]. In the context of fertility preservation, clinicians cannot realistically disclose every possible risk of a procedure—as many of the risks are financial or legal in nature. Instead, these standards offer some guidance about what risk or alternate treatment information a clinic *must* disclose to prevent liability.

But "informed" consent presumes patients actually *understand* the information conveyed to them—which is a lofty goal. Risk is a deceptively simple framework that dictates much of our decision-making. Conceptualizing risk and making decisions based on that information require translating the uncertainty risk implies. Even if a clinician "objectively" reports a numerical risk score, a patient's understanding of that value is inherently subjective. Often, clinicians cannot provide quantified risk scores for each possible outcome in fertility treatment. And even if they could, patients desperate to start families might hear what they want to hear, regardless of the information conveyed by providers.

Concerns about patients hearing what they want to hear apply equally to conversations about the benefits of fertility preservation. Despite a provider framing successful egg retrieval and cryopreservation as the benefit of treatment, a patient might conceptualize successful pregnancy as the benefit instead. What makes these conversations even more fraught is the reality that many patients interact with fertility preservation prior to their first clinical encounter. From "egg freezing parties," where women discuss fertility preservation over martinis, to employers like Apple and Facebook offering subsidized egg retrieval, many patients came to understand the benefits of fertility preservation well before walking into a clinic [36]. In this sense, recruitment starts well before the clinical encounter and raises questions about how to conceptualize the scope of the informed consent process.

In other areas of medicine, informed consent *starts* with recruitment. In the clinical trial context, for example, the FDA considers direct advertising the beginning of informed consent and requires institutional review boards scrutinize recruitment materials to prevent undue coercion [37]. The agency demands heightened scrutiny when a study "involves subjects who are likely to be vulnerable to undue influence" which might compromise informed consent. Contrast this approach with events hosted by fertility clinics at venues like the Beverly Hills hotel. There, clinics pitch egg freezing to childless women as "the smartest thing [they] can do if they are not in a serious relationship"—and they do it over drinks [36]. Clinics try to "make the idea [of egg freezing] less intimidating" and encourage women to conceptualize fertility preservation as "not a medical issue, [but as] a social issue" [36]. It's hard to suggest these environments are not intentionally coercive, especially when clinic specialists admit that "with a glass of wine, everything sounds better" [36].

These encounters undoubtedly shape how patients understand the risks and benefits of fertility preservation and raise fundamental questions about the validity of informed consent once patients enter the more traditional clinical paradigm. But like many things in the realm of fertility preservation, the law remains unsettled in this area. While the next few years

will likely bring litigation challenging these practices, whether such encounters are appropriate prior to the establishment of a doctor-patient relationship remains an open question. Regardless, these encounters are a reality of current practice and likely influence how patients make sense of fertility preservation during informed consent. As the challenges of medical malpractice and "wrongful life" claims give patients limited recourse when things go awry, informed consent represents a vital safeguard for patients.

The Difficultly of Successful Legal Claims

When injuries do arise, a constellation of factors including financial, social, and legal hurdles prevents litigants from successfully bringing their claims to court. Law Professor David Engle notes that, "'more than nine out of ten injury victims' in the United States 'assert no legal claim at all … even in cases where it is likely that a legal duty was breached, and a claim would succeed'" [8] (p. 32). First, litigation is both time-consuming and expensive. Second, states discourage medical malpractice claims by implementing short statutes of limitation (often between 6 months and 2 years), by requiring patients first submit their claims to malpractice review panels, and by capping damage awards. Third, litigation is intrusive; patients may be understandably reticent about putting their medical histories and decisions to use fertility treatment on trial. In addition to these impediments, the doctrine of medical malpractice itself imposes challenges on litigants.

A successful medical malpractice claim requires patients show that the physician or clinic breached a duty of care owed to them and that they were harmed as a result. Traditionally, the law translates this burden into four interrelated elements that plaintiffs must prove by a preponderance of the evidence (i.e., more likely than not): duty, breach, causation, and damages. Each element presents its own challenges in the context of reproductive negligence.

Duty and Breach

The first two elements a patient must prove are (1) that the provider owed them a duty of care and (2) that the provider breached that duty of care. Duties of care exist in the context of many different relationships, from that between parents and children to motorists and pedestrians on the street. These relationships all require people exercise reasonable care to prevent harm to those around them. The duty of care in the context of the doctor-patient relationship is even more acute—which makes duty a relatively easy element to establish in a paradigmatic case. A duty of care is implied once a plaintiff establishes the existence of a doctor-patient relationship, where the doctor was responsible for the patient's health at the time of injury. If the plaintiff then shows the provider failed to exercise that duty of care (i.e., they failed to act as another doctor with a comparable skill level would in the same situation), they will also have established their second element: breach [8].

The context of fertility preservation, however, raises challenges in terms of proving both duty and breach. The introduction of third parties into the equation distances the fertility industry from the paradigmatic doctor-patient relationship. Physicians are generally held to a higher standard of care than nonprofessionals (i.e., gamete banks). The highly regulated nature of medicine produces a high standard of care. In contrast, gamete banks and other third parties involved in ARTs face far less regulation. As a result, these entities are not beholden to the same standard of care. This creates an odd asymmetry: "sperm banks are operating in the reproductive health care realm by providing services for ART procedures, but they are not being held to the same standards as the physicians they work alongside" [38]. As the literature notes, "without similar laws and regulations governing sperm banks, it is difficult to determine what, *if any*, duty or standard of care sperm banks must provide in their relationship with patients or with donors" [38].

Thus, even if courts find that the third party in question owed a duty of care in the given circumstance, proving breach is yet another hurdle plaintiffs must overcome. Courts assess breach in

relation to the relevant industry. Even in the most egregious cases, proving a gamete bank "breached its duty during [the] provider screening, selection, and matching processes [may still be difficult] because the sperm bank's standard of care when conducting these processes will likely be that which is considered reasonable within the industry" [38]. As discussed in section "Insurance Entities as Regulators in a World of Limited Oversight", the piecemeal regulatory environment of ARTs places few requirements on gamete banks. Going beyond the floor set by legislation and guidelines promulgated by professional organizations remains voluntary, meaning what is considered "reasonable" within the industry remains a fairly low standard. As such, proving breach—even in relation to blatant misrepresentations or oversights—may still prove difficult.

Causation

Once patients prove duty and breach, they must establish the third element of medical malpractice: causation. Causation entails two separate showings. A patient must prove that a provider's breach of their duty of care was both (1) the "cause in fact" and (2) the "proximate cause" of their harm. "Cause in fact" simply means that, but for the provider's breach, the patient would not have suffered the harm. "Proximate cause" requires a bit more nuance; at its core, it means showing that the breach directly caused the harm in question, rather than some other intervening event or unforeseeable circumstance. Just as in science, definitively proving causation in the courtroom is a challenge. A patient might develop an unforeseeable complication despite their provider adhering to the appropriate standard of care, or an intervening event might attenuate the provider's liability.

The fertility preservation context is no different. For example, if a lab has suboptimal thawing processes, it is difficult to confirm that step of the process was the cause of the failed fertilization or implantation attempt. These challenges make proving that a provider's breach *caused* the adverse outcome difficult—especially by the "more likely than not" standard required in civil litigation.

Damages

In addition to the difficulties of proving duty, breach, and causation, identifying a cognizable harm and calculating monetary damages also prove challenging for courts and litigants alike. When harms typically occur in the fertility space, they often fall into one of four categories:

1. Implantation of the wrong embryo in a patient
2. Mix ups in the sperm or eggs used to create an embryo
3. Errors in preimplantation genetic testing
4. Damage or destruction of reproductive material in the laboratory or at a cryopreservation facility [39]

When the harm in question deprives an individual of a successful pregnancy or the chance at biological parenthood, courts make their best guess at the sum that would make the patient "whole again." The legal fiction that a monetary award can in fact make someone "whole" underpins civil litigation in the United States. Courts have various methods to calculate damage awards, but reproductive harms are understandably difficult to quantify [39].

Patients seeking recourse for pregnancies that *did* result in children face an additional problem: asserting that a mistake or misrepresentation made by a clinic caused them harm often gets uncomfortably close to a "wrongful life" claim. Courts "generally reject [these claims] as abhorrent" and against public policy, as they "validate pejoration of genetically challenged or interracial children" [40]. These concerns invoke a form of the nonidentity problem, where "as long as the [mistake or misrepresentation] does not produce a child whose life 'is not worth living' we can't say the child has been harmed" [41].

Zelt v. Xytex proves illustrative here. In *Xytex*, a family filed suit claiming that their sperm bank misrepresented their anonymous provider's qualifications. The Zelts selected Donor #9623, described as a PhD candidate with an IQ of 160 and "nearly perfect" medical and mental health history. Yet an administrative mistake at the sperm bank revealed the truth. Instead of a clean bill of health, Donor #9623 had a significant

mental health history, including heritable disorders. He was not a PhD candidate but rather a convicted felon [42]. The Zelts argued that Xytex mislead them. The district court, however, granted a motion to dismiss. The court noted the state of Georgia does not recognize wrongful birth claims, as they are not "legally cognizable injur[ies]" due to public policy reasons [34]. While wrongful birth was not among the 13 state law claims made by the plaintiffs, both the district court and ultimately the 11th Circuit found that the Zelt's argument amounted to one. In this sense, the courts confer on clinics "a misguided immunity under the guise of barring suits for wrongful life" [40].

Professor Dov Fox suggests that for ART-related claims to succeed, courts must establish a new private cause of action [8]. Professor Fox notes that courts have long taken advantage of "tort law's ability to accommodate new technologies by filling the regulatory gap and warning of neglected risks when technological innovation transfers the nature of injuries" [8]. Intentional infliction of emotional distress emerged as a result of mass transportation [8]. Strict product liability came about to deal with defects from innovative goods that harmed their consumers [8]. Privacy torts developed in response to increased technological capacities to surveil [8]. Professor Fox's new private cause of action would address the most common forms of ART harms: the imposition of unwanted pregnancy, the deprivation of wanted pregnancy, or the confounding of efforts to avoid a child born with particular conditions. Perhaps with this tool in their arsenal, plaintiff's suing for reproductive negligence would see greater success in the courtroom.

Regulation of Cryopreservation

Once patients consent to care and undergo the necessary procedure(s), the "preservation" component of fertility preservation brings with it its own unique set of legal issues. When patient tissue reaches cryopreservation, a new regulatory paradigm theoretically steps in to protect patient interests: regulation of cryopreservation. However, the General Assembly of New Jersey said it best: "while technological advances in, and success rates of, IVF have increased since its inception 40 years ago, there is currently little state or federal regulation concerning the storage of embryos in embryo storage facilities" [43]. Limited state and federal oversight allows manufacturers to escape regulation and facilities to function without proper safeguards in place to prevent damage to stored reproductive material.

Storage of Reproductive Material

Federal Regulation

As discussed, the FDA does not regulate fertility preservation *procedures*—instead, the agency regulates the *biologics*, *drugs*, and *devices* used in assisted reproduction. Cryostorage tanks and other equipment used in cryopreservation would seem to fall squarely within the FDA's purview. In fact, Title 21 specifically includes "cryopreservation instrumentation and devices, used to contain, freeze, and maintain gametes and/or embryos at an appropriate freezing temperature" as "assisted reproduction accessories" within the scope of FDA regulation. Yet, a significant loophole exists—one manufactures predictably exploit. The FDA only regulates "when these devices are specifically labeled for use in ART procedures" [44]. If a manufacturer avoids labeling their product as a medical device, the FDA's enabling statutes prevent the agency from asserting jurisdiction. As a result, manufacturers of equipment used in cryopreservation (e.g., cryostorage tanks, dewars, thermostats, etc.) bypass FDA oversight by not labeling their products for ART use.

Equipment malfunctions over the last few years highlight the costs of these regulatory loopholes. One weekend in March 2018 brought with it two unrelated freezer malfunctions [44]. Pacific Fertility Center in San Francisco and University Hospitals Fertility Center near Cleveland both experienced problems with their cryopreservation systems. These problems resulted in the loss of thousands of embryos and eggs [44]. Several

clinic patients filed lawsuits, and at least 150 settled out of court [45]. While the aim of making patients "whole again" underpins settlements and damage awards, in many cases, money cannot make up for the harm caused. For several patients with embryos lost in the California and Ohio incidents, those embryos represented their only chance at biological children—a chance money cannot bring back [45]. This reality underscores the need for more robust regulation to prevent similar incidents from happening again.

State Regulation

The 2018 freezer malfunctions prompted several states to consider legislation regulating embryo storage facilities. Following the incidents, Ohio State Senator Joe Schiavoni consulted with ASRM and the College of American Pathologists ("CAP") to produce detailed operational rules for fertility clinics [46]. These rules included 24-h monitoring requirements, separating a patient's materials into multiple storage tanks to prevent complete loss, and increased liquid nitrogen training for staff [47]. However, the bill failed to get a vote before the end of the legislative session, and Ohio has taken no steps toward regulation since [48].

New Jersey, on the other hand, successfully enacted legislation regulating embryo storage facilities and instituting licensure requirements. The state assembly noted that "it [was] in the best interest of the State to require that the Department of Health promulgate regulations governing the storage of human eggs, pre-embryos, and embryos in embryo storage facilities to guard against catastrophic storage system failures, such as those that occurred in California and Ohio" [43]. The act, which came into effect December 4, 2019, imposes operating standards on the 19 facilities within the state that store reproductive material. These standards include the use of monitoring devices and alarm systems, as well as yearly facility inspections. The act also requires the state's Department of Health to implement a facility licensing system [43].

While New Jersey's act represents a notable step forward in the oversight of embryo storage facilities, the sector remains largely underregulated across the board. Most states fail to give regulation of storage facilities any attention. This nonexistent state regulation, coupled with lax federal oversight, leaves patients vulnerable despite the preventable nature of cryopreservation malfunctions.

Disposition of Reproductive Material

Thus far, this chapter addressed how the legal world makes sense of fertility preservation before a patient seeks services, as they begin the doctor-patient relationship, and during cryopreservation. This, of course, leaves the behemoth of legal issues imbedded in the question of "what happens once a patient finishes treatment?" Patients typically choose one of three disposition options for unused reproductive material: donate unused embryos for procreation (also known as "adoption"), donate for research, or discard the unused material [3]. Once seen as a fourth option, patients may also elect to store their material indefinitely; however, clinics now encourage patients to formalize their disposition decisions rather than delay the inevitable. Legal issues arise when unforeseen circumstances intervene in a patient's disposition decision (i.e., death or divorce) or interested parties disagree down the line. Death, divorce, or disagreement brings questions of what to do with unused reproductive material into the courtroom. Questions about the enforceability of disposition agreements and how to categorize embryos present challenges for successful litigation—challenges courts address through highly variable approaches.

Options for Disposition of Reproductive Materials

Electing to proceed with fertility preservation requires parties make long-term decisions about the fate of unused reproductive material. Most providers offer several disposition options in event of death, separation or divorce, successful completion of IVF treatment, a decision to discontinue IVF

treatment, or failure to pay cryopreservation storage fees [49]. Many clinics use model forms supplied by ASRM and the Society for Assisted Reproductive Technology ("SART"). One such clinic, West Coast Women's Reproductive Center ("WCWRC"), offers the following options to patients:

1. Discarding the cryopreserved embryo(s)
2. Donating the cryopreserved embryo(s) for approved research studies
3. Donating the cryopreserved embryo(s) to another couple in order to attempt pregnancy
4. Use by one partner with the contemporaneous permission of the other for that use [49]

Consistent with California legislation and SART guidance, WCWRC caps storage of cryopreserved embryos at 20 years [49]. While indefinite storage might appeal to patients unsure about family planning, model disposition agreements no longer include indefinite storage as an option. Indefinite storage not only carries a notable cost burden, it "simply put[s] off clear disposition decisions for another day; as people's memories fade, intentions are no longer clearly recalled and more difficult to prove and establish, or former patients cannot be located, so that those decisions often become more challenging as time goes on" [3]. Most states do not institute term limits for cryopreservation storage; though some international regulatory bodies like the United Kingdom's Human Fertilisation and Embryology Authority ("HFEA") follow term limits, after which, clinics must discard unused material [50].

Disposition decisions are not necessarily final; patients may change their disposition decisions with mutual consent. Issues arise when couples disagree on proposed changes or where written disposition agreements do not exist. Mix these disputes with a constantly shifting legal landscape, and the complexities of the legal aspects of reproductive material disposition come as no surprise.

What Happens When Death, Divorce, or Disagreement Intervenes?

When disagreement or unexpected events disrupt disposition decisions, parties often turn to the legal system. With this reality in mind, WCWRC's disposition declarations—and similar agreements at other clinics—include the following warning:

> The law regarding embryo cryopreservation, subsequent thaw and use, and parent-child status of any resulting child(ren) is, or may be, unsettled in the state in which either the patient, spouse, partner, or any donor currently or in the future lives, or the state in which the ART Program is located. [49]

This caution highlights the complexity of litigation in this sector: various legal entities address disposition disputes differently. Limited federal precedent exists in this arena, as the Supreme Court typically leaves issues of family law that do not concern constitutional rights to the states. While courts take varying approaches to adjudicating disposition agreements, Debele and Crockin suggest three models of dispute resolution dominate common law approaches: contractual approaches, contemporaneous mutual consent models, and balancing tests [3]. These approaches are not mutually exclusive; in fact, courts often mix and match. But exploring these models demonstrates the diversity found in courtrooms across the country, even when court's address the same question.

Contractual Model

Perhaps the most straightforward, the contractual approach uses traditional principles of contract law to evaluate disposition disputes. Under this view, courts consider disposition agreements created prior to fertility preservation valid contracts and generally enforce their terms. More specifically, courts often ask the following questions: "(i) did the parties enter into a disposition contract? (ii) is the contract adequate? (iii) should the contract be enforced as a matter of public policy?" [3]. The contractual approach appears relatively common among courts. A survey of notable cases across the country involving embryo disposition between 1992 and 2016 found that in 6 of the 11 cases analyzed, courts utilized a contractual approach [51].

However, despite the relatively straightforward nature of the inquiry, questions often remain. Are boilerplate disposition agreements provided by fertility clinics or storage facilities

enforceable or are more formal agreements akin to prenups required? What constitutes a violation of "public policy" and what societal values should these agreements reflect? The answer to these questions, like many things in the law, depends on the jurisdiction in question—a reality which creates challenges in terms of continuity and guiding practitioners.

Contemporaneous Mutual Consent Model

Similar to the contractual approach, the contemporaneous mutual consent model functions on the premise that the parties who produced the reproductive material retain ultimate decision-making authority over disposition. While each party has an equal say in disposition, the parties must contemporaneously agree to any action—i.e., whether to use, donate, or destroy their reproductive material [3]. By requiring contemporaneous consent, this approach protects parties from unwanted procreation. When disputes arise down the line, courts must determine whether parties achieved contemporaneous mutual consent when they made their disposition agreement [3]. To do this, courts consider whether the disposition agreement included sufficient safeguards to guarantee contemporaneous mutual consent. Yet, even then, enforcement decisions under this model often hinge on whether the agreements included language reserving each party's right to change their mind about disposition.

The contemporaneous mutual consent model is less common among courts than the contractual approach and is not without critics. Professors Glenn Cohen and Eli Adashi argue that, despite claiming to honor the views of both parties, the model "puts in place a veto rule that cannot be overridden: no use of embryos by either party, despite what was agreed to previously, if one party vetoes it now" [51]. Critics argue such a rule violates established principles of contract law, defeats the purpose of disposition agreements made prior to treatment, and essentially pushes final disposition decisions down the line—in opposition to ASRM and SART guidance.

Balancing Tests

The final approach commonly taken by courts rejects the contractual and contemporaneous mutual consent models in favor of weighing the interests of the parties involved. Courts occasionally develop balancing tests in response to cases without prior written disposition agreements or where the circumstances of the parties changed significantly. Certain jurisdictions consider these tests a last resort when no other evidence of an agreement between the parties exists [52]. Prominent cases in this line of thought include *Davis v. Davis* (a 1992 Tennessee Supreme Court decision) and *Szafranski v. Dunston* (before the First District Appellate Court of Illinois in 2015)—both concerning individuals facing iatrogenic infertility after cancer treatment. Both cases lacked formal disposition agreements. In the absence of formal agreements, both courts proceeded to weigh each party's interests in the use, preservation, donation, or destruction of the reproductive material. The court's balancing test in *Davis*, for example, found that the husband's right not to procreate outweighed his wife's interests, as she could start a family through additional IVF or adoption. Importantly, the court advised that in most cases, the party wishing to avoid procreation will prevail. The *Szafranski* court went the other way, awarding the female partner the embryos at issue. An oral agreement tipped the scales in her favor, as the agreement allegedly demonstrated the parties' intent to allow her to use the embryos even absent of her partner's consent [53].

While courts often either explicitly or implicitly invoke balancing tests, these tests arguably provide even less guidance to clinicians and patients than the former two approaches. Balancing tests center on fact-dependent inquiries catered to the parties in dispute. This individuality makes it difficult to extrapolate across cases, even within the same jurisdiction. Such haziness adds additional ambiguity for patients and providers seeking to understand the status of their disposition agreements.

To harmonize the various approaches taken by courts, Professors Cohen and Adashi argue

for a set of uniform codes for embryo disposition [51]. Notably, the code would separate the disposition agreement from the informed consent process. Some clinics combine informed consent for IVF or cryopreservation with the creation of a disposition agreement. Yet Cohen and Adashi point out such conflation undermines the purposes of both forms. Informed consent communicates medical information, while disposition agreements communicate legal information. In this vein, the authors suggest a separate legal form that makes clear to the parties the binding nature of their decision. Additionally, an ideal code would create a presumption that disposition agreements are binding, include a recognition that legal parenthood cannot be imposed on an objecting party, and contain a carve-out for unforeseeable loss of fertility. While by no means perfect, such a code might harmonize the legal landscape and offer some guidance to patients and providers.

State Legislation Addressing Disposition Disputes

To help guide patients and providers in this area, some states enacted legislation designed to clarify questions of disposition in the event of disputes. Both California and Florida require written embryo disposition directives, with the former also requiring the parties to set forth time limits for storage. In cases where no prior agreement exists, Florida vests decision-making authority jointly with the parties that created the material. Louisiana and Arizona stand at the other end of the spectrum. In Louisiana, if a patient surrenders their right to use their reproductive material, the embryos "shall be available for 'adoptive implantation' in accordance with the written procedures of the facility where it is housed or stored" [54]. Concerns about the rights of the "embryo" itself underpin such legislation [3]. Arizona similarly prioritizes concerns about the status of embryos. In July 2018, the state legislature instituted a law that grants "custody" of reproductive material to the party that intends to "develop [the embryos] to birth" if a dispute arises. Like the variability in how courts approach dispute resolution in this area, legislative divergences stem from differences in the legal status granted to reproductive material and the rights (if any) afforded to embryos.

Are Embryos "Property" or "People" Under the Law?

Differences in the legal status granted to embryos explain the high variability in approaches taken by courts and state legislatures. Whether decision-makers see embryos as property, persons, or something in between shifts their calculus. While courts often err on the property side, the so-called personhood movement continues to gain traction in state legislatures. At least 11 states introduced "personhood bills" over the past few years to grant embryo's personhood status in the eyes of the law [55]. Regardless of the approach, the legal status of embryos affects the rights afforded to them and their progenitors and impacts the practices of fertility preservation as a result.

The (Quasi-)Property Approach

Over the past 20 years, as courts developed jurisprudence in response to fertility preservation, a fairly common conceptualization of embryos emerged: one viewing embryos akin to property. As disputes often made their way into the courtroom via divorce or disposition disagreements, viewing embryos in the light of property allowed courts to use familiar methods of dispute resolution [3]. Some states go so far as to codify the property approach. Michigan's legislature specifically classifies embryos as "property" to allow researchers to derive new lines of embryonic stem cells from embryos donated for medical research [3]. Florida takes a similar approach; through statute, the state grants progenitors a "property interest" in their embryos [3].

While some courts make little distinction between embryos and traditional property, others consider embryos sui generis—or a special kind of property deserving particular attention. In a 2017 case, for example, the Missouri Court of Appeals held that the cryopreserved embryos in dispute constituted a unique form of joint or marital property that could not be split down the mid-

dle. The lower court granted each party equal "shares" in the embryos at issue, and the court of appeals affirmed [51, 56]. In its decision, the court expressly rejected arguments that embryos were "children" under a Missouri statute that defines life as beginning at conception [56]. Though the Missouri court rejected this argument, some state legislatures disagree.

The Personhood Approach

Louisiana and Arizona both categorize embryos as persons, and at least 11 states have pending legislation to that effect. In an effort to grant greater protections to cryopreserved embryos, Louisiana expressly rejects a property-based conceptualization and defines a human embryo as a "biological human being which is not the property of the physician who acts as an agent of fertilization or the facility which employs him or the donors of the sperm or ovum" [54]. In terms of disposition disagreements, the best interests of the embryo and its right to life govern [3]. As discussed above, if genetic parents choose not to implant their embryos, then the embryos become available for "adoptive implantation." Arizona follows a similar approach; 2018 legislation grants custody to the party most likely to "develop [the embryos] to birth" in instances of disposition disputes [57].

At least eight states will consider similar "personhood bills" in the 2021 legislative session. On March 18, 2021, the Montana House advanced a measure to change the state's definition of "persons" to the following: "all members of mankind at any stage of development, beginning at the stage of fertilization or conception, regardless of age, health, level of functioning or condition of dependency" [58]. At the hearing, opponents noted that such a definition impacts not only abortion access but the availability of fertility preservation and ARTs. Such arguments fell on deaf ears, as Rep. Sharon Greef responded: "in America, we have a holocaust happening in every state because we are denying that personhood begins at conception" [59]. That said, similar bills advance in the Montana House almost every year, and all have failed to reach the state's voters.

The effects of "personhood legislation" produce real challenges for fertility preservation. As advocacy group, RESOLVE, argues, "at a minimum, [such legislation] would force changes in the practice of reproductive medicine (e.g., limitations on the number of eggs that may be fertilized) that are not in patients' best interests and constitute inferior medical practice" [60]. "Personhood bills" threaten patient care in a variety of ways. In addition to limiting the number of embryos a patient can create, such bills may limit practices such as preimplantation genetic testing or prevent medical research using embryos.

To prevent these harms, advocacy groups and professional organizations argue in support of quasi-property understanding of the legal status of embryos. ASRM notes that "embryos should be afforded 'profound respect' but not the same moral and legal rights that are afforded human beings" [3]. Interim or sui generis status accomplishes this goal by recognizing the moral status of embryos, while preventing the "rights" of the embryo from trumping the rights of the other parties involved. While such sui generis status might circumvent the blatant attack on fertility preservation imbedded in personhood approaches, the feasibility of such an approach remains far from clear. Until a uniform code harmonizes various state approaches or the Supreme Court weighs in on the issue, disjointed jurisprudence regarding the legal status of embryos is inevitable—a reality practitioners and patients alike should be aware of when considering questions of disposition.

Conclusion

Unlike most developed countries—and unlike the robust training, certification, and licensing requirements of other specialties domestically—regulators in the United States have yet to adequately address fertility preservation [61]. Most experts advocate for increased regulation, with some referring to the "United States [as] the Wild West of the fertility industry" [62]. ASRM stands largely alone in asserting that "ART is already one of [the] most highly regulated of all medical

practices in the United States"—a claim not born out when one considers the significant gaps in regulatory oversight [62]. Insurance entities, professional organizations, and state medical boards attempt to fill these gaps. But the lack of cohesive regulation nevertheless creates a host of problems, including those related to insurance coverage, informed consent, cryopreservation, and disposition of reproductive material. This chapter surveyed the legal aspects of fertility preservation in relation to each of these stages but in no way claims to be dispositive. Rather, this chapter highlights some of the most glaring legal and regulatory issues pertinent to the practice of fertility preservation—offering clinicians a lay of the land and demonstrating that patients cannot just "bank" on legal infrastructure to protect their interests in the world of ARTs.

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