Assisted Reproductive Technology and Gamete/Embryo-Fetal Origins of Diseases

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Abstract

Suboptimal intrauterine conditions may produce small for gestational age (SGA), and low birth weight (LBW) babies, that are predisposed to develop cardiovascular and metabolic disease in later life [1]. During assisted reproductive technology (ART) treatment, gametes and zygotes are exposed to a series of non-physiological processes and culture media with increasing evidence that offspring of ART have increasing chances of being low birth weight. An increasing incidence of early-onset hypertension has also been reported in the population of ART offspring. It is therefore important to understand whether ART plays any specific role in disadvantaging a fetus, and, whether any such disadvantage carries long-term consequences.

Methylation of imprinted genes is erased and reestablished during gametogenesis, and, maintained throughout pre- and post-implantation development. Sequence-specific DNA hypomethylation frequently occurs in human sperm in compromised spermatogenesis. Transmission of sperm and oocyte DNA methylation defects occurs, though may be prevented by selection of gametes for ART, or, non-viability of the resulting embryos. In vitro fertilization (IVF), oocyte in vitro maturation (IVM), intracytoplasmic sperm injection (ICSI) and preimplantation genetic diagnosis (PGD) manipulate gametes and embryo at a time that is important for methylation reprogramming, and, may influence epigenetic stability leading to increased risks of adult diseases. Aspects of subfertility may also pose a risk factor for imprinting diseases. In this chapter, we will discuss the evidence related to assisted reproductive technology and embryo-fetal origins of diseases.

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10.1   In Vitro Fertilization

Worldwide, it is estimated that more than five million babies have been born after ART. Children conceived by ART comprise 1–4 % of the newborn population [2]. Although the majority of children born after ART are healthy, some concerns remain regarding the safety of this technology. Multiple pregnancies occur more commonly in ART pregnancies, and, their high frequency increases the risk of low birthweight and preterm delivery [3–5]. Several studies indicate that ART may disrupt epigenetic programming [1]. Animal studies and long-term follow-up studies of ART children suggest there may be an increased incidence of genetic, physical, or developmental abnormalities [4, 5], although there are also observations that contradict these findings [6].

10.1.1   Perinatal Outcome of Assisted Reproduction

Multiple births continue to be the major risk for couples embarking on fertility treatment. Their frequency in different countries ranges from 25 % to nearly 50 %, with the rate of high order pregnancies as high as 40 % (compared with only 3 % of infants among the general birth population) [7]. Transferring two embryos is associated with a threefold increase in the birth rate, and, a 16-fold increase in the twin birth rate [8]. Neonatal complications include low birth weight, preterm delivery, placental dysfunction and congenital malformations [9]. In the general population, the risk of preterm delivery is fivefold in twins compared to singletons, and 50 % of twins are born at low birth weights (<2,500 g) compared to 6 % of singletons [10]. The perinatal mortality is 5–6 times higher among twins compared to singletons [9].

In recent years there has been a developing trend to limit the number of embryos transferred into the uterine cavity per cycle of IVF/ICSI [10]. Single embryo transfer developed remarkably, with the proportion increasing from 10.5 to 12.4 %, and now it is highest in Finland (38.5 %), Sweden (30.5 %) and Australia (25.0 %). Since 2000 the proportions of twin and triplet pregnancies decreased from 26.5 to 25.7 % and from 2.9 to 2.5 % respectively [11]. However, reducing multiple pregnancies will not totally eliminate the increased risk of adverse perinatal outcomes in ART pregnancies. Singleton, ART conceptions appear to be at greater risk than unassisted, singleton conceptions for a number of adverse outcomes. Meta-analyses show that singleton children born after ART (IVF, ICSI, or gamete intra fallopian transfer) have a twofold risk of being born preterm, are at increased risk of having a low or very low birthweight (OR 1.70–1.77 and 2.70–3.00, respectively), and, are most usually born small for gestational age (OR 1.40–1.60) [7]. In addition, risks of preeclampsia, placental abruption, and placenta praevia also appear to increase after ART techniques [12, 13]. Despite a number of evidences indicate increased risks of perinatal complications following ART, its causes are still of no consensus. It is not clear whether ART is culpable, or, whether the adverse outcomes are attributable to factors related to the infertile couple.

The biology of subfertile couples may pose health risks to ART children. Singleton pregnancies in infertile couples after ovarian stimulation with, or without, artificial insemination, have a higher risk of low birth weight when compared with naturally conceived offspring [14]. However, after controlling for the origin of the semen, any differences between IUI and natural conception disappeared [15]. The time to-pregnancy interval has a direct link with the risk of preterm birth, and low birthweight. If the time-to-pregnancy interval was longer than 12 months but no fertility treatment had been used, the risk of low birthweight and preterm delivery increased [7]. Romundstad et al. compared the outcomes of two, consecutive singleton pregnancies, including one child conceived through ART and the other conceived spontaneously. In the sibling-relationship comparisons, there are no significant differences in birthweight, risks of small for gestational age babies, gestational age, and preterm delivery among infants conceived both spontaneously and after ART [16]. These results suggest that adverse outcomes after ART compared to those in the general population, may be attributable to the factors leading to infertility, rather than to factors related to the reproductive technology.

However, studies among subfertile women also show that adverse perinatal risks after IVF cannot be explained completely by subfertility; preterm delivery and lower mean birthweight were found among children born from subfertile women conceived after IVF as compared with children born from subfertile women after spontaneous conception [5]. Imprinted genes are major contributors to fetal growth, maternally expressed imprinted genes act as growth suppressors, whereas paternally expressed imprinted genes are growth promoters [4]. Lower birth weight may reflect increased suppression of paternally expressed (maternally imprinted) genes or conversely, overexpression of maternally expressed genes. According to the Barker hypothesis, babies born preterm and low birthweight after ART may be at risk of cardiovascular disease and diabetes in later life [5]. Given the increased risk of low birthweight in ART babies, specific investigation of the long-term consequences of IVF will be necessary.

10.1.2   Neurological Sequelae

Large, registry-based cohort studies report increased risks of cerebral palsy (CP, OR 1.6–3.7) in IVF children compared to naturally conceived children. The increased risk is largely due to the higher frequency of multiple pregnancies, low birth weight, and preterm delivery in IVF children [17]. The incidence of CP increases in multiple births: twins had risks approximately five times higher and triplets 17 times higher than singletons [9]. Many studies have investigated the neurodevelopmental wellbeing of singleton children born after ART. The reports indicate consistently that there are no neurodevelopmental differences between term singleton children conceived by ART and their naturally-conceived peers. A large European study in five countries assessed neurological outcomes of singleton children age 5 years, born after ICSI, IVF alone, or natural conception, with about 500 infants in each group; the results were reassuring [7]. However, Lidegaard et al., compared 442,349 singleton infants

conceived spontaneously with 6,052 children born after IVF and noted an 80 % increased risk of cerebral palsy in the IVF group [18]. A population-based retrospective cohort study in Sweden showed that children born after IVF singletons had increased risks of neurological sequelae risk (OR 2.8) [19].

The surviving co-twin may account for the increased risk of cerebral palsy in singleton births after IVF – singletons born from IVF originating from twin pregnancies [14]. IVF/ICSI is related to an increased risk of cerebral palsy through the association with preterm birth and plurality [20]. Relatively little evidence is available concerning the outcome of these children when they are older, thus larger, long-term, follow-up studies will be necessary to assess the impact of ART on children through puberty into adult life.

10.1.3   Cancer

Several case control studies suggested a possibly increased incidence of embryonal tumors in children who were prenatally exposed to sex hormones. In 1987, Kramer et al. reported that the risk of neuroblastoma increased following exposure to fertility drugs [21]. Michalek et al. noted that children with neuroblastoma were ten times more likely to have mothers with infertility treatment [22]. In addition, a report from the Netherlands identified a fivefold increased risk for retinoblastoma among children born after IVF [23]. However, a number of published cohort studies do not show increased risk of childhood cancer in ART children when compared with the general population. In an Australian cohort study, no significantly-increased risk of cancer occurred in an IVF group (N=5,249) as compared to the general population [24]. Klip et al. found no increase in childhood cancer after 6 years when an IVF group was compared to the general population, and, spontaneously conceived children born from subfertile parents [22]. In Denmark, Brinton et al. found no significant cancer risk in the children born after ovulation-stimulation after 10.1 years follow-up [14].

Although these cohort studies appear reassuring, there is a continuing need to monitor IVF children to establish the true prevalence of post-IVF childhood cancer, preferably in registry-based studies.

10.1.4   Congenital Malformations

Between 3 and 5 % of all infants are diagnosed with a congenital anomaly soon after birth [25]. A number of meta-analyses indicate a 30 % increased risk of major malformations after IVF or ICSI compared with spontaneous conception, and, there is no difference between the two techniques [2, 7, 10, 26]. The major defects included urogenital cardiovascular, musculoskeletal, and chromosomal anomalies. The increased risk of major malformations may, at least partly, be explained by the higher proportion of multiple births in the IVF group [9]. Mothers of ART children were older and less parous than control mothers [27]. Increasing evidence suggests that parental subfertility may be an important factor, as increasing 'time to pregnancy'

was associated with a greater risk of congenital abnormalities [2, 25]. After adjustment for parental factors, there was no significant association between increased risk of birth defects and IVF. However, the risk of birth defects associated with ICSI remains increased after multivariate adjustment [28].

10.1.5 Epigenetic Mechanisms in ART

Genomic imprinting occurs during gametogenesis and embryogenesis, and, it is possible that techniques used in ART could cause aberrant genomic imprinting and thus imprinting disorders [29, 30]. Since 2002, there is growing evidence that ART procedures may perturb the important epigenetic processes during the preimplantation period and lead to imprinting syndromes [5]. The most extensively studied imprinting disorders related to ART are Beckwith-Weidemann syndrome (BWS). BWS is a congenital overgrowth syndrome characterized by macroglossia, visceromegaly, macrosomia, umbilical and abdominal wall abnormalities including exomphalos, neonatal hypoglycemia and an increased risk of developing embryonal tumors in childhood [31]. BWS has an estimated incidence of 1 in 13,700 live births in the general population [32], a 6–9 times increase among ART offspring as compared with naturally conceived children [33]. While this would represent a dramatic increase in relative risk, the actual incidence of BWS after ART is 1 in 4,000–5,500 [33]. Methylation errors account for 50–60 % of sporadic cases of BWS, and almost 100 % of cases born after ART [31]. These findings are not confirmed by Lidegaard et al. [18], who assessed the incidence rate of imprinting diseases and related disorders in IVF singletons and spontaneously conceived children born in Denmark between1995 and 2001.

Another classic imprinting defect is Angelman's syndrome (AS). AS is a neurological disease affecting 1 in 15,000 live births that is associated with mental retardation, unusual gait, and seizures. AS is caused by loss of maternal UBE3A [22]. However, fewer than 5 % of AS cases are associated with an imprinting defect [29]. The numbers associated with ART are too low to characterize the effects of ART on AS. The first cases of ART-related Angelman syndrome were reported in 2002 and 2003 [19, 20]. Three patients conceived by intracytoplasmic sperm injection were diagnosed with AS, and, found to have aberrant loss of methylation on chromosome xy [2]. The reason for the increased risk of imprinting disorders in ART children is unknown.

Imprints of oocytes are established during the primordial to antral follicle transition and are not completed until just prior to ovulation [34]. For this reason, gonadotrophic hormones used during superovulation may affect imprint acquisition in oocytes. Ovarian stimulation is associated with an increased risk of aneuploidy in artificially matured oocytes [2] and may alter genomic imprinting of both maternally and paternally-expressed genes [35, 36]. Moreover, superovulation can transgenerationally affect the offspring sperm methylation pattern [37].

Preimplantation represents another developmental stage in which changes in DNA methylation take place. As early as 4 h after fertilization, marked demethylation occurs as the developing embryo initiates epigenetic changes, which will lead to

delineation of cell specificity [38]. Animal models indicate that media and other environmental components may cause epigenetic modifications. In-vitro media alterations during the preimplantation period may result in a particular overgrowth syndrome, known as large offspring syndrome (LOS). LOS has substantial phenotypical similarities with Beckwith-Wiedeman syndrome. LOS in sheep is associated with reduced expression of Igf2r through loss of methylation [38, 39]. In addition, alterations in the culture media of in vitro-conceived embryos may also alter methylation of both H19 and Igf2 [38].

10.2   Oocyte in Vitro Maturation (IVM)

10.2.1   Application of IVM

IVM is a technique where immature oocytes are collected from mid-sized, antral follicles, usually from unstimulated ovaries, and cultured, matured and fertilized in vitro to produce embryos [40]. IVM of oocytes was first demonstrated in animals by Pincus and Enzmann in 1934, and later, in humans by Edwards in 1969. The first birth after IVM of immature oocytes occurred from oocytes collected during gynaecological surgery for oocyte donation in 1991. The first reported IVM with patient's own oocytes was used as a treatment for women with polycystic ovaries or polycystic ovarian syndrome (PCOS) in 1994 [41]. Over the past 30 years there has been 1,300 IVM babies [42].

Advantages of IVM include relative ease of treatment, minimal use of fertility drugs, avoidance of ovarian hyperstimulation syndrome, and, low cost. However, various challenges hinder the selection of oocyte IVM as an established means of assisted reproduction. Although good results are reported in some clinics, IVM has not become a mainstream fertility treatment yet. The most important reasons are:

1. *Technical difficulties in retrieving immature oocytes from unstimulated ovaries and their cultivation.* Antral follicles between 2 and 12 mm are usually aspirated though studies show that follicles greater than 6 mm have a greater capacity for yielding immature oocytes that mature in vitro. In unstimulated ovaries, the follicles are small and often widespread throughout the ovarian stroma. Within large antral follicles, the cumulus oocyte complex (COC) is bathed in follicular fluid (FF) until the time of ovulation. FF itself is a serum transudate modified by the thecal and granulosa cell layers that make up the follicle [43]. FF contains undefined factors as well as known proteins, growth factors, steroids, and metabolites, many of which are present in blood plasma [43]. Not surprisingly, the addition of additives such as serum and FSH to maturation media usually results in improvements in maturation and embryo development [44–47]. However, the use of serum in culture systems may be undesirable because of the unknown nature of its contents and the potential for variability [43]. IVM of oocytes is limited by the currently used culture systems, including the doses and duration of hormones and other factors added to the culture media to initiate and coordinate the events of oocyte maturation.

2. *Lower chances of live birth per treatment compared with conventional in vitro fertilization.* In human the maturation rate of IVM is 30–50 %, which is much lower than other species, and, the pregnancies resulting from IVM are limited [48], and, in vitro matured human oocytes have reduced developmental competence. Recent reports document better fertility outcomes after IVM, with live birth rates of 30 % in donor oocyte programmes and clinical pregnancy rates of 21.9 % in patients with PCOS, for whom IVM may be a pivotal treatment strategy [49, 50].

3. *The quality of in-vitro-matured oocytes is suboptimal since immature human oocytes display increased meiotic spindle and chromosome abnormalities.* This observation may account for lower developmental competence of oocytes matured in vitro compared to those matured in vivo. Whether these abnormalities are due to damage induced during the procedure or whether attributable to factors intrinsic to the ovaries of PCOS patients are not known. Alterations in an oocyte's internal structure, in particular spindles and chromosomes, are important in the ability to be fertilized, develop into a normal embryo, and ultimately produce a healthy live birth. Spindles controlled the movement of chromosomes within the oocyte; specialized components of the oocyte infrastructure that are composed of microtubules. This internal infrastructure is a group of moving materials, scaffolding and structures within the oocyte. Li et al. [51] analyzed the appearance of spindles and chromosomes in IVM oocytes of PCOS patients by confocal microscopy and fluorescent immunocytologic staining and compared their results with in vivo matured oocytes of PCOS patients. It is reported that in IVM oocytes chromosome configurations and disorganized meiotic spindle microtubules were more likely to be abnormal. Another significant concern about clinical use of IVM is the potential for perturbations in subsequent fetal and neonatal development that may influence long-term health. Some concern has been expressed regarding the safety of the method, though, chromosomal abnormalities, obstetric and neonatal outcomes were similar for IVM and conventional ART populations [49, 51, 52]. Cha et al. reported obstetric outcomes after IVM in women with PCOS; they reported 38 pregnancies, of which three had congenital anomalies; one case of hydrops fetalis, one omphalocele, and one cleft palate, and, a spontaneous miscarriage rate of 36 % [49]. There are insufficient evidence is available for women with PCOS yet, so IVM can't be the preferred first line treatment for subfertile women. Little is known about long-term outcomes after IVM, as the few children derived from IVM oocytes are not yet of adult age.

10.2.2   Women Suitable for IVM

IVM is a therapeutic option for women with PCOS or PCO in whom there are significant risks of ovarian hyperstimulation syndrome (OHSS) [53]. Also, more women of reproductive age undergo treatment for malignant diseases that are potentially gonadotoxic, and, may wish to preserve their fertility before such treatment.

For survivors from pediatric cancer this is especially important. In vitro culture of primordial follicles contained in cryopreserved ovarian tissue represents another potential method for restoring female fertility [54].

10.2.3   Future Considerations

IVM, with its numerous benefits and pregnancy rates comparable to those in IVF in most centres, is a safe and effective treatment. Progress requires improved culture conditions to further increase implantation rates, and, predictors of embryo competence may help increase implantation rates. Detailed follow-up and assessment of children conceived through IVM will be necessary to establish the safety of this treatment.

10.3   Intracytoplasmic Sperm Injection (ICSI)

ICSI bypasses the normal fertilization process where a single sperm is injected directly into an egg. It involves the introduction of the entire sperm, including the acrosome and its digestive enzymes, directly into the oocyte. This injection of spermatozoa into the oocyte may potentially disrupt the oocyte's cytoskeleton [55]. Introduction of exogenous material is also possible, which may damage other intracellular structures [56]. Imprinting defects resulting from the ICSI technique may have transgenerational effects [57]. There are increasing concerns that manipulations during ICSI may lead to adult diseases in later life. There are many studies that weigh on both sides of these concerns.

Most of the studies giving reassuring data recommend more thorough investigations because the level of congenital defects is statically higher in babies born through ICSI, and, imprinting defects may be preponderant [58]. Evaluation of epigenetic marks and DNA methylation patterns in recent publications show no ill effects stemming from this method of fertilization [59]. Fulka et al. examined differences. in DNA methylation patterns in mouse zygotes produced either by natural fertilization, IVF, or ICSI by looking at global patterns of DNA methylation in paternal pronuclei after labeling with anti-5-methylcytosine (5-MeC) antibody, and, histone methylation patterns in both pronuclei after labeling with dimethylated H3/K9. No differences in global methylation patterns were found among in vivo, in vitro, and ICSI-produced zygotes in mice [59].

Santos et al. evaluated the potential deregulation of DNA methylation in ART-derived human embryos. They evaluated genome-wide DNA methylation together with chromatin organization in human embryos derived by either IVF or ICSI. DNA methylation was assessed using an antibody against 5-methyl-cytidine, and, chromatin organization by DNA staining. Irrespective of the ART procedure, They observed similar errors in both groups, and, there was positive correlation between abnormal chromatin and inappropriate DNA methylation. The ability to develop to the blastocyst stage accompanied with normal chromatin organization

and DNA methylation, reinforcing the pivotal role of epigenetic regulation forming the early lineages of blastocyst. They found blastocysts derived by ICSI and IVF were affected similarly [60].

Tierling et al. examined the effects of ART on the stability of DNA methylation imprints in phenotypically normal children (ICSI, IVF, and spontaneous conceptions) in ten differentially methylated regions (DMRs), including H19, KvDMR1, MEST, SNRPN, GRB10, GNAS NESP55, DLK1/MEG3 IG-DMR, GNAS XL-alpha-s, GNAS NESPas and GNAS Exon1A [61].

Children conceived through ART do not express a higher degree of imprint defect. Feng et al. investigated expression profiles of imprinted genes in IVF-conceived, ICSI-conceived and naturally-conceived children, by microarray and real-time RT-PCR [62]. Hierarchical clustering demonstrated no obvious clustering between the different groups, suggesting similar genomic imprinting expression between the groups. In the majority of the children conceived after ART and spontaneously, the differentially-expressed, imprinted genes remained allele-specific expression. Monoallelic expression of L3MBTL was disrupted in one ICSI case where all CpGs were completely unmethylated [62].

De Waal et al. demonstrated that ICSI can induce epimutations in somatic tissues of adult mice produced by this method. They compared the occurrence of epimutations in mice produced by natural conception, ICSI and somatic cell nuclear transfer. Surprisingly, they observed more epimutations in somatic tissues from ICSI-derived mice. They also observed a delay in reprogramming of the maternal allele of the imprinted H19 gene in spermatogonia from juvenile ICSI-derived male mice. These observations indicated that exposure to exogenous gonadotropins of maternal gametic genome during superovulation may disrupt the normal epigenetic programming of imprinted loci in somatic tissues and/or epigenetic reprogramming in the germ line of ensuing offspring. To prove this hypothesis, they uncoupled superovulation from ICSI through subjecting female mice to gonadotropin stimulation and then let them produce offspring by natural mating. It was found that mice conceived in this way also showed epimutations and/or epimutant phenotypes in somatic tissues and delayed epigenetic reprogramming in spermatogenic cells, providing evidence that gonadotropin stimulation contributes to the induction of epimutations during ART procedures rather than the ICSI manipulation [63].

Imprinting has also been proposed as the link between IVF/ICSI and a possible increased risk for retinoblastoma. No hypermethylation of the RB1 promoter was found in examination of retinoblastoma tumors in seven IVF or ICSI conceived children. This demonstrates that an association between IVF or ICSI and retinoblastoma through this epigenetic mechanism is unlikely [64].

However, plenty of evidence concerning the risks of ICSI indicate that children born following this procedure are at a higher absolute risk of congenital defects (in particular genitourinary defects) and epigenetic syndromes (such as Beckwith Wiedemann) [65]. Bowen et al. compared outcomes of ICSI-conceived, IVF-conceived, and naturally-conceived children at 12 months of age, and, observed a significantly lower mental development index (MDI) in the ICSI group [66]. Sutcliffe et al. compared 123 ICSI-conceived children with naturally-conceived

children at 12–24 months of age. The only difference was a lower eye–hand coordination in the ICSI group [67]. Bonduelle et al. comparing general health status of ICSI-conceived, IVF-conceived and naturally-conceived children at 5 years of age found that ICSI-conceived children presented with increased numbers of major congenital malformations, the most frequent being urogenital [68].

There are several, possible explanations of increased risks associated with ART procedures. Firstly, the use of ICSI suggests the possibility of imprinting defects in paternal sperm cells. ICSI and round spermatid injection (ROSI) may increase the risk of imprinting disorders and disrupt embryonic development through using immature spermatozoa improper imprints or global methylation [69]. Paternal genomic alterations can compromise fertilization rates and embryo viability that may result in increased rates of spontaneous miscarriage and birth defects [70]. In the ejaculate of infertile men the percentage of spermatozoa with damaged DNA increased compared to healthy fertile donors [71]. DNA fragmentation of morphologically normal sperm reduces embryo quality and probability of pregnancy in ICSI cycles [72]. Using spermatozoa with damaged DNA in ICSI suggests that genetic and epigenetic changes during pre-implantation may occur, leading to altered fetal development and, as a consequence, offspring with aberrant growth, behaviour, early aging and tumours [73]. Functional bovine sperm with damaged DNA can normally fertilize oocytes and no significant effects are observed during the first cleavages of the fertilized oocyte. However, embryo fragmentation, apoptosis and aberrant or no signs of mitotic spindle formation may also occur [74].

Sperm with both fragmented and unfragmented DNA can fertilize oocytes with equal efficiency [72]. However, the activation of the paternal genome on day 3 can show imperfections within the paternal genome that were not evident upon fertilization. More often than not, spermatozoa for ICSI originate in men with abnormal semen parameters. Sperm from these men may contain an increased number of chromosomal abnormalities, contributing to the overall risk of ICSI procedures [75, 76]. One such example stemming from recent investigation demonstrates that children conceived via ICSI procedures have significantly shorter fingers, possibly attributable to perturbations in the paternal genome [77].

Secondly, the injection of a spermatozoa into the oocyte can disrupt the oocyte's cytoskeleton and bring about epigenetic defects. The pattern of histone methylation differs between ART and ICSI in mouse [78] and even in human [79]. In rats, demethylation dynamics of the paternal genome at pronuclear-stage are impaired when ART is used [80]. Sperm chromatin remodeling after ICSI is more asynchronous than ART in mouse embryos [81].

Van der Heijden et al. stained monopronuclear zygotes (MPZ) derived from ICSI or IVF with H3K9me3 antibodies, which allows unambiguous identification of parental origin, and, found they originate through fusion of parental chromatin after sperm penetration. Monopronuclear zygotes derived from ICSI contain uni-parental chromatin, and, after both IVF and ICSI androgenic monopronuclear zygotes were found [78].

Aberrant methylation status lead to further embryonic abnormalities, eventually arrest in development, or embryo degenerate. Moreover, A high frequency of

aneuploidy and embryo death is induced by the inhibition of histone deacetylation during meiosis. Manipulative procedures, such as ICSI, may deteriorate the capability of the oocyte to conduct these epigenetic processes correctly, causing abnormal embryo development from nuclear–cytoplasmic interactions [82]. Qiao et al. collected tripronuclear and normally fertilized embryos acquired from patients undergoing ART. Poor morphological appearances of ICSI-derived embryos that were more likely to display H3K9 demethylation than their IVF counterparts [79].

Yoshizawa et al. studied the timing and extent of the active demethylation of paternal genomes among the pronuclear-stage rat zygotes produced in vitro, produced in vivo/cultured in vitro, and produced in vivo. They found that demethylation dynamics of the paternal genome in pronuclear-stage rat zygotes were impaired by routine protocols for in vitro embryo production such as IVF and ICSI [80].

Ajduk et al. compared remodeling of sperm chromatin, the potential of embryos to develop in vitro, synthesis of DNA in mouse embryos gained from IVF and ICSI, and, tested whether pretreatment of sperm prior to ICSI facilitates chromatin remodeling and deteriorates embryo development. The dynamics of sperm chromatin remodeling in ICSI and IVF embryos changed. In ICSI, chromatin remodeling was not as synchronous as in IVF. Sperm capacitation before injection enhanced remodeling asynchrony and delayed pronuclei formation and DNA synthesis [81].

Furthermore, the use of ICSI for achieving fertilization may introduce exogenous materials and create transgenic offspring. The term transgenic refers to any organism whose genome has been altered by the stable integration of a recombinant DNA sequence, regardless of the impact that this integration may have on phenotypic expression. Animal models have been used to introduce exogenous genetic material into the genome via a sperm carrier with the ICSI technique; this has resulted in the creation of transgenic mammals [83], as well as transgenic primates [84]. It is possible for a foreign gene, either DNA or RNA, from protein supplements in culture media to be introduced into the oocyte genome by binding to the sperm surface, or, by needle-carried medium during the ICSI procedure, resulting in the creation of transgenetic embryos. Although verification of transgenesis is lacking in human IVF, the possibility of this occurring inadvertently remains a viable concern, especially following confirmation of unwanted transgenesis in the mouse model [85]. Prostasomal DNA transfer into human sperm confirms this is indeed a cause for concern [86].

Anecdotal reports also suggest that animals conceived through ART that show apparent epigenetic defects do not pass these epimutations to following generations breeding naturally. De Waal et al. analyzed allele-specific DNA methylation and expression at three imprinted genes, H19, Snrpn, and Peg3, in somatic cells from adult mice generated through ICSI. The results confirm that ICSI can result in the formation of epimutations. While such epimutations may persist indefinitely in somatic cells of the ICSI-derived individuals, they are usually rectified in the germ line through epigenetic reprogramming and not transmitted to subsequent generations [87].

Ciapa et al. propose the probability that pathways such as those involving tyrosine kinases, G-proteins or integrins may be activated besides sperm factor

injection, and, there may be upstream mechanisms involved in later embryonic development. Although most reports are reassuring, some recent data suggest a greater incidence of abnormalities in children conceived by ART compared to those conceived normally. Because of the avoidance of membrane fusion and signalling events during ICSI, Spatio-temporal signals may be missing or abnormal. They discuss the hypothesis that potential perturbations during ICSI may have repercussions for epigenetic processes, inducing not only alterations of embryonic development, but also diseases in young children and, perhaps, in adults [88].

10.4   Preimplantation Genetic Diagnosis (PGD)

PGD is a form of prenatal diagnosis in early stage, in which embryos created in vitro are analyzed for a certain kind of genetic defects and only embryos free of the defects are transferred into the womb. The required disruption of cell-cell contacts for biopsy is reported to disrupt its role in embryonic growth [89]. Suboptimal techniques used for biopsy and fixation are believed to interfere with the clinical success of these techniques. Also, research has shown that biopsies of one or two cells from the whole can disrupt the 50 %/50 % ratio of the random X chromosome inactivation balance, an epigenetic function [90]. Disruption of the embryo at any of these preimplantation stages may have a negative impact on the subsequent development of the remaining cells in the embryo.

Logic tells the educated individual that PGD should make for healthier infants born from IVF in conjunction with fewer embryos needed to be transferred to achieve success in ART. However, studies from the human perspective have revealed a range of negative associations and lack of success when associating these techniques with live-birth delivery rates. The rate of live-birth delivery in women <36 years failed to increase following PGD and single-embryo transfer [91]. In fact, another report, this time using women of advanced maternal age, established that PGD not only failed to increase the ongoing pregnancy and live-delivery rates but found that it significantly decreased them [92]. Nevertheless, encouraging results could be found in a report assessing the mental development of 2-year-old children born following these types of biopsies; these children displayed no ill effects of any type of treatment when compared with children conceived naturally [89].

As with all other topics, PGD research applications have been extended to the mouse model. Very recent publication expressed that neither day 3 biopsy nor blastomere biopsy correlates with alterations in preimplantation development or global gene expression [93], although blastomere biopsy is significantly associated with premature and sometimes abnormal hatching due to infringement on the zona [93]. However, in a similar time frame, a report surfaced about the association of a blastomere biopsy with the increased potential for neurodegenerative disorders in the offspring [94]. Again, this research demonstrates the need for greater knowledge concerning this debatable topic before coming to a well-informed conclusion on the risks for adult diseases of its application.

10.5   Cryopreserved/Thawed Gametes and Embryos

Early life events (prenatal and early postnatal events) can initiate long term changes in individual's life, increasing the risk or producing some diseases that could be apparent in adulthood. It was described in Barker's hypothesis "Fetal origin of adult diseases", based on epidemiological studies from birth records in UK, comparing birth weight and physical characteristics at birth and subsequent health status later in the life of the same individuals. Suboptimal condition for fetal development, producing small for age and low birth weight babies, like maternal undernutrition, or disproportionately large babies at birth can change physiology and metabolism predisposing such organism to cardiovascular and metabolic disease later in the life [95].

The methylation of imprinted genes is erased and reestablished during gameto-genesis and is maintained throughout pre- and post-implantation development. ICSI and PGD manipulate gametes during the phase which is crucial for methylation reprogramming, may influence the epigenetic stability, and lead to the increased risks of adult diseases with fetal origin.

10.5.1   Effect of Cryopreservation on the Offspring of ART

Cryopreservation of embryos and zygotes is widely used in assisted reproduction technology (ART), and frozen embryo transfer has become an essential part of ART programmes. Although the studies on perinatal and postnatal outcomes of children born after frozen embryo transfer are limited, previous findings have confirmed the short-term safety of FET.

10.5.2   Different Methods Used to Cryopreservation

Slow cooling is the oldest method using for cryopreservation of embryos, and it is far-ranging used recently. During slow cooling, embryos are usually pre-equilibrated in either 1.5M dimethyl sulfoxide or 1.5M propylene glycol at room temperature. During the next steps, the concentration of cryoprotectants inside and outside the embryos will gradually increase. When the concentration is high enough to support glasslike solidification of the cells and the outside solution, at temperatures approaching −33 to −40 °C, the cells could then be rapidly exposed to much lower temperatures such as −150 °C or lower. To complete these, it costs more than 2 h [95]. Vitrification is a new method which is now commonly applied in cryopreservation of embryos in ART. Vitrification is an ultrarapid method of cryopreservation whereby the embryo transits from 37 to −196 °C in <1 s, resulting in extremely fast rates of cooling [96]. For cryopreserving embryos in a vitrified, glass-like state, high concentrations of cryoprotectants and rapid cooling rates are essential. During vitrification, higher rates of cooling and rewarming are key points to successes.

A research proved that comparing to slow cooling, vitrification using DMSO/sucrose had a higher survival rate, while implantation rates were almost identical [97]. But another study showed that the post-thaw survival of vitrified embryos was significantly better than those of embryos resulting from slow freezing process. And a better pregnancy rate per thawed embryo cycle was observed following vitrification [97]. Using vitrification, the process of embryo freezing is simplified as well as the mechanical injury to embryos during the formation of intra- and extra-cellular ice crystals can be avoided. So a successful vitrification can significantly improve the embryo recovery rate and integrity.

10.5.3 The Birth Weight

It was postulated that infants born after frozen-thawed embryo transfer (FET) have a higher mean birth weight (BW) than those born after transfer of fresh embryos [98, 99]. A study about obstetric outcome in singletons after IVF with cryopreserved/thawed embryos [100] found that the rates of large for gestational age (LGA) and birth weight >4,500 g were significantly increased for the singletons from the cryopreservation cycles, both in comparison with the general population and in comparison with singletons from fresh cycles.

A large cohort study of infant outcome of 957 singletons born after frozen embryo replacement from 1995 to 2006 was done by Pinborg A. from Denmark [101]. This study showed that the mean BW was 205 g higher in Cryo ($3,578 \pm 625$ g) as compared with the fresh group ($3,373 \pm 648$ g). But in first-born children, the mean BW difference between the Cryo and fresh group was 187 g, and it was 86 g when compared with the non-ART group. The risk of low birth weight (LBW; <2,500 g) decreased significantly in Cryo versus fresh group in the adjusted analyses, but for very low BW (VLBW) risks, there were no statistically significant differences between singletons from frozen and fresh cycles. An analysis of 25,777 children in the national assisted reproduction registry of Japan [102] found that the mean birth weight after FET was significantly higher compared with fresh ET and all Japanese births ($3,100.7 \pm 387.2$ g, $3,009.8 \pm 376.8$ g, and $3,059.6 \pm 369.6$ g, respectively). For sex, the risk of LBW was higher in female neonates compared with male neonates. Another study by Shih W [103] found that the mean birth weight in first births was significantly lower, and LBW were more frequent for IVF ($3,166 \pm 676$ g, LBW = 11.7 %) and ICSI ($3,206 \pm 697$ g, LBW = 11.5 %) than for FET ($3,352 \pm 615$ g, LBW = 6.5 %) and non-ART conceptions ($3,341 \pm 634$, LBW = 7.1 %).

Although a higher rate of very low birth weight was observed among IVF twins from frozen cycles than children from fresh cycles, Cryo ICSI twins had a significantly higher mean birth weight than their fresh peers [99]. In addition, there was no difference in very low birth weight rate between cryo IVF and cryo ICSI.

In Australian and Swedish studies the low birth weight rate in singletons was significantly lower for children born from cryopreserved embryos than children born after fresh cycles [103, 104]. In the former study, singleton first babies born

after fresh embryo transfer had a lower mean birthweight than those born after cryopreservation [103]. And for twins, low birthweight rates in frozen cycles were lower than fresh cycles [103].

Many factors may result in low birthweight, including fetal sex, birth defects, parity and maternal age. Culture media may also affect perinatal outcome including low birthweight [105]. Culture in Cook's medium after fresh transfer resulted in singletons with lower mean birthweight, and, more singletons with low birthweight, when compared to singletons born after culture in Vitrolife AB medium. Inter-related health, lifestyle and socio-economic factors may also be important but the reason why the risk for low birthweight in FET was significantly lower than fresh ET continues to be studied. In FET cycles, supplements of oestrogen and progesterone may improve the uterine environment leading to better development of the placenta, subsequent fetal growth, and heavier birth weight. Ovarian stimulation is one of the causes of differences in mean birthweight following fresh ET and FET cycles. Birthweight was also affected by the duration of embryonic culture, the medium for culture and the procedure of freezing and thawing.

10.5.4 Preterm Birth Rate

A systematic review of children born after cryopreservation of embryos [106] shows that the preterm birth rate (PBR; <37 weeks) for cryopreserved singletons varies between 9.2 and 12.0 %. And the corresponding PBR for fresh IVF/ICSI singletons varies between 7.4 and 14 %. In the Swedish and the Australian studies [103, 104], the PBR in singletons was significantly lower for children born after cryopreservation than children born after fresh cycles. Although in other studies, there were no significant differences in preterm birth rates.

The Danish cohort study showed that, in singletons after frozen cycles, gestational age was 2.4 days longer when compared with the fresh group. The risk of preterm birth decreased significantly in cryo versus fresh groups, but for very preterm birth (<34 weeks), there were no significant differences. A systematic review and meta-analysis of perinatal outcomes in singleton pregnancies resulting from FET versus fresh ETs [107] shows that relative risks (RR) of having a delivery at <37 weeks was 0.84 in singleton pregnancies after FETs, when compared to those after fresh ETs with a risk of 2 %. However, when compared to singletons from the general population, significantly higher rates of extreme preterm birth (<28 weeks) were found in singletons from cryopreserved cycles [100].

For twins, the PBR for infants born after cryopreservation, varies between 33 and 62 %, while for "fresh" twins, it was between 47.6 and 61.3 %. Although there were no significant differences between "frozen" and "fresh" ICSI, a study found a significantly higher PBR for "frozen" versus "fresh" IVF twins [99]. But in other studies no significant differences were found between these groups of twins.

To explore the association between infertility and preterm birth, Basso et al. [108] examined the relationship in various subsets of the study population, after combining data for primiparas and multiparas. When excluding women aged

>30 years or restricting the samples to women with a BMI of 20 ± 24.99, or with regular menstrual cycles of 27 ± 31 days, or nonsmokers, the results were similar to those before. It is proposed that the fetus determines the length of gestation, whereas mother's size determines the size of the baby at birth.

10.5.5   The Sex Ratio

The sex ratio of neonates may be associated with genetics, differential survival of male fetuses in utero, and, other intrapartum factors. In the Danish cohort study, the male sex ratio of Cryo-ICSI was 57.0 %, and it was 48.9 % in the fresh-ICSI group [101]. In previous reports, the use of ICSI was associated with a decrease in the sex ratio of male infants [109] though the reasons for the sex-ratio imbalance remain unclear. One report suggests over-use of ICSI as there is a general disruption in genomic imprinting during the process of ICSI, while others suggested differential death of male and female embryos in the early stages of embryogenesis. Sperm separation procedures or culture media may also affect the sex ratio of the children from cryo and fresh ETs [110]. A study of the effect of energy sources during culture on the development of in vitro bovine embryos, demonstrates that the energy substrate during in vitro culture affects both the production and viability of blastocysts. Furthermore, manipulating the metabolic profile of embryos during in vitro culture may have an impact on sex ratio [111].

   If some component of the fresh-ICSI procedure are likely to cause reductions in the male sex ratio, other components drawing in the opposite direction may cause an increased male sex ratio in Cryo-ICSI children. A comparison of the offspring sex ratio between fresh and thawed blastocyst transfer (BT) find a significant sex-ratio imbalance towards female offspring from thawed BT, compared with those from fresh BT. In the subgroups of either singleton or multiple deliveries, the female-to-male ratio increased significantly in the thawed BT group [112]. Such findings may be associated with iatrogenic factors including the embryo morphology selection strategy. Because top-quality embryos are selected by experienced embryologists and then given priority for fresh cycle transfer, most of the thawed embryos are also fresh cycle transfers.

10.5.6   Birth Defects and Mortality

A summary of 17 studies show that the rate of congenital malformations in all frozen cycles varies between 0.7 and 8.6 %, and, the corresponding figures for fresh cycles varied between 0.7 and 8.7 % [102]. Although adjusted risk differences in malformation rates between cryopreserved and fresh or non-ART were not found in the Danish cohort study, there was a tendency for highest rates in fresh and lowest rates in non-ART, with cryopreserved somewhere in between [101]. The rates of total malformations, particularly cardiac and musculoskeletal, increased in fresh and cryopreserved versus non-ART though there were no significant

differences of weeded malformations or any specific organ system problems between cryo-ICSI and cryo-IVF. There were no significant differences in rates of mental retardation and cerebral palsy in cryopreserved compared to either fresh or non-ART groups. The Swedish study showed similar results, and, no significant difference was found between frozen and fresh cycles when adjusting for year of birth, maternal age and number of infants born [104]. But in the Belgian study, malformations were more frequent in cryo-ICSI liveborns (6.4 %) compared to cryo-IVF liveborns (3.1 %) and fresh-ICSI liveborns (3.4 %) [99]. And there was a significantly higher rate of malformations in the total cryopreserved group, compared to the total fresh group. Compared to fresh-ICSI, pre- and post-natal rates of chromosome aberrations in cryo-ICSI were similar. In the study by Olson [113], compared with fresh IVF/ICSI twins, only in cryo IVF/ICSI twins higher malformation rates were found.

With regard to perinatal mortality, there were no differences between singletons and twins from fresh and frozen cycles in a UK study [103], while a significantly higher perinatal mortality rate for singletons from fresh versus frozen cycles in an Australian study. Although the crude figures for most obstetric measures were better for cryo-ICSI compared with cryo-IVF, no statistically significant differences were found between the two groups.

An increased risk of congenital malformations after IVF is largely independent of preterm births, which is associated with the upper respiratory tract infections, convulsions, and accidents. It seems unlikely that IVF itself increases the risk of these problems. Compared with parents of spontaneously conceived children, parents of IVF children are more likely to ask for medical help for less severe conditions, and that may affect hospitalization for these conditions and contribute to elevated risks for children born preterm.

10.5.7 Growth and Childhood Morbidity

Data are limited for growth, childhood morbidity and mental development, and, few differences were found between children born after frozen and fresh embryo transfer. In a Swedish study, 255 children from cryopreserved embryos were followed up to 18 months, and growth was normal. No differences were found between children from fresh and frozen embryos or spontaneous pregnancies. In the questionnaire study by Nakajo et al. including 78 born after IVF, 343 children born after ICSI and 81 after cryopreservation, the children were followed up to 2 years old [114]. For singletons, growth was similar to non-ART children, however, compared with naturally-conceived singletons, the growth of IVF, ICSI and cryo multiples was significantly delayed but had caught up by 6 months of age.

In a study of 16,280 children born after IVF with a median follow-up time of 5.5 years, which included 1,474 children from frozen embryos, three children in this group had a cancer diagnosis. In another study, there was no difference in the prevalence of chronic disease at 18 months of age among the cryopreserved, fresh IVF and naturally-conceived groups.

10.5.8 Cryopreservation of Zygotes

Clinical applications for oocyte cryopreservation include fertility preservation in cancer patients, oocyte accumulation in low-responder patients, ovum donation programs, fertility preservation for social reasons, minimization of ovarian hyper-stimulation syndrome risk, and surplus oocyte storage after controlled ovarian stimulation when embryo cryopreservation is not feasible [115]. There is no significant difference in the potential of fertilization, embryogenesis, and pregnancy between from oocytes derived from vitrification warming cycles and from that of fresh oocytes. But there are rare reports of offspring's ill-health following oocyte cryopreservation.

Children born after cryopreservation have a better outcome as compared to those born after fresh transfer. The risks of low birthweight and preterm birth are significantly lower in infants from FET than those from fresh ETs. The caesarean section rate is significantly higher in the cryopreserved than the fresh groups, and is almost doubled compared with the non-ART group. The difference of sex ratio between frozen and fresh transfer is mostly in ICSI and blastocyst. For the birth defects and mortality, there were no significant differences for children from frozen and fresh transfer, but all have a higher risk than non-ART children. The long term follow-up investigations of FET children have found no significantly adverse outcomes; however, the data are limited. Prospective studies are necessary to fully evaluate the outcomes of FET.

10.6 Conclusions

Superovulation, in vitro culture, IVM, ICSI, embryo biopsy and cryopreservation, may all contribute to increased risks of adult diseases. Large, prospective, epidemiological studies to systematically assess the potential risk factors associated with adult diseases will be necessary to establish precise risks.

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