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Elisabetta Baldi
Monica Muratori *Editors*

Genetic Damage in Human Spermatozoa

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Editors
Elisabetta Baldi
Department of Experimental and Clinical Biomedical Sciences
University of Florence
Florence, Italy

Monica Muratori
Department of Experimental and Clinical Biomedical Sciences
University of Florence
Florence, Italy

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To our mentor, professor Mario Serio

Preface

The goal of male gametes is to deliver a fully intact and functioning paternal genome to the oocyte. To fulfill this aim, the process of chromatin maturation during spermiogenesis must be correctly completed to guarantee DNA protection during the long journey to reach the oocyte and to properly decondense and form the male pronucleus after fertilization. In this scenario, any condition or agent that can interfere with this complex process represents a threat to safe embryo formation and development and to the birth of healthy offspring. Concerns about these risks acquire particular relevance in the present era of assisted reproduction techniques that bypass several, if not all (as in the case of intracytoplasmic sperm injection), natural barriers designed to prevent fertilization by sperm that has significant genetic damage.

This book presents an overview of the various types of damage that may affect the genetic material of the male gamete. Genetic damage in spermatozoa can occur during spermatogenesis or during transit in both male and female genital tracts, or it may be due to aging, environmental or iatrogenic conditions, or to the protocols used in cryopreserving and selecting spermatozoa in assisted reproduction techniques. Two chapters of the book are dedicated to the "hot topic" of sperm epigenome and epigenetic damage that may generate important and unexpected transgenerational effects. In addition, since so far the only proposed therapy to prevent sperm DNA damage is the administration of antioxidant compounds, the last chapter of the book is dedicated to this controversial issue.

We wish to thank all the authors for their invaluable contributions to the book. They are all expert scientists in the field, and we appreciate their willingness to offer their knowledge in this important branch of reproductive medicine. We hope that this book will help researchers in the field of reproduction and serve as a reference for medical and technical staff working in laboratories investigating assisted reproduction techniques.

Florence, Italy

E. Baldi
M. Muratori

Contents

Contributors

Lars Björndahl Centre for Andrology and Sexual Medicine, Clinic for Endocrinology, Karolinska University Hospital, Huddinge, Stockholm, Sweden

Centre for Andrology and Sexual Medicine, Department of Medicine, Karolinska Institutet, Huddinge, Sweden

Sara Brilli Andrology Unit, Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy

Douglas T. Carrell Division of Urology, Department of Surgery, Department of Obstetrics and Gynecology, Department of Human Genetics, University of Utah School of Medicine, Salt Lake City, UT, USA

Chiara Chianese Andrology Unit, Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy

Graçiela Estela Cognigni GynePro Medical Centers, Reproductive Medicine Unit, Bologna, Italy

Marc Cohen Procrelys, avenue Rockefeller, Lyon, France

Brian Dale London Fertility Associates, London, UK

Centre for Assisted Fertilization, Naples, Italy

Benjamin R. Emery Division of Urology, Department of Surgery, University of Utah School of Medicine, Salt Lake City, UT, USA

Don Evenson South Dakota State University, Brookings, SD, USA

Marco Filicori GynePro Medical Centers, Reproductive Medicine Unit, Bologna, Italy

Laura Gambera Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy

Center for Diagnosis and Treatment of Couple Sterility, S. Maria alle Scotte Hospital, Siena, Italy

Loredana Gandini Department of Experimental Medicine, University Laboratory of Seminology – Sperm Bank, University of Rome "La Sapienza", Rome, Italy

John R. Gannon Division of Urology, Department of Surgery, University of Utah School of Medicine, Salt Lake City, UT, USA

Carlos Guerrero-Bosagna Center for Reproductive Biology, School of Biological Sciences, Washington State University, Pullman, WA, USA

Timothy G. Jenkins Division of Urology, Department of Surgery, University of Utah School of Medicine, Salt Lake City, UT, USA

Meritxell Jodar Human Genetics Research Group, IDIBAPS, University of Barcelona, Barcelona, Spain

Csilla Krausz Andrology Unit, Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy

Ulrik Kvist Centre for Andrology and Sexual Medicine, Clinic for Endocrinology, Karolinska University Hospital, Huddinge, Stockholm, Sweden

Centre for Andrology and Sexual Medicine, Department of Medicine, Karolinska Institutet, Huddinge, Stockholm, Sweden

Andrea Lenzi Department of Experimental Medicine, University Laboratory of Seminology – Sperm Bank, University of Rome "La Sapienza", Rome, Italy

Sheena E.M. Lewis Centre for Public Health, Queen's University Belfast, Belfast, Northern Ireland, UK

Institute of Pathology, Belfast, Northern Ireland, UK

Francesco Lombardo Department of Experimental Medicine, University Laboratory of Seminology – Sperm Bank, University of Rome "La Sapienza", Rome, Italy

Yves Menezo London Fertility Associates, London, UK

Laboratoire CLEMENT, Paris, France

Cristian O'Flaherty Division of Urology, Department of Surgery, McGill University and the Research Institute-McGill University Health Centre, Montréal, Québec, Canada

Rafael Oliva Biochemistry and Molecular Genetics Service, Clinic Hospital, Villarroel, Barcelona, Spain

Faculty of Medicine, University of Barcelona, and Institut d'Investigacions Biomèdiques, Spain

Donatella Paoli Department of Experimental Medicine, University Laboratory of Seminology – Sperm Bank, University of Rome "La Sapienza", Rome, Italy

Lodovico Parmegiani GynePro Medical Centers, Reproductive Medicine Unit, Bologna, Italy

Paola Piomboni Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy

Center for Diagnosis and Treatment of Couple Sterility, S. Maria alle Scotte Hospital, Siena, Italy

Michael K. Skinner Center for Reproductive Biology, School of Biological Sciences, Washington State University, Pullman, WA, USA

Anita Stendardi Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy

Chapter 1
Structure of Chromatin in Spermatozoa

Lars Björndahl and Ulrik Kvist

Abstract The specialized structure of the sperm chromatin has a dual function – first to protect the DNA from damage during storage and transport to the oocyte, and then to enable a rapid and complete unpacking of the undamaged paternal genome in the ooplasm. It is evident that zinc has a pivotal role in maintaining the structural stability and in enabling a rapid decondensation at the appropriate time. It is important for the sperm chromatin structure that the spermatozoa are ejaculated together with the zinc-rich prostatic secretion. Early exposure to zinc-binding seminal vesicular fluid can deplete the sperm chromatin of zinc and most likely induce surplus formation of disulfide bridges, likely to cause incomplete and delayed decondensation of the sperm chromatin in the oocyte. A premature decrease in sperm chromatin structure stability is likely to increase the risk for damage to the DNA due to increased access to the genome for DNA damaging compounds. The status of the sperm chromatin structure can vary in vitro depending on the exposure to zinc-depleting conditions when spermatozoa are stored in semen after ejaculation. When sperm DNA damage tests are evaluated and validated, it is therefore essential to also take into account the dynamics of zinc-dependent and zinc-independent sperm chromatin stability.

Keywords Sperm chromatin structure • Sperm DNA • Zinc • Ejaculation • Prostatic secretion • Seminal vesicular secretion • Sperm DNA fragmentation • Sperm DNA damage • Disulfide bridges • Protamine thiols

L. Björndahl (✉) • U. Kvist
Centre for Andrology and Sexual Medicine, Clinic for Endocrinology,
Karolinska University Hospital, Huddinge, S-141 86, Stockholm, Sweden

Centre for Andrology and Sexual Medicine, Department of Medicine, Karolinska Institutet,
Huddinge, S-141 86, Stockholm, Sweden
e-mail: lars.bjorndahl@ki.se

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Significance and Consequence of the Unique Sperm Chromatin Structure

The spermatozoon is a highly specialized cell. It is the only type of cell designed to transfer from one individual to another. Due to the challenges imposed on cells able to survive such a transition, a number of important adaptations of cell organization and functions must take place to make it possible for the cell to survive and fulfill its mission. The basic functions were evolved some 600 million years ago, when meiosis and reproduction with gametes first arose. The detailed mechanisms of these functions are still not fully understood, but their significance can be imagined: maintenance of cell homeostasis (water content, electrolyte composition) in an environment where conditions can vary and jeopardize, for instance, the best adaption of sperm motility or preparations for contacting and passing the coronary cells, penetrating of the zona pellucida in mammalians and the final connection to the oocyte, and the following fusion of cell membranes and delivery of sperm messages to the oocyte. The specific mission of the spermatozoon is to transfer the haploid genome undamaged to the oocyte. It appears that this is accomplished through a chromatin structure that is completely different from that of somatic cells. The safekeeping of the DNA is based on the sperm-specific packaging of the DNA. In somatic cells the transcription of the DNA code is controlled by epigenetic modulations of DNA as well as modifications of histone tails. In the mature spermatozoon, DNA replication and transcription are terminated due to an exchange of DNA binding histones for, in human, two types of protamines (P1 and P2, respectively). Besides inactivating DNA functions, the incorporation of protamines allows a tighter packaging of the DNA fibers, rendering the mature sperm nucleus a volume estimated to be approximately one-sixth that of a somatic cell nucleus. The condensed state is believed to create an almost water-free, crystalline structure. Absence of water is important to decrease the risk of damage to the DNA. For instance, ionizing radiation is known to create reactive compounds from water, compounds that, in turn, due to the high degree of chemical reactivity, can cause DNA strand breaks. Another risk with a high content of water within a DNA-containing cell organelle is that water-soluble compounds are more likely to obtain access to the DNA and thereby have an increased ability to react with the DNA molecules.

From this background the importance of the condensed state of the sperm nucleus for a successful sperm function is obvious. But it is not only the formation of the condensed state that is important. Once the sperm nucleus has been delivered into the ooplasm, the condensed nucleus must decondense rapidly to release the DNA for formation of a paternal pronucleus. Any abnormal change in the structural organization can cause delays or defects in the delivery of the paternal DNA. Furthermore, any damage to the DNA during the transition from the testicle to the oocyte cannot be repaired until the DNA is accessible for DNA repair systems in the ooplasm. The risk of error during the repair process increases with the number of DNA strand breaks in an individual sperm nucleus.

The human sperm chromatin structure is thus based on the incorporation of protamines 1 and 2 in the nuclear matrix. Still, in humans, it appears that not all histones are exchanged in this process, and it has been hypothesized that incomplete histone exchange could explain the lower degree of chromatin compaction found in human spermatozoa. There are investigations indicating that subnormal levels of histone exchange can be more common among subfertile men and, therefore, one of several possible causes of decreased fertility. Another possible interpretation of remaining histones in the human sperm chromatin is that "normal" histones can be located in minor DNA structures that link protamine-organized, larger parts of the DNA (Ward 2010).

Thus, abnormal sperm chromatin packing could cause problems by two different mechanisms. One is the increased vulnerability of the DNA due to subnormal condensations and stabilization of the chromatin. The other is what could best be described as a supernormal compaction or superstabilization. Supernormal compaction of the sperm chromatin would jeopardize the timing of the rapid delivery of the sperm DNA in the ooplasm. It is therefore essential that both factors that reduce and those that increase the normal compaction of the sperm chromatin be considered to understand the causes of reduced male fertility potential. Comprehensive knowledge and understanding of the normal male reproductive physiology and disorders that can interact with normal sperm function is essential if progress is to be made in the area of male contribution to subfertility.

Agents Interacting with Sperm Chromatin Structure

First an overview of agents that can contribute to changes in the normal structural stability of the human sperm chromatin.

Sperm Protamines

Protamines in mammals contain both free $–NH_2$ and –SH groups. This gives a chemical basis for salt bridges where, for instance, Zn^{2+} can participate. *Arginines* are the dominating component (45–48 %) of both protamines (P1 and P2) in human sperm chromatin (Gusse et al. 1986). Arginines introduce a profusion of positively charged $–NH_3^+$ groups into the protamines – groups that neutralize the negative charges of the phosphate groups of the DNA backbone. In this way, a high degree of compaction of adjacent chromatin fibers can be achieved (Balhorn 2007). *Histidines* introduce imidazole groups, and *cysteines* introduce thiol (–SH) groups. These groups form the basis for ion bridges involving zinc ions. In the absence of Zn^{2+}, thiol groups can form disulfide bridges between them (Kvist et al. 1980; Bal et al. 2001). *Serines* and *threonines* have the propensity to become phosphorylated, i.e., these amino acids can bind negatively charged phosphate groups. Thus, when

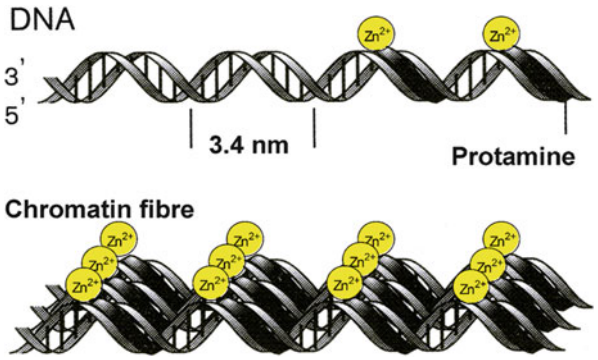

Fig. 1.1 Schematic view of DNA double helix in sperm chromatin and its relation to protamine compounds, indicating possible role of zinc to connect protamines: one zinc per protamine molecule for every ten base pairs (turn of the helix) of the DNA. If negative charges of phosphate groups in DNA are neutralized by protamines, tight packing of sperm DNA chromatin fibers is possible. With permission from Revised guidelines for human embryology and andrology laboratories (2006)

phosphorylated serines and threonines can provide a basis for negatively charged repulsive forces and when dephosphorylated, they do not hinder the compaction of adjacent chromatin fibers. Phosphorylation of serine and threonine units can therefore be an important mechanism for allowing a rapid decondensation by repulsion of chromatin fibers while unpacking the DNA in the oocyte.

Zinc

Zinc is assimilated into the sperm chromatin during spermiogenesis at the stage where the compaction of the nucleus is initiated. A general first sign of zinc deficiency is an arrest of the formation of spermatozoa at a certain point of spermiogenesis, leading to a total lack of elongated spermatozoa (Barney et al. 1968). The content of zinc in the chromatin is on a magnitude of 8 mmol Zn^{2+}/kg (Kvist et al. 1985). Using a method to determine subcellular levels of atomic elements, X-ray microanalysis, the sperm head was found to contain up to one zinc atom for every five sulfur atoms. Since each human protamine molecule contains approximately five sulfur atoms, the zinc-to-sulfur ratio is 1/5 – one zinc ion for every protamine molecule. One protamine compound can provide 20 positively charged groups ($-NH_3^+$) that can neutralize the 20 negatively charged phosphate groups of 10 base pairs of the DNA. Since one turn of the DNA-protamine helix equals ten base pairs, the human sperm chromatin has one zinc ion for each turn of the DNA (and therefore also for each protamine molecule). It is therefore not unreasonable to presume that zinc ions can contribute to the DNA-protamine structure by linking protamines with salt bridges (Bench et al. 2000; Kjellberg 1993) (Fig. 1.1).

Another piece of evidence for the idea that interaction between zinc and protamine thiols could constitute a base for a stable but still rapidly reversible chromatin

stability lies in the finding that the detectable amount of chromatin thiols decreases during sperm maturation in the epididymis and reappears after sperm exposure to DTT(dithiothreitol). The original interpretation of these findings was that thiols become engaged into S-S bridges due to the fact that DTT is a compound that can break strong covalent disulfide bridges (Calvin and Bedford 1971). However, an alternative interpretation is more valid: Zn^{2+} interacting with thiols in, for instance, salt bridges could also make the thiols undetectable. Furthermore, exposure of the sperm chromatin to DTT can bind zinc, and therefore free thiols could be detected after DTT exposure. In favor of this interpretation is that epididymal spermatozoa show more thiols if preexposed to acid or ethylenediaminetetraacetate (EDTA) treatment, both of which can act as zinc binding agents (Calvin and Bleau 1974; Calvin et al. 1973; Kvist and Eliasson 1978). Furthermore, spermatozoa exposed to DTT are deprived of zinc but not magnesium (Kvist and Eliasson 1978).

Chromatin of Ejaculated Spermatozoa

Immediately after ejaculation the chromatin of human spermatozoa can be rapidly decondensed by simple zinc removal with EDTA, which chelates cations like Zn^{2+} (Kvist 1980a, b; Roomans et al. 1982), simultaneously with exposure to the anionic detergent sodium dodecyl sulfate (SDS), which removes membranes and forces a repulsion of chromatin fibers (Björndahl and Kvist 1985). Since disulfide breaking agents are not required to achieve chromatin decondensation in spermatozoa immediately after ejaculation, it is justified to conclude that *at ejaculation* the human sperm chromatin has a zinc-dependent chromatin stability.

In vitro, the inherent, zinc-dependent stabilization of the chromatin is rapidly lost and replaced by another type of stability (Kvist and Björndahl 1985). The second type of stability (superstabilization) is more likely based on disulfide bridges since agents specifically able to break those covalent bindings are required to achieve decondensation, while simple Me^{2+} binders cannot induce unpacking of the chromatin. This alteration of the structural stabilization is enhanced when zinc is removed from the sperm chromatin in vitro. Furthermore, the change into a superstabilized state of the chromatin is largely counteracted when spermatozoa are stored in an environment with high bioavailability of Zn^{2+}.

A reasonable mechanism that could explain a dual role for zinc, both (1) stabilizing the chromatin structure and (2) preventing the development of a superstabilization, is that zinc forms salt bridges between protamine thiols (Fig. 1.2). A salt bridge that comprises Zn^{2+} and thiols has a binding strength equivalent to that of a covalent disulfide bridge. Therefore, a zinc-thiol-based salt bridge could serve a double purpose as a temporary, reversible stabilizer of the sperm chromatin structure. Simultaneously the to thiols prevents the oxidization of these thiols into disulfide bridges. Furthermore, removal of zinc by agents in the ooplasm would allow a rapid decondensation of the sperm chromatin structure without an excessive need for disulfide breaking agents.

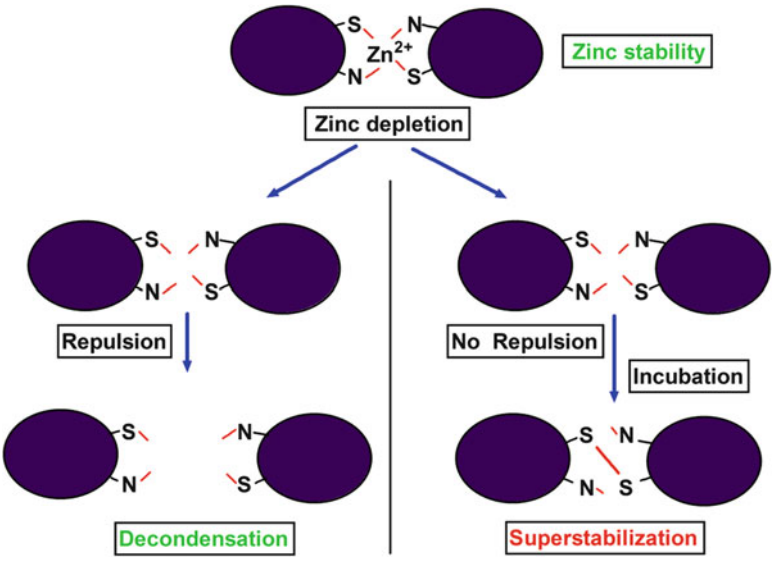

Fig. 1.2 Schematic representation of dual action of zinc: Zn^{2+} (Zn) *stabilizes* the structure and simultaneously *prevents* formation of disulfide bridges: formation of salt bridges with thiols (–S) of the protamines. Binding of zinc (e.g., by EDTA in vitro) enables two biologically opposite outcomes: *immediate decondensation* if chromatin fibers are induced to repel (e.g., repulsion induced by addition of SDS in vitro); otherwise, thiols freed from Zn^{2+} can become engaged into disulfide bridges (-S-S-) creating a *superstable chromatin*. With permission from Björndahl and Kvist (2010)

Alteration of Sperm Chromatin Structural Stability In Vitro

Normally, spermatozoa are expelled with the prostatic fluid most likely directly onto the cervical mucus protruding from the cervix. Therefore, the prostatic fluid must be regarded as the physiological vehicle for ejaculated spermatozoa. However, when the ejaculate is collected for ART purposes or for research, the common practice is to collect the whole ejaculate in one single container. Thus, the secretion from the seminal vesicles is also included and able to interact with the spermatozoa. The content of zinc in the sperm head and the nature of the chromatin stabilization therefore will be affected by the more or less random and always altering properties of the seminal fluid that each ejaculated spermatozoon is surrounded by (Björndahl and Kvist 2003). It is too often ignored that ejaculate is not a homogenic and homeostatically regulated fluid like blood plasma. (The unfortunate term seminal *plasma* has probably misled scientists to make an erroneous parallel between blood plasma and seminal plasma.) Ejaculate is just a heterogeneous mixture of discharged secretions from the prostate, the seminal vesicles, the epididymis, the testes, and a diversity of small glands in the male reproductive tract. The contents of the seminal plasma actually vary between different parts of the ejaculate, during ejaculation, during liquefaction, and after ejaculation. Moreover, the composition varies among

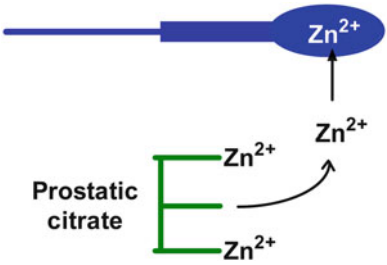

Fig. 1.3 Schematic overview of zinc availability in prostatic fluid. Normally prostatic fluid has a high availability of zinc due to the secretion of free zinc and citrate-bound zinc. Spermatozoa ejaculated in prostatic fluid retain their chromatin zinc due to the high availability of zinc in the fluid. With permission from Björndahl and Kvist (2010)

men and among ejaculates from the same man. Therefore, the golden standard for semen laboratories (World Health Organization 2010), with a collection of the entire ejaculate in one single container allowing a mixture of all secretions and interactions with the spermatozoa, will cause largely increased heterogeneity of the structural stabilization of the sperm chromatin.

To better understand the remodeling in vitro of the sperm chromatin structure stabilization after ejaculation, it is important to acknowledge the significance of the sequence of ejaculation and how the zinc content of the sperm chromatin content is affected by prostatic fluid and seminal vesicular fluid (Björndahl et al. 1991; Björndahl and Kvist 1990). Normally, most spermatozoa are ejaculated in the first third of the ejaculate, together with the slightly acidic, zinc-rich prostatic secretion. The prostatic fluid has a high biological availability of Zn^{2+} that is likely to prevent a loss of chromatin zinc (Fig. 1.3). The last two-thirds of the ejaculate contain mainly seminal vesicular fluid. The addition of secretion from the seminal vesicles raises the pH, which causes an increase in the affinity of citrate for zinc, resulting in less free zinc. Moreover, the seminal vesicular fluid also contains high molecular weight (HMW) zinc ligands, which have a high affinity for zinc (Fig. 1.4). Thus, during liquefaction in vitro seminal plasma develops into a zinc-chelating medium able to deplete spermatozoa of zinc (Arver and Eliasson 1982; Björndahl and Kvist 1990; Björndahl et al. 1991). A magnitude of the zinc binding property of the seminal plasma is the percentage of seminal zinc content bound to HMW ligands. Among fertile donors the proportion of HMW bound zinc was less than 10 %. In contrast, the proportion varied between 2 % and 67 % among 115 men in subfertile couples (Kjellberg 1993). Altogether, seminal vesicular secretion changes the seminal plasma into a zinc-binding medium, although the total concentration of zinc in seminal plasma may appear to be normal.

In conclusion, spermatozoa exposed to seminal plasma are exposed to highly variable conditions. The variations appear between different samples, and by the duration of exposure to the seminal plasma, due to variations in the zinc-containing prostatic fluid and the zinc-chelating seminal vesicular fluid and the dynamics in the mixture of these fluids (Lundquist 1949; Arver 1982a, b).

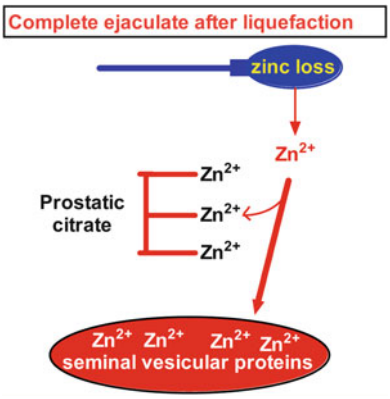

Fig. 1.4 Schematic overview of zinc availability in whole ejaculate in vitro after liquefaction of seminal fluid. Seminal vesicular fluid contains high molecular weight proteins with high affinity for zinc, and its pH is alkaline, which increases the zinc binding capacity of citrate. The availability of zinc therefore decreases after addition of seminal vesicular fluid because this fluid creates a zinc-binding medium. With permission from Björndahl and Kvist (2010)

It is of clinical interest that some men in subfertile couples have an abnormal sequence of ejaculation, where most spermatozoa are ejaculated together with mainly seminal vesicular fluid, leading to a loss of zinc from the sperm chromatin (Björndahl et al. 1991; Björndahl and Kvist 1990). This can in turn lead to changes in the accessibility of the DNA. A likely cause of this disorder could be an obstruction of the ejaculatory ducts (Fisch et al. 2006) or prostatic oedema (Kjellberg 1993). In both cases the spermatozoa will not reach the urethra until forced by the contractions and emptying of the seminal vesicles. Such an abnormal sequence of ejaculation cannot be detected by routine semen analysis, although the main finding would be decreased sperm motility. Indeed, whether the spermatozoa are mainly expelled in the last ejaculate fractions together with the seminal vesicular fluid or the first, sperm-rich ejaculate fractions are dominated by seminal vesicular fluid, the result is a decreased sperm motility. Examination of a split ejaculate with an assessment of accessory sex gland markers is the only means to unmask an abnormal sequence of ejaculation (Björndahl and Kvist 2003).

Implications for the Interpretation of Sperm DNA Damage Tests

An increasingly popular topic is the study of sperm DNA damage or DNA fragmentation. The basis for this interest is very sound since it focuses on the message the sperm is supposed to deliver to the oocyte. The classic basic semen analysis would probably not yield any information that is directly linked to the status of the DNA

of spermatozoa that are likely to participate in fertilization. Of course, a very poor efficiency of sperm production and transport is likely to give rise to spermatozoa with poorly protected DNA. In contrast, it cannot automatically be presumed that sperm with good motility and morphology will always have an undamaged message.

However, when interpreting results from all sorts of sperm DNA damage tests, the structural organization of the sperm chromatin must be acknowledged. Basically, tests originally developed for somatic cells (with a completely different chromatin structure) have been modified to provide access to the DNA within the sperm nucleus. No real attention has been given to the highly variable structural stability in different spermatozoa – alterations of methods to get access to the DNA can actually cause damage to the DNA, especially if the structure is already compromised by premature zinc extraction. In contrast, a superstabilized chromatin is less likely to give a correct response in a test system – a false negative result can occur as a result of decreased access to sperm DNA (Pettersson et al. 2009; Tu et al. 2009; From Björk et al. 2009).

Conclusion

- The freshly ejaculated human sperm chromatin is stabilized by salt bridges in which zinc binds between thiols and possibly imidazole groups of histidine. This type of salt bridge resists chromatin decondensation by exposure to the detergent SDS in vitro.
- Zinc binding to thiols prevents the oxidation of thiols into disulfide bridges.
- Partial or premature withdrawal of zinc, simultaneously with partial decondensation of the chromatin, can leave the DNA more exposed to agents that can damage the DNA.
- Withdrawal of zinc from the sperm chromatin within the ooplasm, or as part of an in vitro experiment close to ejaculation, enables a rapid chromatin decondensation if chromatin fibers are simultaneously induced to repel.
- Withdrawal of sperm chromatin zinc without simultaneous repulsion of chromatin fibers may result in oxidation of thiols into disulfide bridges (superstabilization), which could delay the delivery of DNA in the oocyte and thereby cause a defect zygote development. Furthermore, decreased access to sperm DNA due to excess formation of disulfide crosslinking is also likely to hinder detection of DNA breaks by assays like Comet, Tunel, and SCSA techniques and give false negative results.

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Chapter 2
Genomic Changes in Spermatozoa of the Aging Male

Chiara Chianese, Sara Brilli, and Csilla Krausz

Abstract Modern society is witnessing a widespread tendency to postpone parenthood due to a number of socioeconomic factors. This ever-increasing trend relates to both women and men and raises many concerns about the risks and consequences lying beneath the natural process of aging. The negative influence of the advanced maternal age has been thoroughly demonstrated, while the paternal age has attracted comparatively less attention. A problematic issue of defining whether advanced paternal age can be considered an independent risk factor, not only for a man's fertility but also for the offspring's health, is represented by the difficulty, linked to reproductive studies, in characterizing the impact of maternal and paternal age, separately. Researchers are now trying to overcome this obstacle by directly analyzing the male germ cell, and emerging data prove this sperm-specific approach to be a valid tool for providing novel insights on the effects of aging on the spermatozoa and, thus, on the reproductive outcome of an aging male. The purpose of this chapter is to summarize most of what is known about the relationship between male aging and changes in the spermatozoa, giving special focus on the events occurring with age at the genomic level.

Keywords Aging male • Spermatozoa • Genomic anomalies

Introduction

History provides fascinating episodes of men fathering children at very old ages. In 1935, a 94-year-old man was the oldest age-of-paternity case reported by a scientific publication (Seymour et al. 1935). Other examples of greatly aged fathers

C. Chianese • S. Brilli • C. Krausz (✉)
Andrology Unit, Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy
e-mail: csillagabriella.krausz@unifi.it

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have appeared more recently, such as that of two Indian farmers, Nanu Ram Jogi, who fathered his twenty-first child at age 91 in 2007, and Ramjit Raghav, who became the world's oldest dad, giving birth to his first child at the age of 94.

Beyond anecdotal curiosity, it is interesting to note that in industrialized countries delayed parenthood is becoming an increasingly widespread social phenomenon. Many factors account for this trend, but referring to literature citations increasing life expectancy, economic stability, and career ambitions represent the most relevant issues for parenthood postponement, raising not a few concerns about age-associated risks and consequences. Indeed, the process of aging can be ascribed to a number of endogenous and environmental factors inducing DNA damage.

As demonstrated by epidemiological data, the last decades saw a considerable increase in the mean age of childbearing mothers, reflecting for women a birth rate shift toward 35 years of age and older. A similar tendency shows up in the paternity rate, which has been continuously rising since 1980, in parallel with a decrement of paternity among men between 25 and 29 years old (Martin et al. 2007). In fact, the percentage of men fathering children over 35 years old has been markedly rising from 15 % to 25 % during the last 40 years, as has the number of men aged between 50 and 54 desiring to conceive children (Fisch 2009). In line with these data, a rise in the number of fathers over 60 is predicted to happen over the next 10–20 years (US Census Bureau 2005).

The effect of delayed motherhood has been studied to such an extent that it is now possible to acknowledge advanced maternal age as the most important risk factor for infertility, spontaneous abortions, and genetic defects among offspring. Only lately has male age attracted more attention in this regard, but whether a comparable age-dependent effect also exists for delayed fatherhood remains to be fully elucidated. There is suggestive epidemiological evidence that paternal age correlates with an increased incidence of abnormal reproductive outcomes and heritable defects (Tarin et al. 1998; de la Rochebrochard and Thonneau 2002), including several types of genomic modification. In particular, there is growing evidence that advancing male age is associated with an increased frequency of certain genetic and chromosomal defects in spermatozoa (Crow 2000; Shi and Martin 2000; Tiemann-Boege et al. 2002; Bosch et al. 2003; Sloter et al. 2004). The last-named authors prove that the male germ cell represents a direct target of study for a straightforward identification of merely paternal risk factors, overcoming the difficulty, often present in reproductive studies, in distinguishing between the impact of maternal and paternal ages. Nevertheless, it is as yet impossible to even define a general consensus of what can be considered *advanced* paternal age, although some studies and the precautionary measures taken for sperm donations destined for assisted conception advise a threshold of 40 years old (de la Rochebrochard et al. 2003).

This chapter aims at summarizing data available on male age-related effects in spermatozoa, describing with specific focus how the sperm genome can be modified during meiosis of aging men.

Chromosomal Alterations in the Aging Male

Chromosomal aberrations were among the first observed manifestations of decreased genome integrity with age. Studies comprising chromosomal analyses from human peripheral blood lymphocytes corroborate these observations, demonstrating that the occurrence of various genomic changes (i.e., aneuploidies, translocations, acentric fragments, chromosomal loss, micronuclei formation) increases linearly with the age of the individuals (Sloter et al. 2004). In light of these data, concerns have arisen that certain types of chromosomal alterations may increase with age in male germ cells as well.

Aneuploidies

In humans, aneuploidies represent the most common heritable chromosomal anomaly, with approximately 0.3 % of infants born with an altered number of chromosomes (Hassold 2001). This genomic rearrangement derives mainly from nondisjunction during meiotic divisions [both meiosis I (MI) and meiosis II (MII)] and its primary reproductive consequence is spontaneous abortion. Analyses on fetal material retrieved from abortuses show that 60 % of all aneuploidies consist in 45, XO monosomy and trisomies of chromosomes 16, 21, and 22 (Hassold 2001). A subset of aneuploidies involving sex chromosomes as well as trisomies 13, 21, and 18 make it to birth and lead to typical developmental and morphological defects.

Several studies proved maternal age to be a risk factor for trisomy formation, although the nature of such a relationship is not unequivocally definable: in fact, the effect of maternal age is differently exerted among chromosomes, increasing either linearly (e.g., trisomy 16) or exponentially (e.g., trisomy 21). The characterization of such an age-dependent connection for individual trisomies has not progressed much for men due to the relatively low number of trisomy cases determined to be paternally derived. Moreover, whether paternal age is associated with the generation of aneuploidies is still controversial due to the paucity of data available on the affected offspring because most abnormal embryos are lost and because studies performed so far were limited by the difficulty in separating paternal from maternal effects (Wiener-Megnazi et al. 2012). Cytogenetic data from human oocytes, fertilized eggs, preimplantation embryos, and spermatozoa allowed to compensate for the issue of discriminating the pure paternal effect. The approach of targeting solely the germ cell genome, extracting parent-specific information, indicates that most constitutional aneuploidies are generated de novo during parental meiosis.

The first data about the chromosomal content of human spermatozoa date back to 1978 and derive from the insemination of hamster eggs with human spermatozoa, a cytogenetic method known as the hamster-egg penetration test (Rudak et al. 1978).

This study showed that 2–3% of spermatozoa from normal men carried an aneuploid karyotype. Other large hamster-egg studies followed, but an effect of the donor's age on the rate of aneuploidies was not found (Estop et al. 1995; Brandriff et al. 1988); this was probably due to a bias related to the study population, which included only a few men aged over 40 years old. Finally, two more studies based on the same method reached divergent conclusions: in one, a significantly negative correlation was found between the rate of aneuploidy and the donor's age (Martin and Rademaker 1987), while in the other study the authors observed a significantly higher incidence of hyperploid spermatozoa from a comparison between seven old donors (aged between 59 and 74 years old) and five young donors (aged between 23 and 39 years old) (Sartorelli et al. 2001).

In the early 1990s, fluorescent in situ hybridization (FISH) replaced the hamster-egg method for the detection of sperm aneuploidy, introducing the advantage of a quicker labour- and cost-saving analysis of greater amounts of spermatozoa. With regard to sperm autosomal aneuploidies, FISH data show modest evidence for a paternal age effect. One study (Martin et al. 1995) reported an increase with age in disomy 1 in the spermatozoa of men aged from 21 to 52 years old, although none of the following studies confirmed their result. Another study found a significant correlation between a decreased incidence of chromosome 18 sperm disomy and increased age (Robbins et al. 1997). In contrast, studies on chromosome 21 sperm disomy all converge on the independence of such a rearrangement from men's age, with the exception of only one small study (Rousseaux et al. 1998) in which the authors found that advanced age correlated with a higher incidence of sperm disomy 21.

A different scenario is offered by FISH studies on sex chromosomes, for which more distinct evidence exists for an age-related increase in aneuploidies in male germ cells. In the literature, 11 sperm FISH studies are available on the effects of paternal age on the frequency of aneuploidy formation within spermatic X and Y chromosomes, and only 2 reached a negative conclusion with respect to the age–aneuploidy link. The rest provide evidence that errors in MI and MII are more likely to occur with the advancement of age. As for nondisjunctions in both MI and MII, the literature reports a general positive paternal age effect by which men aged over 50 have about a two- to threefold higher risk of carrying spermatozoa with a 24, XY karyotype as a consequence of MI errors (Guttenbach et al. 2000; Bosch et al. 2001; Asada et al. 2000; Griffin et al. 1995; Lowe et al. 2001) and a two to threefold higher frequency of producing X or Y disomic spermatozoa as a consequence of MII errors (Kinakin et al. 1997; Martin et al. 1995; Robbins et al. 1997; Rubes et al. 1998).

Multiprobe FISH analyses have proven their utility in the detection of sperm diploidy, the incidence of which in association with paternal aging is still controversial. For instance, the literature offers a number of studies reporting an age-related increase of the frequency of diploid sperm in older men compared to younger men with about a twofold increased risk. However, several other studies do not reach the same conclusion because they report no association between sperm disomy formation and advanced paternal age (Sloter et al. 2004).

Structural Aberrations

Although the incidence of structural chromosomal abnormalities is lower compared to aneuploidies at birth (0.25 % versus 0.33 %, respectively) (Hassold 1998), a study based on chromosome heteromorphisms first estimated that 80 % of such de novo rearrangements are of paternal origin (Olson and Magenis 1988). These data were supported by subsequent findings on paternally derived aberrations. Thomas et al. observed a paternal derivation of de novo unbalanced structural chromosomal abnormalities detectable by light microscopy, with 84 % interstitial deletions and 58 % duplications and rings (Thomas et al. 2006). Even more recently, de novo microdeletions associated with de novo reciprocal translocations as well as cases of complex chromosomal rearrangements were determined to be paternally derived in all cases. Likewise, array comparative genomic hybridization (aCGH) analyses helped in determining that all de novo deletions identified in men carrying balanced translocations and abnormal phenotypes derived from the fathers (Baptista et al. 2008). Other studies reported that both a recurrent de novo translocation, i.e., t(11;22), and nonrecurrent balanced reciprocal translocations were almost entirely of paternal origin, with 100 % for the former and 96 % for the latter being inherited from the fathers (Kurahashi et al. 2009; Ohye et al. 2010; Thomas et al. 2012).

Concerning the aging male, the literature provides conflicting evidence of a paternal age effect for structural rearrangements. The incongruence emerges between case studies noting that structural aberrations spontaneously occur in children conceived by older fathers and population-based studies in which such a correlation does not appear to be real (Sloter et al. 2004). Although the paternal contribution still seems rather high, information on structural aberrations in human male gametes is still scarce. This is partly due to the overall lower occurrence of such rearrangements among live births that render the paternal effect enormously complicated to define. However, the mounting development of assays that allow the detection of structural chromosomal aberrations directly within spermatozoa represents an important incentive for the evaluation of those factors, such as age, that will potentially increase the formation rate of anomalies in a man's sperm population.

The first clue of a relationship between the incidence of structural aberrations in male germ cells and paternal age comes from rodent studies. There is consistent evidence that structural aberrations in rodent spermatozoa increase with age, although the pre- and postmeiotic spermatogenetic compartments seem differently affected, with late-step spermatids, and not primary spermatocytes, showing a greater fold increase in the frequency of abnormalities between old and young mice (Pacchierotti et al. 1983). These data are supported by two micronucleus assays (Allen et al. 1996; Lowe et al. 1995) focusing on the age effect on the frequency of aberrations in round spermatids of mice and hamsters, respectively, and both leading to the conclusion that older animals have a significantly higher frequency of unstable aberrations in their spermatids compared to young animals.

Concerning human semen, the hamster-egg method in the first instance revealed itself as a relevant tool for the detection of spermatozoa bearing structural

chromosomal abnormalities such as unrejoined breaks and acentric fragments, of which 75 % resulted in unstable aberrations. The examination of 1,582 sperm chromosomal complements from 30 fertile men divided into six age groups ranging from 20 to 24 years to older than 45 years reported a fourfold increase in the total structural chromosomal abnormalities for older men (Martin and Rademaker 1987). The reanalysis of these data by Sloter et al. (2004) demonstrates that this effect is mainly due to the significant increase in chromosomal breaks, but not in acentric fragments, indicating the greater susceptibility to aging of postmeiotic DNA-repair-free spermatids. Another human-sperm/hamster-egg study (Sartorelli et al. 2001), including several men between 59 and 74 years old, reported a significantly higher frequency of acentric fragments and of complex radial figures in sperm complements of older donors compared to younger donors.

Notwithstanding its importance in producing the aforementioned results, the hamster-egg method is inefficient to measure the frequency of deletions and duplications as well as of the so-called stable rearrangements, i.e., translocations, inversions, insertions, isochromosomes, small deletions, and small duplications, in the spermatozoa and thus has been replaced by the FISH strategy. An age-related effect was observed for the frequency of centromeric deletions of chromosome 1 in a cohort of 18 men aged 20–58 years old (McInnes et al. 1998); likewise, a significant age-related increase was reported for the frequency of spermatozoa with duplications and deletions at the centromeric and subtelomeric regions of chromosome 9 in a cohort of 18 men aged 24–74 years old (Bosch et al. 2003). Another FISH-based analysis demonstrated a significant increase in the frequency of spermatozoa carrying breaks and segmental duplications and deletions of chromosome 1 among older men compared to younger men. In particular, older men showed twice the frequency of segmental duplications and deletions in chromosome 1 in their spermatozoa. Similarly, the researchers found a significant age-related increase in the frequency of spermatozoa carrying breaks within the 1q12 fragile-site region that was almost doubled in older men (Sloter et al. 2007). A more recent study based on a multicolor, multichromosome FISH strategy was performed on the semen of ten male donors 23–74 years old and found that older patients had a higher rate of structural abnormalities (6.6 %) compared to younger men (4.9 %); interestingly, although both duplications and deletions occurred more frequently in older men, an excess of duplications versus deletions was observed in both groups. In addition, the authors demonstrated a nonlinear distribution of duplications and deletions along the chromosomes and observed an inclination toward a higher susceptibility to rearrangements in larger chromosomes (Templado et al. 2011).

Overall, both human and animal studies suggest that the increased trend of delaying fatherhood could predict an augmented risk of delivering offspring liable to paternally derived genetic diseases resulting from chromosomal aneuploidies or structural aberrations, assuming that spermatozoa bearing such rearrangements are as capable of fertilizing as normal spermatozoa. However, animal models provide evidence for a paternal age effect mostly on chromosomal breaks, duplications, and deletions rather than chromosomal numeric alterations. Along these lines, duplications appear to occur more frequently than deletions, suggesting a mitotic rather than meiotic

origin for some of these sperm de novo abnormalities. As for stable rearrangements, it has been hypothesized that they would originate during spermatogenic mitotic divisions or during meiosis.

Doubtlessly, further research is needed to identify whether there are specific environmental or paternal host factors that are associated with paternally transmissible chromosomal abnormalities. Another fascinating challenge is posed by the lack of knowledge about whether there exist specific types of chromosomal abnormalities that are produced at a specific stage of germ cell production, the relative contribution that spermatogenetic mechanisms might exert on the development of chromosomal aberrations, and how both processes are affected by age.

Sperm DNA Damage

What makes DNA damage an extensively investigated topic is the irreplaceable nature of the DNA molecule. Vital information about cellular content and function is sheltered in the DNA, rendering it a crucial target for age-related degeneration. For instance, damage in the DNA can cause cell cycle arrest, cell death, or mutations the accumulation of which may lead to deregulation of transcription pathways, reduced fitness, and, ultimately, the aging phenotype. In spermatozoa, DNA damage could show up in the form of DNA fragmentation, abnormal chromatin packaging, and protamine deficiency potentially leading to cell impairment, and a number of studies have contributed to our understanding of whether an association with male aging exists. Higher levels of double-stranded DNA breaks were reported in older men (Singh et al. 2003), and a gradual age-related upward trend has been proposed for DNA fragmentation (Wyrobek et al. 2006) since the DNA fragmentation index (DFI) more than doubled in men between 20 and 60 years old. As for DNA fragmentation, data in the literature are not completely homogeneous concerning its relationship with paternal age, but there is undeniable ever-increasing evidence for a DFI augment with advancing age (Belloc et al. 2009; Plastira et al. 2007; Schmid et al. 2012; Vagnini et al. 2007).

In the myriad of DNA changes that occur as a consequence of aging, several theories collocate oxidative stress among those mechanisms predicted to play a causal role. Similarly to somatic cells, the continuous generation of reactive oxygen species (ROSs) produces oxidative damage, especially in spermatozoa, because of their high content of polyunsaturated fatty acid in the cell membrane (Aitken and Krausz 2001). Since ROSs production is likely to increase with age, it is plausible to hypothesize that, in men of advanced age, growing oxidative stress might be responsible for the age-related augment in sperm DNA damage. Moreover, changes in the efficiency of mismatch repair, base excision repair, nucleotide excision repair, and double-strand break repair mechanisms might endure the effect of aging and present themselves as cofactors in age-inflicted DNA damage. In conclusion, paternal age does indeed appear to be an additional factor that is positively correlated with an increase in DNA damage in spermatozoa deriving from men of both fertile

and infertile couples (Sartorius and Nieschlag 2010). Clearly, further research should be conducted to better define not only the nature but also the mechanisms underlying age-related changes in DNA and the extent of the damage that could be consequently produced.

Effect of Father's Age on Disease Risk

It is now fully recognized that children born from older parents are exposed to a much higher risk of having genetic disorders. This has been extensively proven for women delivering children at advanced ages, as witnessed by the strong maternal age effect for Down syndrome. However, there is an ever-growing evidence that paternal age also confers to offspring a susceptibility to a broad range of conditions, including spontaneous dominant disorders, congenital anomalies, neurological diseases (i.e., schizophrenia and autism), and some types of childhood cancers.

De Novo Mutation Rate in Male Gametes

Paternal aging is considered the major cause of new mutations in human populations (Crow 1999). Indeed, it is common knowledge that male germ cells undergo continuous cell divisions, which clearly accumulate with age, consequently leading to an accelerated mutation rate in spermatozoa. This could be due to several mechanisms, the first of which are the alterations of age-sensitive processes such as DNA replication and repair (Crow 2000). Moreover, the accumulation of mutagens from either external or internal sources, which would certainly increase with age, might also contribute to the increased occurrence of DNA replication inaccuracy.

Information available on de novo mutation rates mainly derives from studies in which the direct examination of parent-to-child transmission is limited to testing specific genes or regions, whereas the innovative whole-genome, sequencing-based studies are still inadequate to address this question. A recent study by Kong et al. addressed this issue by performing an estimate of the genome-wide mutation rate by sequencing the entire genomes of 78 parent-offspring trios (Kong et al. 2012). In particular, they focused on single nucleotide polymorphisms (SNPs), showing that the transmission of mutations to children is mainly due to fathers, and this behavior seemed closely linked to the paternal age. Considering that in this study the father's average age was 29.7 years old, the mean of the de novo mutation rate of SNPs was 1.20×10^{-8} per nucleotide per generation (Kong et al. 2012). This effect increased with the father's age (approximately two mutations per year), and the risk that children carrying harmful mutations, which could potentially lead to pathological conditions, increased proportionally.

Although in some circumstances the evidence for an association with advanced paternal age is not always consistent and reproducible, this is not the case for a small group of conditions known as paternal age effect (PAE) disorders, of which Apert syndrome and achondroplasia are considered the best representative examples. PAE disorders include some other disorders due to specific mutations in the fibroblastic growth factor receptor (*FGFR*) genes: mutations in *FGFR2* cause Apert, Crouzon, and Pfeiffer syndromes, mutations in *FGFR3* cause achondroplasia, thanatophoric dysplasia, hypochondroplasia, and Muenke syndrome. All these conditions are caused by substitution: transition/transversion at CpG dinucleotides or transition/transversion at non-CpG dinucleotides at key points within the growth factor receptor-RAS signaling pathway. These syndromes are characterized by autosomal dominant transmission; 1:30,000/130,000 birth prevalence for new mutations; paternal origin of mutations; strong paternal age effect; and a high apparent germ line mutation rate. Apert syndrome is a form of acrocephalosyndactyly, characterized by malformations of the skull, face, hands, and feet. In most cases there are two different mutations in the germ line occurring in the *FGFR2* gene: C to G at position 755, and C to G at position 758, which cause, respectively, a serine to tryptophan and a proline to arginine change in the protein (Wilkie et al. 1995). Achondroplasia is a common cause of dwarfism, and more than 99 % of the cases are caused by two different mutations in the *FGFR3* gene. In about 98 % of the cases, a G to A point mutation at nucleotide 1138 of the *FGFR3* gene causes a glycine-to-arginine substitution (Rousseau et al. 1996) and a G to C point mutation at nucleotide 1138 causes about 1 % of cases. These point mutations originate from unaffected fathers, suggesting that these events take place in the spermatogonial stem cells during spermatogenesis. The common explanation for these effects is the copy-error hypothesis, which postulates an accumulation of recurrent mutations in specific DNA hotspots. Although this process may play a specific role, alone it cannot explain these paternal age effects (Goriely and Wilkie 2012). Using a new polymerase chain reaction approach, it was possible to reveal that, although the mutational events in Apert syndrome are rare, when they take place, they become enriched because the encoded mutant proteins confer a selective advantage on spermatogonial cells, originating the so-called protein-driven *selfish* selection (Goriely et al. 2005). This mechanism is better known for somatic mutations that occur during neoplasia rather than in germ line diseases. In fact, if the same mutations that take place in PAE disorders occurred in somatic cells, they would lead to neoplasia: 755C>G and 758C>G substitutions in the *FGFR2* gene define endometrial cancer, and 1138G>A and 1138G>C in the *FGFR3* gene cause bladder cancer (Goriely and Wilkie 2012). Thus, it is important to consider that these mutational events may lead to both a specific syndrome and an oncogenetic process.

In conclusion, what are the long-term consequences of selfish selection? It seems that with age, spermatozoa of all men are progressively enriched with PAE mutations, even though PAE disorders fortunately have a low reproductive fitness; conversely, other mutations that define mild syndromes can be transmitted over many generations, representing a contribution to genetic variability.

Telomeres: The Bright Side of Aging

Telomeres are specific DNA sequences enclosing a number of $(TTAGGG)_n$ repeats located at the ends of all chromosomes. Although their function is not yet fully established, it is widely known that telomeres are involved in the protection of chromosomes from fusion, recombination, and degradation. In many tissues, telomere length (TL) is shortened by successive cell divisions, and consequently it tends to progressively diminish with age. Therefore, TL changes are believed to be implicated in cell senescence and aging as well as tumorigenesis and DNA repair (De Meyer et al. 2007; Unryn et al. 2005). Consistent with this, elderly people, whose leukocytes display shorter telomeres due to their advanced age, are presumably subjected to a higher morbidity and reduced life expectancy.

Although it is well known that TL reduces with age in most proliferating tissues, spermatozoa represent the exception to the rule. For instance, there is substantial evidence that sperm TL dynamics follows a fascinating divergent trajectory that entails the elongation of sperm telomeres with age (Aston et al. 2012; Baird et al. 2006; Kimura et al. 2008). This notion provides a completely novel facet of the effects that might be exerted by paternal age and demonstrates that sometimes clouds do have a silver lining. In fact, emerging data provide growing evidence that older fathers will transmit to their offspring longer leukocyte telomeres (Arbeev et al. 2011; De Meyer et al. 2007; Unryn et al. 2005). In addition, a recent study performed on delayed human reproduction found that such an association between paternal age and offspring's TL is cumulative across multiple generations since the paternal grandfather's age predicts longer telomeres in grandchildren at their father's birth ($p = 0.038$) (Eisenberg et al. 2012). The most common explanation for telomere lengthening among offspring of older fathers is the high telomerase activity in the testes (Baird et al. 2006; Kimura et al. 2008). Aston et al. (2012) suggest that sperm TL elongation is dependent on an overactivation of telomerase in male germ cells, leading to TL lengthening at every replication cycle (estimated value = 2.48 bp/replication). However, it remains to be defined why testicular telomerase would lead to the progressive lengthening of sperm telomeres rather than just maintain a stable length. Kimura et al. (2008) proposed that testicular telomerase exerts a preferential effect on long telomeres. This might depend on the fact that spermatozoa displaying short telomeres undergo a negative selection that with age progressively leads to their disproportional extinction (Kimura et al. 2008).

In light of these data, telomere lengthening might be considered the bright side of aging because delayed fatherhood would not only imply negative consequences, but could also confer positive traits to future generations conceived by aged fathers such as higher survival and lower risk of developing TL-related diseases.

Conclusions

Human aging includes a number of time-related processes occurring throughout adult life that guide a wide range of physiological changes that increase an individual's vulnerability to death and weaken normal functions and intensify one's susceptibility to a number of diseases. The ever-spreading phenomenon of postponing parenthood till older ages represents one of the multiple aspects of the aging process, given the recognition of advanced parental age effects.

While extensive evidence has proven maternal age to be a major and independent negative factor for fertility, the effects of paternal age remain poorly understood. However, there is growing evidence that advanced paternal age correlates with a number of complications, and 40 years old has been proposed as the "amber light" in a man's reproductive life. Reproductive studies suggest that male aging does not affect a couple's fecundity as an independent factor but that its effects manifest themselves in combination with maternal age or in the presence of altered spermatogenesis. This information might depend on the difficulty in discriminating paternal from maternal age effects, implying the need to direct further research straight to the male germ cells. This sperm-specific approach helped to define a pure paternal age effect on a multitude of issues discussed throughout this chapter (Fig. 2.1). Current data in the literature suggest that the spermatozoa of aged men apparently more frequently

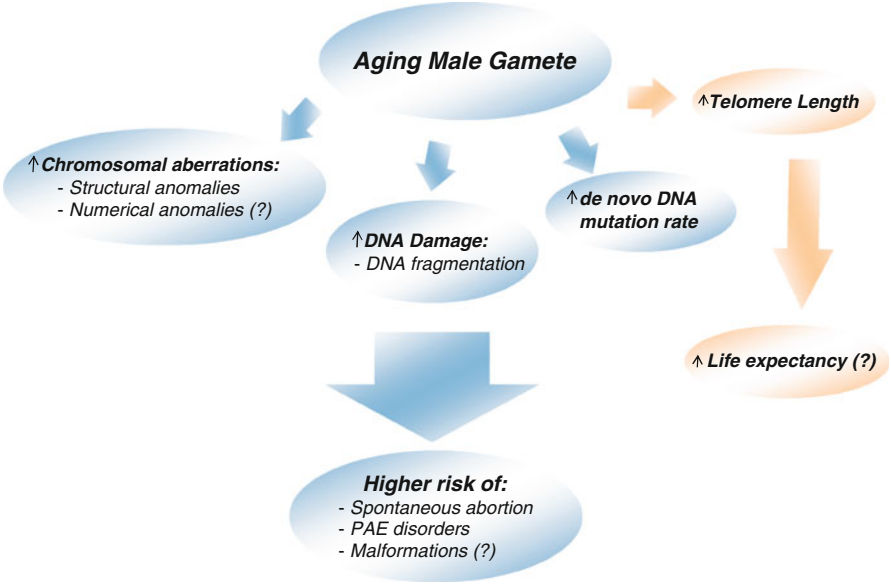

Fig. 2.1 Graphical summary of various consequences reportedly derived from the process of aging in the male gamete

undergo age-related modifications, potentially leading to various consequences. The occurrence of such alterations in male germ cells has rather important implications because any damage to reproductive cells might produce permanent effects not only on the fertility status of the questioned patient but also on the health and viability of the offspring, with potential consequences on the fitness of future generations.

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Chapter 3
Chromosomal Aberrations and Aneuploidies of Spermatozoa

Paola Piomboni, Anita Stendardi, and Laura Gambera

Abstract Chromosomal abnormalities are relevant causes of human infertility, affecting 2–14 % of infertile males. Patients with seminal anomalies could be affected by improper meiotic recombination and increased sperm chromosome aneuploidy. Since the transmission of a haploid chromosomal asset is fundamental for embryo vitality and development, the study of sperm chromosomes has become fundamental because intracytoplasmic sperm injection allows fertilization in cases of severe male infertility.

In this chapter we summarize the data on the incidence of sperm aneuploidy, detected by fluorescence in situ hybridization (FISH), in infertile men with normal or abnormal karyotype. The possibility of reducing sperm chromosomal imbalance is also reported.

Among control males, the lowest aneuploidy rate was detected (range: 0.09–0.14 % for autosomes; 0.04–0.10 % for gonosomes). In infertile patients with normal karyotype, the severity of semen alteration is correlated with the frequency of aneuploidy, particularly for X and Y chromosomes. Among patients with abnormal karyotype, 47,XXY and 47,XYY carriers showed a high variability of sperm aneuploidy both for gonosomes and autosomes. In Robertsonian translocation carriers, the increase in aneuploidy rate was particularly evident for total sex disomy, and resulted mainly from interchromosomal effect (ICE). In reciprocal translocation carriers, a high percentage of unbalanced sperm (approximately 50 %) was detected, perhaps mostly related to ICE.

P. Piomboni (✉) • L. Gambera
Department of Molecular and Developmental Medicine, University of Siena,
Viale Bracci 14, 53100 Siena, Italy

Center for Diagnosis and Treatment of Couple Sterility, S. Maria alle Scotte Hospital,
Viale Bracci 14, 53100 Siena, Italy
e-mail: paola.piomboni@unisi.it

A. Stendardi
Department of Molecular and Developmental Medicine, University of Siena,
Viale Bracci 14, 53100 Siena, Italy

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Sperm chromosomal constitution could be analyzed to obtain more accurate information about the causes of male infertility. It would be worthwhile to evaluate the benefits of a therapy with recombinant Follicle Stimulating Hormone (rFSH) on sperm chromosome segregation in selected infertile males.

Keywords FISH • Sperm aneuploidy • Male infertility • Abnormal karyotype

Introduction

Chromosomal abnormalities are a relevant cause of human infertility, affecting 2–14 % of infertile males and have been clearly demonstrated to increase proportionally with the severity of the spermatological phenotype. Both numerical and structural chromosomal aberrations are major contributors to pregnancy loss (Egozcue et al. 2000a), perinatal death, congenital malformations, mental retardation, and behavioral anomalies (Hook 1985; Hecht and Hecht 1987), the latter accounting for the 0.8–1 % of live births (Gardner and Sutherland 2004).

Among the abnormalities, trisomy of chromosomes 13, 18, and 21 and aneuploidies of the sex chromosomes constitute the most important load of congenital abnormalities. In most cases, autosomal trisomies originate in maternal germ cells, whereas sex chromosome aneuploidies are frequently of paternal origin, occurring during spermatogenesis (Sloter et al. 2004).

The term spermatogenesis indicates the processes by which primordial germ cells, namely spermatogonia, become haploid sperm cells. Spermatogonia divide by mitosis, giving rise to primary spermatocytes, which undergo a meiotic process. Meiosis includes two successive cell divisions without DNA replication. During the first and second meiotic divisions, homologous chromosomes separate to form haploid gametes. At the end of spermatogenesis, the haploid spermatid nucleus contains 23 chromosomes with one chromatid. Soon after, they are transformed into spermatozoa by a morphogenetic process, spermiogenesis. Recently, several lines of evidence have linked unexplained male infertility to meiotic defects in pairing, synapsis, and recombination and to an increase in aneuploid sperm (Tempest and Martin 2009).

Errors during mitotic or meiotic divisions may lead to aneuploid gametes, in which autosomes or the sex chromosomes are affected. Aneuploidy, the most frequently detected cytogenetic abnormality, is defined as the condition of having fewer or more than the euploid number of chromosomes. Aneuploidies in male gametes may be caused by two main mechanisms: (1) nondisjunction of chromatid pairs during mitosis or meiosis II or nondisjunction of homologous chromosomes during meiosis I; (2) chromosome lagging near the equator at anaphase followed by chromosome loss (Ford et al. 1988).

The incidence of sperm aneuploidy increases proportionally with the severity of the male-factor sterility, including Y chromosome microdeletions, as confirmed by various studies suggesting that, in selected cases, the paternal contribution to aneuploidy in the developing conceptus could be more relevant than expected from

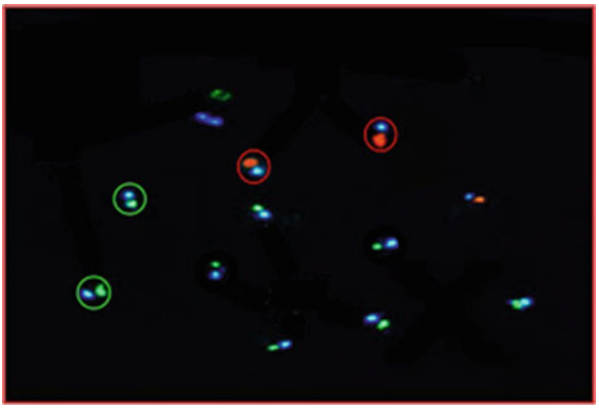

Fig. 3.1 Fish analysis using different centromeric-DNA probes for the simultaneous detection of chromosome 18 (*aqua*), X (*green*) and Y (*red*). Euploid sperm nuclei with 18,X (*green circles*), and 18,Y (*red circles*) complements are observed

general data on aborted fetuses and live births (Gianaroli et al. 2005; Magli et al. 2009; Mateu et al. 2010; Kahraman et al. 2006; Harton and Helen 2012).

This is particularly true in cases of assisted reproductive techniques, such as intracytoplasmic sperm injection (ICSI), that have improved the chances of achieving pregnancy also for patients with severe seminal anomalies (Van Steirteghem et al. 1993, 1996), despite an increased incidence of embryo aneuploidies (Verpoest and Tournaye 2006; Tesarik and Mendoza 2007). In particular, it appears that men with severe factor infertility treated by ICSI have an increased risk of generating offspring with unbalanced chromosomal constitution (Rimm et al. 2004; Wen et al. 2012).

The clinical use of aneuploidy screening should be recommended in patients affected by Klinefelter syndrome, structural rearrangement of karyotype, severe teratozoospermia, nonobstructive azoospermia, as well as in patients with recurrent pregnancy loss or unexplained repeated in vitro fertilization (IVF) failures.

The development of the FISH technique, which uses a chromosome-specific DNA probe detected by fluorescence microscopy, and its application to the study of sperm aneuploidy has made possible the screening of a large number of germ cells in a relatively short time. In addition, the simultaneous use of different probes allows for the evaluation of aneuploidy frequency for different human chromosomes in normal (Fig. 3.1) and pathological conditions (Figs. 3.2 and 3.3) (Egozcue et al. 1997; Downie et al. 1997; Guttenbach et al. 1997a; Rives et al. 1999; Carrel 2008; McLachlan and O'Bryan 2010). Since 1990, this technique has been used to analyze chromosome aneuploidies in sperm, and many papers have been published on sperm aneuploidies even in control individuals (Downie et al. 1997; Guttenbach et al. 1997a; Egozcue et al. 1997; Rives et al. 1999). Nevertheless, FISH has its limits due, for example, to the degree of chromatin condensation since condensation efficiency is directly correlated with fluorescent signal quality (Vidal et al.

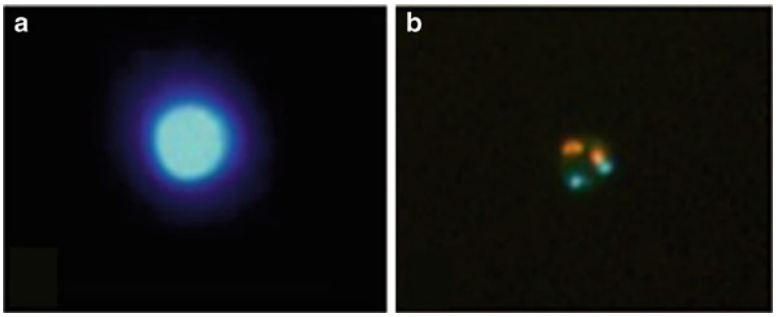

Fig. 3.2 DAPI counterstaining (**a**) and FISH analysis (**b**) showing a disomic 18,YY sperm

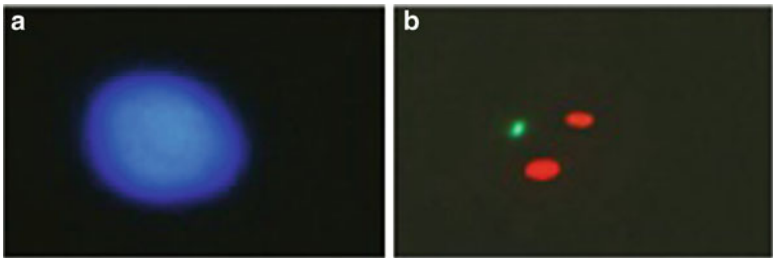

Fig. 3.3 DAPI counterstaining (**a**) and FISH analysis (**b**) showing a diploid 18,18,YY sperm

1993; Egozcue et al. 1997). Moreover, FISH does not enable one to appreciate the difference between nullisomy (the absence of a chromosome) and the absence of hybridization, despite the fact that if one of the probes gives a correct signal, the absence of a signal from the other probe may be considered evidence of nullisomy (Holmes and Martin 1993; Bischoff et al. 1994). Furthermore, FISH on decondensed sperm head only provides information about anomalies in the chromosome number, while the analysis of chromosome structural rearrangement is still complex due to the use of locus-specific probes not always able to hybridize. In these latter cases, sperm karyotyping, single-sperm polymerase chain reaction, or single-sperm typing should be applied to improve the identification of recombination in specific genome areas (Martin 2008).

Sperm karyotyping through a fusion assay was originally used, with the drawback of the technique being that it is laborious, technically difficult, and only allows for studying sperm that are able to fuse with a hamster oocyte, yielding results on a relatively small number of cells. The advent of FISH revolutionized the study of sperm chromosome constitution, mitigating many of these problems, except that it no longer allowed the whole chromosome complement to be studied in a single cell or structural aberrations to be picked up without the use of specially designed probes.

Nevertheless, not all 24 chromosomes (22 autosomes plus X and Y chromosomes) can be easily assessed in a single cell, and a limited number of fluorocromes are available; therefore 3–5 signals at most are technically feasible.

FISH Analysis in Normozoospermic Males as Reference Value for Aneuploidy Frequency

Sperm chromosomal aneuploidies have been investigated by FISH in normal donors by various authors. Unfortunately, it is not always easy to compare the results of these studies and identify a reference value, mainly because of the differences in the frequency and distribution of aneuploidies. This heterogeneity can be ascribed to differences in application of the technique, such as sperm decondensation, scoring criteria, storage of samples, types of probe, number of sperm scored, or data analysis and reporting and donor selection criteria (e.g., *normozoospermic*, *normozoospermic and fertile*, *fertile*, or *healthy* men).

To provide some useful information on this topic, we compared data from 19 different studies selected on the basis of donor characteristics and techniques (Gambera et al. 2011). According to donor selection criteria, we distinguished three subgroups: *normozoospermic* – a total of 55 donors with reported normal semen parameters according to the World Health Organization (WHO) (1999, 2010) or Kruger morphology (1986) criteria but without information about fertility; *normozoospermic and fertile* – including 57 normozoospermic donors of proven fertility; *fertile* – including 37 donors with proven fertility but without information about semen parameters. In these studies sperm aneuploidies were evaluated using specific probes for all chromosomes, with the most investigated being 13, 18, 21, X, and Y chromosomes mainly because aneuploidies of these chromosomes do not preclude embryo development and survival. Overall information about sperm chromosome quantitative alteration was given as the total frequency of aneuploidies. The weighted mean of autosomal disomy for each chromosome ranged from a minimum of 0.05 % to a maximum that varied somewhat in the different groups: 0.14 ± 0.06 % in *normozoospermic and fertile*, 0.23 ± 0.08 % in the *normozoospermic* group, and 0.09 ± 0.09 % in *fertile* (Table 3.1).

Disomies for chromosomes 13, 18, and 21 have been extensively studied and provide a statistically significant background for the interpretation of results. The disomy rate for each chromosome varied only slightly in the three groups and between groups. However, chromosome 21 disomy had a significantly higher incidence in the *normozoospermic* group, suggesting greater susceptibility to nondisjunction for this chromosome.

Sex chromosome disomies were also investigated, and XY disomy frequency increased more than twice in comparison with XX and YY disomies. Therefore, it seems that errors in meiosis I, giving rise to sperm with XY chromosomes, should be more frequent than errors in meiosis II, which generate X or Y disomic sperm (Fig. 3.2). On the other hand, it must be considered that errors in meiosis I give rise to two disomic and two nullisomic sperm, while errors in meiosis II may produce a disomic sperm and a nullisomic one. The diploidy rate, evaluated by three fluorescent probes, one for autosomes and two for sex chromosomes, was not statistically different among the three groups.

Table 3.1 Aneuploidies and diploidies in fertile and infertile men ICSI candidates (Modified from Gambera et al. 2011)

	Autosomal								Sexual						
	Disomy (%)								Disomy (%)						
	1	7	8	12	13	18	21	Dipl.[a] (%)	XX	YY	XY	Tot.D[b] (%)	Dipl.[a] (%)	Tot.A[c] (%)	Tot.N[d] (%)
Sperm aneuploidy frequency in normospermic fertile men															
Normozoospermic															
Mean ± Standard deviation			*0.11 ± 0.07*	*0.05 ± 0.00*	*0.12 ± 0.04*	*0.12 ± 0.06*	*0.23 ± 0.08*	*0.15 ± 0.08*	*0.07 ± 0.05*	*0.07 ± 0.03*	*0.14 ± 0.08*	*0.15 ± 0.03*	*0.21 ± 0.11*	*1.01 ± 0.99*	*0.07 ± 0.00*
Normozoospermic and fertile															
Mean ± Standard deviation					*0.13 ± 0.05*	*0.09 ± 0.08*	*0.14 ± 0.06*	*0.12 ± 0.08*	*0.04 ± 0.02*	*0.05 ± 0.03*	*0.10 ± 0.05*	*0.34 ± 0.13*	*0.17 ± 0.11*	*0.50 ± 0.25*	*0.27 ± 0.13*
Fertile															
Mean ± Standard deviation						*0.09 ± 0.09*		*0.12 ± 0.02*	*0.14 ± 0.08*	*0.16 ± 0.10*	*0.32 ± 0.17*		*0.25 ± 0.10*	*1.01 ± 0.35*	*0.10 ± 0.03*
Sperm aneuploidy frequency in men with alterations of sperm parameters and normal karyotype															
Sperm concentration															
Oligozoospermia (5-20×10⁶)															
Mean ± Standard deviation						*0.08 ± 0.05*			*0.09 ± 0.03*	*0.09 ± 0.04*	*0.42 ± 0.22*		*0.52 ± 0.27*		
Severe oligozoospermia (<5×10⁶)															
Mean ± Standard deviation						*0.10 ± 0.09*			*0.10 ± 0.03*	*0.20 ± 0.13*	*0.57 ± 0.11*		*0.69 ± 0.36*		
Sperm morphology															
Teratozoospermia															
Mean ± Standard deviation			*0.62 ± 0.48*	*2.40 ± 4.75*	*0.51 ± 0.27*	*0.72 ± 1.32*	*0.68 ± 0.29*		*0.43 ± 0.74*	*1.05 ± 0.79*	*1.71 ± 1.91*		*0.50 ± 0.28*		
Teratoastenozoospermia															
Mean ± Standard deviation									*0.52 ± 0.45*	*0.46 ± 0.37*	*0.23 ± 0.10*		*0.06 ± 0.04*		
Oligoteratozoospermia															
Mean ± Standard deviation									*0.49 ± 0.23*	*0.44 ± 0.21*			*0.14 ± 0.15*		
Globozoospermia															

Mean ± Standard deviation					*0.95 ± 2.51*	*0.24 ± 0.26*	*0.10 ± 1.49*		*0.28 ± 0.19*	*0.16 ± 0.16*	*1.51 ± 2.18*		*0.50 ± 0.35*	
Sperm motility														
Asthenozoospermia														
Mean ± Standard deviation						*0.12 ± 0.14*			*0.09 ± 0.02*	*0.10 ± 0.01*	*0.23 ± 0.09*		*0.39 ± 0.10*	
Oligoastenoterato-zoospermia														
Mean ± Standard deviation	*0.28 ± 0.14*	*0.74 ± 0.83*	*1.63 ± 0.30*	*0.59 ± 0.24*	*0.46 ± 0.84*	*0.35 ± 0.45*	*0.54 ± 0.80*		*0.42 ± 0.80*	*0.41 ± 0.67*	*0.79 ± 3.81*		*0.45 ± 0.67*	
Genetic asthenozoospermia														
Mean ± Standard deviation						*0.10 ± 0.04*			*0.12 ± 0.09*	*0.14 ± 0.12*	*0.28 ± 0.22*		*0.43 ± 0.23*	
Sperm aneuploidy in male ICSI candidates														
Oligoastenoteratospermia														
Mean ± Standard deviation			*1.53 ± 0.29*	*0.37 ± 0.43*	*0.87 ± 0.72*	*0.64 ± 0.62*	*0.74 ± 0.80*	*0.37 ± 0.33*	*0.35 ± 0.28*	*0.40 ± 0.14*	*0.27 ± 0.11*	*1.13 ± 0.80*	*1.12 ± 1.08*	*0.89 ± 0.98*
Azoospermia														
Mean ± Standard deviation			*0.69 ± 0.22*		*0.79 ± 1.04*	*1.05 ± 0.81*	*1.49 ± 1.91*	*4.28 ± 4.97*	*0.95 ± 1.30*	*1.21 ± 1.43*	*1.64 ± 2.50*	*2.53 ± 1.54*		*3.35 ± 4.57*

[a]Diploidy
[b]Total disomy
[c]Total aneuploidy
[d]Total nullisomy

Few extensive studies have reported high aneuploidy frequencies for chromosomes 14, 21, 22, X, and Y (Shi and Martin 2000a; Templado et al. 2005). A review of the available literature revealed only the disomy frequency of 21, X, and Y in reference subjects increased. This finding could be explained by the hypothesis that chromosomes 21, X, and Y may be prone to recombination reduction or failure during meiosis (Shen et al. 1998) because they have a single chiasmata (Sun et al. 2004), which increases the probability of incorrect segregation during meiosis I (Koehler et al. 1996; Templado et al. 2005).

A lower incidence of the mean of total aneuploidies in male *normozoospermic and fertile* subjects (0.50 ± 0.25 %) is not unexpected since normozoospermia alone does not necessarily indicate fully fertile status. Moreover, it is well known that donor age and lifestyle, including aspects such as smoking, alcohol consumption, and exposure to toxic substances, can affect semen quality. Therefore, the sperm chromosomal aneuploidy rate probably varies significantly in time; in addition, the fertile status cannot be considered constant throughout a person's life, according to several published studies on the effects of age and environmental factors on sperm aneuploidy rate (Bosch et al. 2001, 2003; Naccarati et al. 2003; Templado et al. 2011b).

FISH Analysis in Men with Alterations of Sperm Parameters

Nearly all studies investigating sperm aneuploidy in infertile men have demonstrated a significant increase in aneuploidy levels compared to their fertile counterparts (Templado et al. 2005; Sarrate et al. 2005; Miharu 2005). The majority of studies report around a threefold increase in the sperm aneuploidy rate in infertile men.

Increases in sperm aneuploidy are strongly correlated with an increasing severity of infertility: the highest level of aneuploidy was reported in men with severe oligoasthenoteratozoospermia and in cases of nonobstructive azoospermia where sperm are retrieved from testicular tissue (Vidal et al. 2001; Egozcue et al. 2003; Miharu 2005).

Furthermore, FISH data have been reported for all seminal phenotypes, including oligozoospermia (low concentration), asthenozoospermia (poor motility), and teratozoospermia (poor morphology) in infertile males with normal karyotype (Bernardini et al. 1997, 1998; Lahdetie et al. 1997; McInnes et al. 1998; Storeng et al. 1998; Pang et al. 1999; Ushijima et al. 2000; Vegetti et al. 2000; Calogero et al. 2001a; Shi and Martin 2001; Vidal et al. 2001; Egozcue et al. 2003; Rives et al. 2004; Miharu 2005; Sarrate et al. 2005; Templado et al. 2005).

In particular, a negative correlation has been reported between sperm aneuploidy rate and progressive motility (Ushijima et al. 2000; Vegetti et al. 2000; Celik-Ozenki et al. 2004; Collodel et al. 2007), normal morphology (Bernardini et al. 1998; Calogero et al. 2001a; Ryu et al. 2001; Lewis-Jones et al. 2003; Carrell et al. 2004), and nuclear maturity (Kovanci et al. 2001).

Nevertheless, seminal alterations such as oligozoospermia, asthenozoospermia, and teratozoospermia are often detected simultaneously in the same ejaculate because a damaged seminiferous epithelium produces fewer sperm, generally with abnormal morphology and, therefore, with decreased motility, as in oligoasthenoteratozoospermic (OAT) patients. A high percentage of aneuploid sperm could be produced as a result of the negative influence of any testicular pathology on spermatogenetic processes (Martin et al. 1993; Bischoff et al. 1994; Spriggs et al. 1995; Calogero et al. 2001a).

Among sperm alterations, reduced sperm concentration is reported to be the most strongly associated with chromosomal aneuploidies (Ohashi et al. 2001; Martin et al. 2003a; Nagvenkar et al. 2005) because severe quantitative impairment of spermatogenesis has been related to qualitative alterations of chromosome recombination and segregation during spermatogenesis (Egozcue et al. 2005; Miharu 2005; Sarrate et al. 2005).

Among chromosomes, gonosomes are the most susceptible to nondisjunction because X and Y are generally involved in only one crossover in the pseudoautosomal region, and thus if this process remains incomplete, normal disjunction does not occur.

Among autosomes, disomies of chromosomes 13, 18, and 21 were found to increase in OAT patients with a frequency of more than 3 times higher in comparison with *normozoospermic and fertile* controls (Table 3.1). Sex chromosomes were particularly affected by OAT condition: the mean disomy rates resulted in significantly increases in all gonosomes and mainly for XY disomy, which was approximately eight times higher than in *normozoospermic and fertile* controls (Table 3.1).

Taken together, all these data suggest that men with impaired spermatogenesis should have reduced genome-wide recombination leading to chromosome-specific sperm defects.

Sperm morphology is considered one of the main criteria for sperm selection before an assisted reproductive procedure. Lee et al. (1996) analyzed the chromosomal constitution of human sperm injected into mouse oocytes. Sperm with abnormal head morphology showed a frequency of structural chromosomal aberrations approximately four times higher than those with normal morphology. The statement that teratozoospermic patients have an aneuploidy rate significantly higher than controls has been confirmed by several authors using multicolor FISH analysis: the frequency of chromosome 18 disomy was approximately eight times greater than in *normozoospermic and fertile* controls (Table 3.1). Teratozoospermic samples also showed a significant increase in the frequency of disomy for sex chromosomes: some morphological abnormalities may be more closely associated with chromosome imbalance, particularly those involving the sperm head (Sun et al. 2006).

When a high percentage of macrocephalic, multinucleate, and multiflagellate sperm are detected, autosome and gonosome frequencies show an approximately tenfold increase, as reported by various authors (Table 3.1). Globozoospermia, a peculiar sperm head alteration of genetic origin, seems to be strictly associated with a higher incidence of sperm aneuploidies (Carrell et al. 1999, 2001; Moretti et al. 2005).

While a consensus exists on the role played by severe oligozoospermia and teratozoospermia on sperm aneuploidy and diploidy, whether isolated asthenozoospermia affects sperm aneuploidy is less clear. It is often difficult to group data from asthenozoospermic samples into separate categories due to the concomitant alteration of other sperm parameters, such as concentration and morphology.

Isolated asthenozoospermia has been reported by few authors (Aran et al. 1999; Bernardini et al. 2005; Collodel et al. 2007) analyzing a range of autosomes (1, 4, 8, 12, 13, 18, and 21) and sex chromosomes: on the whole, increased sperm disomy and diploidy rates were detected with respect to the controls.

In other cases of absolute asthenozoospermia characterized by systematic sperm anomalies of the flagella, such as stump tail syndrome and Kartagener syndrome, abnormal aneuploidy and diploidy rates were confirmed by different authors (Rives et al. 2005). In the case of fibrous sheath dysplasia, some studies reported that the mean frequency of diploidy (0.43 ± 0.23 %) and sex chromosome aneuploidies increased sharply in comparison to controls group (Baccetti et al. 2005; Moretti and Collodel 2006; Piomboni et al. 2007).

Sperm Aneuploidy in Infertile Male ICSI Candidates to ICSI

The advent of ICSI (Palermo et al. 1992) revolutionized the treatment of male infertility by allowing patients with severely compromised semen parameters to achieve fatherhood. Although sperm with the "best" morphological features are selected for injection into the oocyte, this is not an absolute indicator of a normal genetic constitution (Ryu et al. 2001; Burrello et al. 2004; Celik-Ozenci et al. 2004), and the transmission of chromosomal abnormalities to offspring is possible.

Various researches have shown that prenatal karyotypes of embryos obtained by ICSI have higher sex chromosome aneuploidy rates (0.6 % versus 0.2 %) and higher autosomal structural alterations (0.4 % versus 0.07 %) than the general neonatal population (Veld et al. 1997; Bonduelle et al. 1998, 2002; Van Steirteghem et al. 2002). Several clinical studies suggested a strong correlation between the aneuploidy rate of male gametes and ICSI outcome: implantation failure, decreased pregnancy, and increased miscarriage rates after ICSI have been reported in OAT male when FISH analysis demonstrated abnormal aneuploidy and diploidy frequencies.

Few studies published so far have found an effect of sperm aneuploidies on the outcome of ICSI (Colombero et al. 1999; Calogero et al. 2001b) and reported comparable fertilization rates, clinical pregnancy rates, pregnancy losses, and occurrence of neonatal malformations in males with both normal and abnormal semen parameters. However, these authors concluded that, although the overall ICSI outcome was not significantly correlated with sperm aneuploidy, a tendency to a lower aneuploidy rate was underlined in the male partner of pregnant women.

The evaluation of the influence of chromosome abnormalities in men with altered semen parameters undergoing an Assisted Reproductive Technologies (ART) procedure could be biased by semen selection methods. Many reports consistently found an increase in aneuploidy rates in subfertile men, underlining that conventional sperm separation techniques are not able to exclude aneuploid gametes from fertilizing pools (Samura et al. 1997; Pfeffer et al. 1999; Van Dyk et al. 2000). More recently, a selection technique based on hyaluronic acid (HA)–sperm binding was demonstrated as being able to recover a high percentage of euploid sperm: the advantages of HA-mediated sperm selection in relation to ICSI outcome improvement could be due to the decreasing frequency of chromosomal disomy and diploidy, which results in a four- to sixfold reduction in comparison with whole semen samples (Jakab et al. 2005; Huszar et al. 2007).

With regard to clinical practice, sperm aneuploidy screening may be recommended especially in those countries, such as Italy, where preimplantation diagnosis can be performed only in selected cases and therefore FISH on male gametes seems to be the only possible technique for determining the risk of generating unbalanced embryos.

ICSI with Testicular or Epididymal Sperm

Sperm extracted from the epididymis (MESA) or testicular tissue (TESA/TESE) have a substantially increased risk of chromosomal abnormalities, and therefore FISH investigation appears even more suitable. A high aneuploidy rate in testicular sperm recovered from nonobstructive azoospermic (NOA) patients has been widely reported (Bernardini et al. 2000; Levron et al. 2001; Burrello et al. 2002; Mateizel et al. 2002; Palermo et al. 2002; Rodrigo et al. 2004; Gianaroli et al. 2005; Vozdova et al 2012). These data have not been confirmed by Martin et al. (2000), who analyzed aneuploidy frequencies for chromosomes 13, 21, X, and Y in sperm from three men with nonobstructive azoospermia. The authors concluded that NOA patients may not have a substantially increased risk of chromosomally abnormal sperm, in comparison to healthy men. Nevertheless, in these cases it could be take into consideration that only a small number of testicular sperm are available for FISH analysis, and this could affect the accuracy of the estimated aneuploidy rate.

A higher incidence of chromosomal anomalies in epididymal than in ejaculated sperm has also been reported (Bernardini et al. 2000; Burrello et al. 2002; Palermo et al. 2002; Levron et al. 2001; Rodrigo et al. 2004).

We therefore can conclude that chromosomal abnormalities affect the ICSI outcome when sperm are obtained by MESA and TESE, decreasing the fertilization and pregnancy rates and increasing the miscarriage rate.

On the whole, embryos originated by azoospermic patients have an increased rate of chromosomal abnormalities, and therefore appropriate genetic counseling should be offered before ICSI.

Sperm Aneuploidy in Infertile Male Carriers of Chromosomal Alterations

The incidence of constitutional chromosomal abnormalities is approximately 2 % in males with combined indications of infertility (Meschede et al. 1997), 5 % in oligozoospermic, and 14 % in azoospermic men (Johnson 1998). The most common karyotype abnormalities in infertile men include numerical sex chromosome alterations and Robertsonian translocations (Shi and Martin 2001).

Numerical Sex Chromosome Abnormalities

47,XYY

The extra Y chromosome in 47,XYY males may arise by at least two mechanisms: paternal nondisjunction at meiosis II after normal chiasmata in meiosis I (84 %) or postzygotic mitotic error (16 %) (Robinson and Jacobs 1999; Rives et al. 2003a). Males with an extra Y chromosome are generally fertile, and meiotic studies carried out in these patients indicated that the extra Y chromosome is frequently lost during the premeiotic stages (Thompson et al. 1967; Chandley et al. 1976). Nevertheless, in some cases one X and two Y chromosomes have been detected during prophase I as an X univalent plus a YY bivalent (Hulten and Pearson 1971; Speed et al. 1991; Blanco et al. 1997). No increase in the frequency of any category of sex chromosomal aneuploidy was found in 47,XYY patients by Han et al. (1994). In contrast, several authors (Martini et al. 1996; Mercier et al. 1996; Morel et al. 1999; Lim et al. 1999a; Martin et al. 1999; Giltay et al. 2000; Wang et al. 2000; Moretti et al. 2007) reported a moderate increase in sex chromosome disomies.

Globally, the frequencies of sperm with an abnormal number of sex chromosomes range from 0.04% to 19 % depending on the study: the mean XY disomy rate increased sharply (4.43 ± 6.03 %) as shown by an evaluation of comparable data from different studies (Table 3.2). The general finding is that the persistence of an extra chromosome in germ cells of 47,XYY males can impair spermatogenesis, determining a low sperm count. Since most children of 47,XYY fathers have a normal karyotype, the extra Y chromosome may presumably be lost during meiosis (Shi and Martin 2000b, 2001). Nevertheless some XYY germ cells can complete meiosis and produce mature aneuploid sperm.

Recent review studies (Sarrate et al. 2005; Rodrigo et al. 2010) indicated that 3.7 % of the spermatozoa analyzed by FISH carry an extra sex chromosome and that diploid sperm ranges from 0 to 3.35 %.

On the other hand, males with a mosaic 47,XYY/46,XY showed a lower cumulative rate of sex chromosome aneuploidy in sperm than XYY patients. The mean gonosome disomy resulting from comparable data reported in the literature ranged from 0.17 ± 0.16 % for XX to 0.48 ± 0.31 % for XY (Table 3.2). As regards the risk

Table 3.2 Sperm aneuploidy frequency in infertile male carriers of chromosomal unbalance (Modified from Gambera et al. 2011)

	Autosomal disomy (%)			Sexual disomy (%)		
	13	18	21	XX	YY	XY
47, XYY						
Mean ± Standard deviation				*1.65 ± 2.31*	*1.54 ± 1.53*	*4.43 ± 6.03*
Mosaic 47,XYY/46,XY						
Mean ± Standard deviation				*0.17 ± 0.16*	*0.50 ± 0.45*	*0.48 ± 0.31*
47, XXY Klinefelter syndrome						
Mean ± Standard deviation				*4.64 ± 2.56*	*0.30 ± 0.46*	*11.1 ± 6.89*
Mosaic 47,XXY/46,XY						
Mean ± Standard deviation				*0.40 ± 0.31*	*0.42 ± 0.49*	*1.22 ± 0.71*
Robertsonian translocations						
Mean ± Standard deviation	*2.65 ± 2.73*	*0.22 ± 0.27*	*0.62 ± 0.89*	*0.65 ± 0.92 (Total sex disomy %)*	*1.20 ± 1.91 (Diploidy %)*	

assessment of the transmission of chromosomal aberration to embryos, Gonzalez-Merino et al. (2007) analyzed 47 preimplantation embryos and reported a total aneuploidy rate of 32 %.

47,XXY, Klinefelter Syndrome

Infertile males affected by Klinefelter syndrome (KS) are approximately 3 %, increasing up to 11 % among azoospermic men (Foresta et al. 1999). These subjects are rarely naturally fertile, although assisted reproductive procedures such as ICSI offer them a chance at fatherhood. The sperm phenotype among KS males is widely heterogeneous, ranging from azoospermia to normozoospermia.

The extra X chromosome in males with KS may arise by a paternal nondisjunction at meiosis I in approximately 50 % of cases (Hall et al. 2006). During spermatogenesis, the extra sex chromosome appears to be eliminated (Shi and Martin 2001). On the other end, many studies carried out in 47,XXY males detected marked increases in sex chromosome disomies and diploid sperm (Guttenbach et al. 1997b; Estop et al. 1998; Foresta et al. 1998, 1999; Okada et al. 1999; Rives et al. 2000; Morel et al. 2003; Ferlin et al. 2005; Sarrate et al. 2005; Templado et al. 2011a). The mean disomy rate increased sharply for XX (4.64 ± 2.56 %) and XY (11.1 ± 6.89 %),

with an average incidence of 6.3 % (Table 3.2). Diploid sperm in these patients also increased, ranging from 0.03% to 4.2 %, as did autosomal aneuploidies, reaching 6.2 % for chromosome 21 (Templado et al 2011a).

In patients with mosaic KS, the frequency of sperm aneuploidy varied according to the percentage of 47,XXY cells. Various FISH studies (Chevret et al. 1996; Martini et al. 1996; Lim et al. 1999b; Okada et al. 1999; Rives et al. 2000; Ferlin et al. 2005) have demonstrated an increased frequency of sex chromosome disomy, ranging from 0.40% to 1.22 % for XY (Table 3.2).

Chromosomal Translocations

Balanced chromosomal translocations are characterized by breakpoints in two chromosomes and repair of the chromosomal fragments with transpositions of genetic material between them, without loss of genetic material.

Male carriers of these structural alterations generally have a normal phenotype while showing a reduced fertility and an increase in spontaneous miscarriage and birth defects.

Robertsonian Translocations

Robertsonian translocations are the most common chromosomal anomaly among infertile men, characterized by the centric fusion of two acrocentric chromosomes (13, 14, 15, 21, 22) and resulting in a 45 chromosome karyotype. The most frequent reorganization are t(13q;14q) and t(14q;21q), with an estimated frequency of 0.97 and 0.20 %, respectively (Frydman et al. 2001). Before the report of Plymate et al. (1976), testicular function defects were only associated with sex chromosome abnormalities (Paulsen et al. 1968). Since 1976, many studies have shown that carriers of chromosome anomalies, especially translocations, have an altered spermatogenesis characterized by severe oligozoospermia (Chandley et al. 1976; Veld et al. 1997; Ogawa et al. 2000). In addition, unusual ultrastructural sperm anomalies related to immaturity were observed in carriers of Robertsonian translocation (Baccetti et al. 2002). Spermatogenetic alterations could be a consequence of a chromosomal anomaly: the pairing of the reorganized chromosomes during meiotic prophase I gives rise to a trivalent configuration that is prone to segregate in an alternate way, producing normal or balanced sperm (Sybenga 1975; Vidal et al. 1982; Luciani et al. 1984). Unbalanced sperm are generated by an adjacent segregation pattern, and they could be responsible for miscarriages or aneuploid offspring (Egozcue et al. 2000b).

In Robertsonian translocation carriers, FISH analysis demonstrated a percentage of normal or balanced sperm ranging from 73.6 up to 91 % (Escudero et al. 2000; Morel et al. 2001; Anton et al. 2004; Roux et al. 2005; Nishikawa et al. 2008). Contrasting results showing a high percentage of unbalanced sperm derived from adjacent segregation, ranging from 3 % to 36 % (reviewed by Harton and Tempest 2012).

Reciprocal Translocations

Exchanges of genetic material between two or more chromosomes characterize the reciprocal translocation. A wide range of different situations is included in this structural chromosomal anomaly, each of them unique in individual families, depending on the chromosome involved, the size of the translocated regions, and the probability of recombination within these regions (Harton and Tempest 2012). Reciprocal translocations are the most frequent (1/600) structural chromosomal anomalies in humans (Estop et al. 1997). Among infertile males these chromosomal reorganizations are approximately ten times more frequent than in the general population (Van Assche et al. 1996), and a high level of unbalanced gametes are reported in various studies ranging from 29 % up to 81 % (Harton and Tempest 2012), with an average of 50 % (Shi and Martin 2001).

The meiotic behavior of reciprocal translocations depends on the chromosomes involved in the rearrangement, the position of the breakpoints, the presence of crossovers in the translocated chromosomes, and the morphological characteristics of the rearranged chromosomes. During meiosis I, segregation of the quadrivalent formed among the translocated chromosomes and their normal homologs produces a variety of balanced and unbalanced gametes. In the alternate segregation pattern, where homologous centromeres move to opposite poles, chromosomally balanced or normal gametes are produced. Unbalanced gametes are produced by the other segregation patterns, specifically adjacent I, adjacent II, and 3:1 segregation.

Alternate segregation is the most common meiotic behavior, occurring with a frequency of 44–51 %; adjacent I segregants have a frequency of 16–40 %, while adjacent II segregants have a lower mean frequency of approximately 9 % (Shi and Martin 2001), which varied inversely with the length of the shorter centric segment (Faraut et al. 2000). Finally, 3:1 segregants occur with a mean frequency of 11 % (Shi and Martin 2001) even if, in some cases, 3:1 segregation is the preferential pattern (Jalbert et al. 1980; Estop et al. 1999; Van Assche et al. 1999) with an unusually high rate up to 23.5 % as reported in four different reciprocal translocation carriers (Nishikawa et al 2008).

An analysis of familial cases confirmed that segregation patterns were specific for a given translocation, as demonstrated by detection of the same profile of meiotic segregation mode in each family (Rousseaux et al. 1995; Cora et al. 2002; Anton et al. 2004; Morel et al. 2004; Wiland et al. 2007).

Interchromosomal Effect

The possibility that chromosome rearrangements could interfere with the meiotic behavior of chromosomes not involved in translocation led to the concept of interchromosomal effect (ICE), postulated for the first time in humans by Lejeune (1965).

Meiotic segregation of sex chromosomes and autosomes was investigated directly on sperm nuclei by FISH by various authors (Rousseaux et al. 1995; Blanco et al. 2000; Vegetti et al. 2000; Morel et al. 2001; Anton et al. 2004), and the results

suggested that ICE was generally restricted to translocation carriers with abnormal semen parameters.

In six carriers of Robertsonian translocations t(13;21) and t(14;22), the interactions between chromosome rearrangements and ICE were studied by evaluating aneuploidy and diploidy frequencies of chromosomes 18, X, and Y: the mean percentage of sex chromosome disomy as well as the frequency of diploid sperm were significantly higher than in controls (Baccetti et al. 2005)

Therefore, the increase in sperm aneuploidies among Robertsonian translocation carriers could be related to ICE, as suggested by many studies (Blanco et al. 2000; Vegetti et al. 2000; Morel et al. 2001; Baccetti et al. 2002, 2005; Anton et al. 2004; Ogur et al. 2006; Chen et al. 2007). However¸ a negative effect of an altered testicular environment on the meiotic process cannot be excluded in any of these studies because none of the enrolled subjects with translocations was classified as normozoospermic.

The question of ICE in reciprocal translocation carriers is still controversial. Some authors did not report any evidence of ICE in several reciprocal translocation carriers (Van Hummelen et al. 1997; Honda et al. 1999; Estop et al. 2000; Rives et al. 2003b; Oliver-Bonet et al. 2004). Some of the analyzed patients had normal semen parameters, and therefore the authors suggested that ICE in translocation carriers could be restricted to patients with abnormal semen parameters (Vegetti et al. 2000; Pellestor et al. 2001).

On the other hand, many reports detected an ICE in different reciprocal translocation carriers (Blanco et al. 2000; Oliver-Bonet et al. 2001, 2002; Baccetti et al. 2003; Douet-Guilbert et al. 2005; Wiland et al. 2007; Vozdova et al. 2008).

All reports support the occurrence of ICE in particular cases of structural chromosome reorganization, depending on the type of reorganization and on the chromosome or chromosomal region involved. However, the increase in aneuploidy and diploidy rates in infertile translocation carriers could be feasibly due to altered semen quality, as previously reported for infertile males of normal karyotype with oligoasthenoteratozoospermia.

Sperm Aneuploidy and Hormone Treatment

As highlighted so far, errors in sperm chromosome segregation are often observed in infertile males, and this is especially negative for candidates for assisted fertilization, increasing the failure rate and risk of generating offspring with chromosome imbalance. Therefore, it would be useful to develop methods for reducing the rate of aneuploidy in sperm.

Follicle stimulating hormone (FSH) is known for its role in the initial development of Sertoli cells and their stimulation to control spermatogenesis. FSH can therefore be used to improve spermatogenesis and fertilizing competence of oligozoospermic males, increasing both spermatogonial population and sperm production (Acosta et al. 1991, 1992; Foresta et al. 1998, 2002, 2005; Baccetti et al. 1997,

2004; Ben-Rafael et al. 2000). The administration of FSH can be useful in hypogonadotropic hypogonadism and when sperm alterations associated with normal gonadotropin levels suggest functional gonadotropin deficit.

In selected male patients with serum FSH less than 8 mIU/mL and a frequency of sperm aneuploidies greater than 0.6 % according to FISH analysis, 3 months of recombinant FSH therapy improved sperm quality and significantly decreased the frequency of sperm chromosomal alterations. The average percentage of total aneuploidies dropped by 31.8 %. The general improvement in sperm chromosome segregation was predominantly due to the decrease in diploidies and sex chromosome disomies (Piomboni et al. 2009).

The effect of FSH therapy on spermatogenesis may be explained by findings indicating that gonadotropins act as survival factor for spermatogonia and spermatocytes regulating the intrinsic and extrinsic apoptotic pathways, by which germ cells die in normal adult seminiferous epithelium (Ruwanpura et al. 2008).

Conclusions

Multicolor FISH in decondensed sperm nuclei using probes for sex chromosomes and autosomes, particularly chromosomes 1, 13, 18, and 21, allows an accurate evaluation of the incidence of sperm aneuploidy and is an appropriate way to analyze several thousand cells as well as a few cells in the case of severe oligozoospermic or azoospermic patients undergoing testicular biopsies. This technique, developed in the 1990s, may be applied for clinical or research aims. By pooling all published data from FISH analysis on sperm nuclei, it has become a useful tool in reproductive counseling for infertile couples (Gambera et al. 2011).

Using multicolor FISH, errors in chromosomal segregation have been found in sperm from normozoospermic or fertile men with a mean incidence ranging from 0.6 % to 1.45 %. Moreover, the percentage of numerical chromosomal aberrations increases in relation to sperm phenotype as in OAT men, suggesting that the risk of chromosome malsegregation events increases depending on the severity of testicular failure. This is also true for infertile males with abnormal karyotypes, which can produce a high percentage of gametes with unbalanced chromosomes. Sperm carrying chromosome abnormalities generally have a reduced fertilization potential; however, the development of assisted reproductive techniques such as ICSI revolutionized the treatment of male infertility, enabling these patients to procreate but increasing the risk of generating embryos with chromosomal unbalances.

Therefore, on these bases, information about meiosis and the incidence of eventual meiotic abnormalities should be useful in couples undergoing assisted reproduction for male infertility factor.

No technical procedure of sperm selection can guarantee a choice of gamete without chromosomal imbalance; in that case, knowledge of the chromosomal constitution of the male gametes in selected cases might suggest the need for a preimplantation or prenatal genetic diagnosis.

Further information on the relationships between sperm chromosome unbalance and human male infertility could help to promote a correct diagnostic and therapeutic approach to infertile couples, even when the cause of infertility is unknown, as in idiopathic diagnosis.

As regards the progressive improvement of the technique, in the future, the introduction of automated systems for multicolor FISH scoring would save time in the evaluation of results, which actually implies many hours of microscope viewing, which could depend on interoperator variability.

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Chapter 4
The Sperm Epigenome: Implications for the Embryo

John R. Gannon, Benjamin R. Emery, Timothy G. Jenkins, and Douglas T. Carrell

Abstract Recent advances, including the human genome project and numerous studies of cancer and other diseases, have shown that the genetic code is not simply limited to the sequence of the four bases of DNA but also includes epigenetic programming, heritable changes that affect gene expression [Riggs A, Martinssen R, Russo V (2007) Introduction. In: Riggs A, Martinssen R, Russo V (eds) Epigenetics mechanisms of gene regulation. Cold Spring Harbor Press, New York]. The science of epigenetics is important in understanding many diseases and biological processes, including in identifying the causes of disease and better understanding the mechanisms by which the environment can affect gene expression [Carrell Fertil Steril 97 (2):267–274, 2012]. This chapter will focus on the epigenome of sperm and particularly highlight the potential role of the sperm epigenome in embryogenesis.

The sperm epigenome is unique and highly specialized because of the unique nature and function of sperm and because of the diverse requirements for successful fertilization. Due to the need for motility, sperm chromatin must be compacted and highly organized. During spermiogenesis the chromatin is packaged tightly into the sperm head by the replacement of most histones with protamines. This allows for protection of the DNA from the hostile environment in the female reproductive tract. Remaining histones can have chemical modifications to the tails of the protein that either facilitate or repress gene transcription. Sperm, like embryonic stem cells, have a unique pattern of histone modifications that includes both activating and silencing marks in the promoters of genes associated with development. These bivalent

J.R. Gannon, M.D. • B.R. Emery, M.Phil. • T.G. Jenkins, B.S.
Division of Urology, Department of Surgery, University of Utah School of Medicine, Salt Lake City, UT, USA

D.T. Carrell, Ph.D., H.C.L.D (✉)
Division of Urology, Department of Surgery, Department of Obstetrics and Gynecology, Department of Human Genetics, University of Utah School of Medicine, 675 S. Arapeen Dr. #201, 84108 Salt Lake City, UT, USA
e-mail: douglas.carrell@hsc.utah.edu

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marks, along with DNA hypomethylation, comprise a unique state in which the key genes are “poised” for possible activation in embryogenesis. Sperm epigenetic abnormalities have been linked with multiple diseases including male factor infertility and poor embryogenesis.

Keywords Epigenetics • Protamine • Histone • DNA methylation • Embryogenesis

Introduction

Embryogenesis, the process by which an embryo develops, includes a complex uniting and interplay of the maternal and paternal haploid genomes (Ostrup et al. 2012). It is well established that the haploid genomes include the DNA sequence coding for genes and unique “marks” on both the DNA and the histones that comprise the nucleosomes required for packaging the chromatin (Rivera and Ross 2013). These marks, which regulate gene activation and transcription via chemical modifications to the DNA or histones, are termed epigenetic modifications. Epigenetic programming helps define the cell function, and abnormalities are often associated with diseases, such as cancer.

The sperm nucleus is a highly organized and complex structure and unique compared to all other cell types (Ward 2010). The DNA in somatic cells is wrapped around octamers of histones, the protein responsible for complex packaging and organization of DNA. Histones function to regulate, protect, and organize DNA in both somatic and germ cells. Male germ cells are the only cells that express protamines, a secondary nuclear compaction protein that provides an even higher order of chromatin compaction. In the human, most of the sperm genome is packaged by protamines rather than histones (Brewer et al. 2002). However, approximately 5–15 % of the genome remains bound to histones (Hamatani 2012). The retention of sperm histones allows the possibility that the histones may have a programmatic function through the epigenetic modification of the histones as well as methylation of the DNA itself. This concept has initiated an exciting field of study to describe and evaluate the sperm epigenome and its possible role in embryogenesis. This chapter will explore those studies.

Histones, Protamination, and the Nuclear Matrix

The evaluation of the sperm genome must begin with the unique manner in which sperm DNA is packaged. Three structural elements in sperm function to create sperm chromatin, the protamines, retained histones, and the nuclear matrix (Conaway 2012). Each element has a specific and distinct role in paternal genetic influences on fertilization and embryogenesis. Sperm chromatin is by necessity a tightly bound, highly organized structure, but the DNA must also be packaged in a

manner that allows rapid decondensation and integration of the DNA into the zygotic genome, including the possibility of gene expression in the early stages of embryogenesis (Rivera and Ross 2013).

Histone-bound DNA is associated with the nuclear matrix in both somatic and gamete cell lines. Histones are alkaline proteins, which serve as a structure for DNA to wrap around, which they do approximately 1.6 times. Histones have five different subtypes (H1/H5, H2A, H2B, H3, and H4) that, when bound together, are known as a nucleosome (Conaway 2012). Nucleosomes are linked together by short stretches of linker DNA consisting of approximately 50 nucleotides. Human sperm are unique in that during the late stages of spermiogenesis, most of the histones are replaced by protamines (Fig. 4.1). Protamination is a multistep process that involves numerous enzymes and regulators, but it is essential for normal fertility.

Protamines are large, charged proteins containing positively charged arginine amino acids that can bind to the negatively charged phosphorus in DNA (Hud and Vilfan 2005). The interaction between the positively charged arginine fragments and the DNA backbone lead to tight coiling of the DNA, causing it to nearly appear hidden in the protamine; this structure is known as a toroid (Hud and Vilfan 2005) (Fig. 4.1). Protamine compaction is further achieved via disulfide bonds between cysteine bases. This further arranges the protamine into a tightly packed structure of toroids "stacked side to side like a package of Life Savers" (Hud and Vilfan 2005; Mudrak et al. 2009). The highly compacted protamine structure of sperm chromatin facilitates protection of the sperm chromatin. The protamines in mature spermatozoa are not found in any other type of somatic cell. When compared to the more loosely bound histone segments of the paternal DNA, the protamine structure appears more stable and less prone to modifications.

In fertile males between 5 % and 15 % of the spermatozoa chromatin remains bound to histones rather than protamines (Carrell 2012; Hammoud et al. 2011; Ward 2010; Jenkins and Carrell 2012). Through the process of specific histone modification that either facilitates or represses gene expression, histone retention in sperm may facilitate important epigenetic programming for regulation of the paternal genome during early embryogenesis. It is now known that histone placement and retention in the sperm is nonrandom (Hammoud et al. 2011). The work done by Hammoud et al. in 2009 and 2011 showed that in mature sperm histones were retained in regions of the genome that were related to gene promoters of developmentally important genes, including developmental genes, transcription factors, miRNAs, and imprinted genes. Furthermore, specific modifications to those histones correlated with an increased facility to be "accessible" to transcription factors, thus potentially poising the paternal genome for activation in the early stages of embryogenesis. Histones, specifically H3 and H4, have long tails that may be modified with covalent bonds; these modifications include methylation, acetylation, ubiquitination, and other chemical modifications that will be discussed in more depth throughout this chapter. The modifications have the ability to activate or suppress the activity of the DNA bound to the histone (Zentner and Henikoff 2013). When specific segments of the paternal DNA are activated or suppressed, we can see epigenetic effects on the offspring.

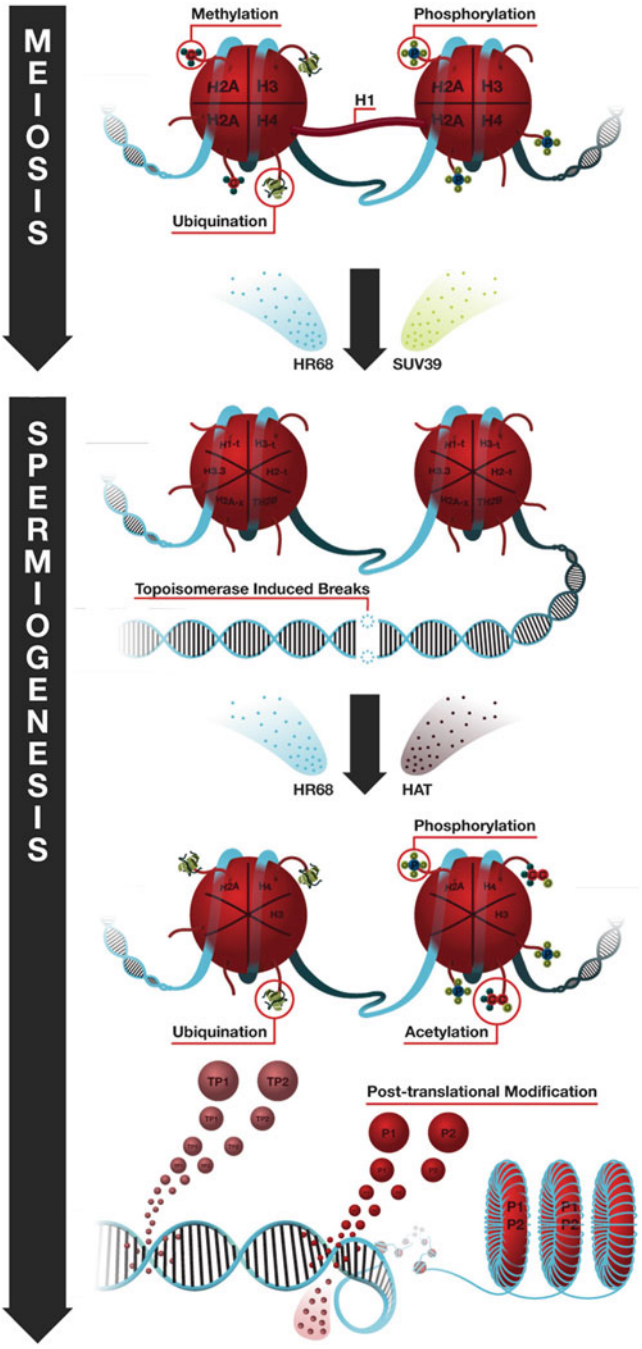

Fig. 4.1 (continued)

After successful fertilization of oocytes, sperm protamines are replaced with histones. Studies have shown that replacement of paternal protamines with maternal histones is achieved within the first 2–4 h after sperm penetration of the oocyte, with minimal initial activity of these particular encoding regions of DNA (Hammoud et al. 2011; van der Heijden et al. 2006). Paternal histone-bound segments are largely not initially replaced in the maternal oocyte, unlike the protamine segments (Ward 2010).

In addition to protamine and histone structure, there is a third method by which the paternal chromatin is organized within the nucleus. DNA not involved in a protamine toroid or histone solenoid is commonly referred to as a linker segment and is attached to the sperm nuclear matrix at matrix attachment regions at 50 kb intervals (Ward 2010). These linker segments are known as matrix attachment regions (MARs). The MARs appear to be involved as a checkpoint for sperm DNA integrity since sperm devoid of MARs fail DNA replication after intracytoplasmic sperm injection (ICSI) in the mouse model (Yamauchi et al. 2007a; Shaman et al. 2007). In addition to this role, MARs appear to act as promoters for the formation of the paternal pronucleus after fertilization (Ward 2010). The nuclear-matrix-attached regions serve as a required organizational step as the fertilized oocyte begins to divide (Shaman et al. 2007).

The nuclear matrix is a functional proteinaceous scaffold that also plays a role in gene expression (Pederson 2000). The scaffolding properties of the matrix are an intertwining of cytoskeletal elements such as actin and vimentin (Capco et al. 1982). The matrix is attached to the nuclear lamina and adds structural support to the chromatin and nucleus as a whole, but further investigation into the nature of MARs, chromatin organization, and matrix-associated mRNAs has shown a functional component as well (Cockerill and Garrard 1986). Recent studies indicate that this structural arrangement allows transcription machinery access to the open configuration of DNA within the nucleus; this has also been demonstrated in sperm (Kramer and Krawetz 1996; Ward et al. 1989). The loop organization of the chromatin is anchored to the matrix at the MARs every 20–120 kb (Vogelstein et al. 1980). This structure allows for one protamine-bound toroid per loop domain (Ward 1993). The strongest evidence for the functional nature of the sperm nuclear matrix in sperm is from a 1999 study by Ward et al. (1999) where mammalian sperm with a disrupted nuclear matrix were unable to support embryonic development. This study, and other

Fig. 4.1 The function of mature sperm requires extensive nuclear remodeling. The removal of many somatic canonical histones and the replacement with testis-specific histone variants is required for the early stages of germ cell differentiation, termed spermatogenesis. These testis-specific substitutions are also hallmarked by changes in expression regulation (epigenesis), such as the modification of histone tail marks and DNA methylation. The sperm morphology and chromatin modification are even more dramatic during spermiogenesis (late-stage spermatogenesis), where there is a stepwise replacement of the majority of canonical and testis-specific histones with transition proteins. Transition proteins are completely removed and replaced with protamines as the chromatin is wound into tightly packaged toroids. The toroid structure facilitates DNA protection, transcriptional quiescence, and efficient sperm motility. The DNA will remain in this state until nuclear decondensation during fertilization

functional studies (Yamauchi et al. 2007a, b), have shown that the sperm nuclear matrix and an organized chromatin configuration are indeed required for normal embryogenesis in mammalian models.

Sperm DNA Methylation and Histone Modifications

Methylation in the mature sperm nucleus is greatly reduced from other mammalian somatic cell types and cell lines. Methylation can regulate gene transcription by direct modification of DNA at cytosine residues (5-mC) that are located within gene regulatory regions, termed cytosine-phosphate-guanine dinucleotide islands (CpG islands) (Portela and Esteller 2010) or by histone tail modification (Jenuwein and Allis 2001). These types of marks are strong epigenetic regulators that can poise a gene or gene region for activation or suppression.

DNA hypomethylation (i.e., low levels of 5-MC) is a gene activation mark, while DNA hypermethylation causes interference of gene transcription machinery. Therefore, removal of methylation is necessary, but not sufficient, for gene expression. Enzymes that can actively remove DNA methyl marks in sperm have been surmised due to the temporal progression of demethylation during spermatogenesis, but these enzymes have yet to be elucidated (Ooi and Bestor 2008). The DNA methyltransferase 1 (DNMT1) is found in spermatogenesis and is responsible for de novo methylation and maintenance 5-MC (Eden and Cedar 1994).

While the majority of the sperm nucleus has been remodeled with sperm-specific protamines, 5–15 % of the chromatin remains histone-bound (Tanphaichitr et al. 1978; Wykes and Krawetz 2003). This fraction of the genome has been shown to be relevant to gene poising in the embryo and contains methylated histone marks for activation, inactivation, and gene poising (Hammoud et al. 2009). The most abundant histone methylation mark for gene activation is H3K4, while H3K9 and H3K27 modifications are gene repressors (Fig. 4.2). It has been shown that, in sperm, bivalent histone methylation (H3K9 and H3k27, both found in the same region) causes gene poising (Hammoud et al. 2009). Gene poising is the notion that genes can be *preprimed* for gene expression by setting up gene promoters for activation (Orford et al. 2008).

RNA in Sperm: An Epigenetic Factor?

There are approximately 10–400 femtograms of RNA present in mature spermatozoa (Miller and Ostermeier 2006). These cytoplasmic mRNAs are not actively translated due in part to the fact that there is an irregular distribution of the ribosomal subunits needed for mRNA translation within the mature spermatozoa cytoplasm (Miller and Ostermeier 2006). The mRNAs present in mature sperm appear to be selectively retained, and the profile of retained mRNAs has been shown to be

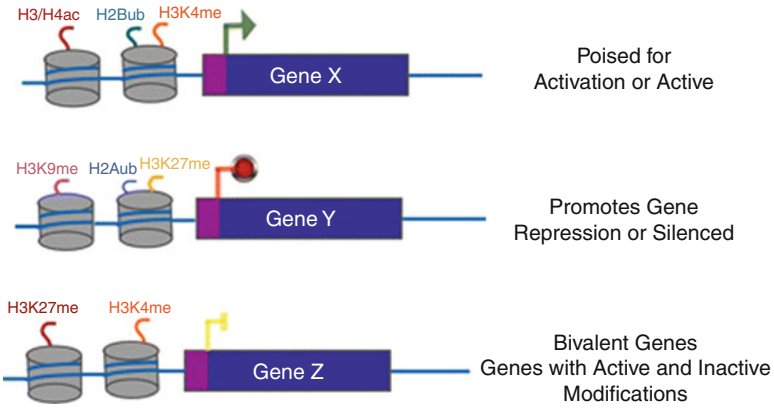

Fig. 4.2 Role of histone tail marks in epigenetic regulation. Histones play a critical role in gene activation, repression, and gene poising. This figure summarizes specific histone modifications that repress or activate transcription

reproducible when repeat analyses have been conducted on the same patient. Numerous studies in both hamsters and mice have demonstrated the inheritance and activity of spermatozoa-specific mRNAs in the early embryo and in offspring. This has perhaps best been elucidated by studies of the *Kit* locus, which codes for a tyrosine kinase receptor. When this was present, or inserted into the pool sperm mRNA, there was a clear and demonstrated inherited phenotype based upon the mRNA present while the DNA code was unchanged (Rassoulzadegan et al. 2006). This and other studies have shown that the select mRNA present in mature spermatozoa may have an epigenetic effect on the embryo. However, unlike other types of epigenetic regulation, RNA effects would not be heritable.

The exact epigenetic role mRNA plays in concert with the sperm chromatin is unclear. Whether they have an active role, helping to stabilize and promote the remaining histone-bound portions of the sperm chromatin, or they are passive bundles awaiting translation in the fertilized oocyte has not been clearly elucidated (Hamatani 2012). Further research on smaller noncoding segments of RNA, including miRNA and piRNA, have shown that they additionally may play a role as promoters or regulators of early embryogenesis. This is an important area for further development and research.

Postfertilization Epigenetic Remodeling

Following successful fertilization of the oocyte in the fallopian tube dynamic changes take place in both paternal and maternal chromatins in order for embryonic development to proceed. These dynamic and rapidly evolving changes transform two haploid cells into a diploid embryo. This transformation requires protamine

removal from the paternal chromatin and reestablishment of nucleosome-bound DNA with maternally derived histones. This complex transition is poorly understood, though we are beginning to gain intriguing insights into the process. Embryonic epigenetic reprogramming is essential in facilitating tissue-specific gene expression profiles requisite for proper embryo development (Jenuwein and Allis 2001).

Protamines located in the paternal chromatin are replaced by maternal histones soon after the fertilization event. Many studies have placed this timing within the first 4 h after fertilization, though the data are difficult to interpret due to the impossibility of directly studying healthy human embryos immediately following fertilization. As a result, our current understanding of the dynamics of human paternal pronuclear deprotamination in the early zygote is derived from mammalian studies of these events as well as heterologous (ICSI) with human sperm (Rodman et al. 1981; Nonchev and Tsanev 1990; Shimada et al. 2000). Though the precise timing is still controversial and poorly characterized, we do know that paternal chromatin relaxation (decondensation) occurs rapidly following fertilization and is likely driven directly by the removal of protamines. This event is essential in the future development of the embryo and is completed prior to syngamy (Wright and Longo 1988; Jones et al. 2011). Interestingly, the protamination of the paternal pronucleus, resulting in a quiescent chromatin structure, is incomplete as a result of events during spermatogenesis (Hammoud et al. 2009). The result is a few select regions of paternal histone retention. It is now believed that, because these regions of chromatin are in a more "relaxed" state, they may have the potential to be of great consequence in the early embryo. In fact, it has been demonstrated that the histone modifications in these regions of retention are in a poised state, similar to that of embryonic stem cells (Hammoud et al. 2009; Arpanahi et al. 2009). Current studies are attempting to elucidate the early activity of histone- and protamine-bound segments. The decondensation of the paternal chromatin through the protamine-to-histone transition results in the formation of the paternal pronucleus.

In addition to the swift decondensation previously discussed, paternally derived DNA must also undergo a dramatic demethylation to facilitate normal embryonic development (Ooi and Bestor 2008). The maternal DNA also undergoes demethylation; however, this is done through a passive, replication-dependent process (Eden and Cedar 1994). Importantly, it should be noted that the active demethylation in the paternal pronucleus is incomplete (Abdalla et al. 2009a, b). Multiple regions are known to escape this active process, including imprinted clusters and retrotransposons (Abdalla et al. 2009b). This creates an environment where new methylation marks that are tissue specific can be laid down in each given cell type as fates are determined, but it also suggests that the paternal epigenome is of some consequence to the developing embryo and, likely, to the offspring. The transition from gamete-derived epigenetic landscapes to that seen in the embryo is a critical step required for the growth and success of the embryo.

It is known that gene regulation is highly governed by the epigenetic landscape in each cell type. It is also well established that the sperm epigenome is among the most unique found in any cell in the human body, due both to its unique DNA methylation marks and nuclear protein content. It follows, then, that many of these

unique epigenetic features must be removed, oftentimes by dramatic mechanisms, to enable the paternal pronuclei to be capable of contributing to embryonic totipotency. However, it must also be noted that while many of the sperm-specific epigenetic marks are removed, some remain, and of those already studied, it appears that these marks are important for the embryo and the offspring. This is in stark contrast to the previously held dogma that the sperm was limited in its capacity to effect change in the embryo due to its epigenetic specificity. The dynamic nature of embryonic epigenomes makes the study of epigenetics in the early embryo difficult. Much of our knowledge is therefore derived from failures of embryogenesis and the subsequently identified epigenetic abnormalities (Eden and Cedar 1994). Other aspects of our knowledge stem from recognized imprinting errors of the paternal epigenome and genome, which have been linked to several severe diseases including Beckwith-Wiedemann syndrome (Hammoud et al. 2011). These diseases appear to be more prevalent in fetuses derived from in vitro fertilization (IVF)/ICSI, which has led to in-depth study of epigenetic factors in this population (Reefhuis et al. 2009). Clearly, much work is still required to further our understanding of the epigenetics of embryonic development; however, we have gained a great deal of insight into these processes in recent history.

Sperm Epigenetics and Infertility

As described previously, successful fertilization requires dramatic changes in the sperm chromatin, including protamination, proper histone retention, specific histone modifications, and fidelity in the maintenance of DNA methylation, as well as the likely role that retained RNAs may play in embryogenesis. Complicating this process even further is the reprogramming that must occur to the male pronucleus following fertilization. The presence of the paternal epigenetic landscape is necessary for complete embryogenesis, and data are now beginning to demonstrate epigenetic abnormalities in infertile patients (Jenkins and Carrell 2012).

Numerous studies have demonstrated a strong association of abnormal protamination with male infertility (Aoki et al. 2006a; Depa-Martynow et al. 2012). In humans, protamination results in the placement of two protamines, protamines 1 and 2, at a ratio of 1:1. It has clearly been shown that fertile men exhibit a tight distribution of the protamine ratio around 1.0, while in infertile men the ration can vary dramatically but is generally associated with decreased sperm quality, diminished sperm fertilizing capacity, and poorer embryogenesis in patients undergoing IVF (Aoki et al. 2006b). Studies indirectly evaluating protamination via indirect staining with aniline blue or chronomycin A have demonstrated a similar relationship (Sakkas et al. 1998).

The underlying causes of abnormal protamination have not been well elucidated, but gene polymorphisms of the transition protein and protamine genes are not the cause of abnormal expression in most men with abnormal protamination (Aoki et al. 2006c; Hammoud et al. 2007). Numerous enzymes involved in protamination

have been shown to be essential and may be related to infertility in some men. For example, impaired spermatogenesis has been associated with aberrant acetylation of the histone 4 domain, and hyperacetylation of this domain has been documented in infertile men with Sertoli cell-only syndrome (Faure et al. 2003). Abnormal phosphorylation of the P2 domain has been linked to male infertility, DNA damage, and poor sperm quality (Wu and Means 2000; Balhorn et al. 1988). Further studies have shown that the downregulation or upregulation of protamine with epigenetic modifications caused failure of spermatogenesis, arrest of transcription, translation, and additional abnormalities in embryogenesis (Kleene 2003).

Hammoud et al. (2011) recently completed a genomewide analysis of seven infertile men evaluating the location of regions of the genome enriched for histones, as well as histone modification. This genomewide evaluation was performed in two types of selected patients, those with known abnormalities of protamination and those with unexplained, repeated poor embryogenesis. This seminal study clearly showed marked abnormalities in the retention of histones at some promoters of developmentally related genes in many of the patients. When specific histone modifications were evaluated, more subtle results were seen. This study is the only study thus far that has evaluated histone modifications in sperm from infertile men, but it highlights the potential role that defects in this process may play in infertility.

DNA methylation, facilitated by DNA methyltransferase (DNMT), is an essential sperm epigenetic mark. Knockout mice with no DNA methyltransferase activity have decreased male fertility (Kato et al. 2007). Additional studies from couples undergoing assisted reproductive technology have also shown that changes, specifically hypomethylation, in spermatozoa DNA methylation is linked with decreased pregnancy rates, decreased sperm maturation, and embryogenesis (Benchaib et al. 2005). Additionally, numerous studies have found abnormal methylation of imprinted genes in sperm from oligozoospermic men (Marques et al. 2008; Kobayashi et al. 2007). Additionally, aberrant methylation of specific CpG islands in sperm DNA has been found to be elevated in sperm from men with abnormal protamination (Hammoud et al. 2010). Initial studies attempting to screen for aberrant methylation in sperm have been met with variable success; however, specific patients can be identified with broad methylation abnormalities (Aston et al. 2012; Nanassy and Carrell 2011).

As described earlier, noncoding and mRNAs may represent another epigenetic factor that could be related to infertility. Since mRNA is responsible for encoding protein synthesis during the translation process, the lack of mature sperm proteins, or their lack of function, may lead to infertility. Studies published thus far have demonstrated consistent differences in the RNA profiles of some infertile men, which may lead to the possible use of mRNA screening in infertile men as a tool to determine the cause of infertility (Hamatani 2012).

Screening for mRNA or other epigenetic defects could be conducted using commercially widely available methods for genetic amplification, and microarray screening could be used to help identify specific mRNA sequences or deficiencies that correlate with infertility. Significant differences have been noted, aside from the

protamine ratios, when comparing the mRNA proteins of fertile and infertile men on microarray analysis (Garrido et al. 2009). Among the differences cited in this study and others is underexpression of mRNA sequences that code for enzymes responsible for DNA repair and embryogenesis. mRNA screening has been further emphasized by comparing the semen analysis of known fertile men with patients having a history of heavy cigarette smoking and cryptorchidism. In each of these analyses, patients with decreased fertility have been identified by an underexpression of mRNA genes (Hamatani 2012).

Numerous studies, some of which were briefly discussed and cited earlier, have shown that disruption of the paternal epigenetic landscape leads to infertility and an inability for the embryo to proceed with appropriate development. The paternal genome and its epigenetic factors are crucial in numerous steps that lead to the successful formation of a fertilized and growing embryo. As Dada et al. said, "Both the complex path of sperm production and the delicate balance of epigenetic and genetic factors during sperm maturation contribute to the formation of a mature sperm with the ability to fertilize an oocyte and contribute to the developing embryo" (Dada et al. 2012). Therefore, a change or disruption of this balance may then lead to male infertility, as the required epigenetic factors for successful embryogenesis would be absent.

Conclusions

The spermatozoa chromatin is a highly specialized and unique structure and appears to be critical in facilitating normal fertilization and embryogenesis. Protamination, a process unique to sperm, results in a greatly compacted and quiescent sperm nucleus. Alterations to protamination appear to affect other epigenetic processes, such as DNA methylation, and are associated with reduced fertility. Classic epigenetic modifications to the retained sperm histones are consistent with a programmatic role for poising developmentally related genes for early embryogenesis. These modifications include regulatory modifications to the retained histones and DNA demethylation at the poised sites. Together, these changes suggest a string role for the sperm epigenome in embryogenesis.

Maternal changes to the male genetic code occur after fertilization. These modifications include removal of the protamines during pronucleus formation, replacement with maternal histones, and an active demethylation of most of the paternal genome. How these changes interplay with the marks set in the sperm epigenome during spermiogenesis is a key area of interest for further study.

Preliminary studies have clearly demonstrated epigenetic abnormalities in many infertile patients, including abnormal DNA methylation and aberrant histone enrichment in developmentally relevant genes. However, studies thus far are almost entirely descriptive and not mechanistic. Therefore, caution must be urged in evaluating the data from this important field of study.

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Chapter 5
Environmental Epigenetics and Effects on Male Fertility

Carlos Guerrero-Bosagna and Michael K. Skinner

Abstract Environmental exposures to factors such as toxicants or nutrition can have impacts on testis biology and male fertility. The ability of these factors to influence epigenetic mechanisms in early life exposures or from ancestral exposures will be reviewed. A growing number of examples suggest environmental epigenetics will be a critical factor to consider in male reproduction.

Keywords Testis • Spermatogenesis • Sperm • Epigenetics • Transgenerational • DNA Methylation • Epimutations

Introduction

Environmental toxicants present in the environment, from either synthetic or natural origins, can influence physiological responses and developmental processes in organisms. Some of these compounds interfere with the action of endogenous hormones at several physiological levels and so are categorized as endocrine disruptors (Schug et al. 2011). Industrialization and the progressive accumulation of synthetic endocrine disruptors in the environment has altered the ecological balances in natural populations and affected human health (Balabanic et al. 2011). These compounds are present in cosmetics, food items and containers, packaging materials, toys, agrochemicals, and in practically every manufactured product with which humans have contact. These toxicants are often associated with increased incidence of reproductive disease (Balabanic et al. 2011; Caserta et al. 2011; Fowler et al. 2012). Research has demonstrated that exposure to environmental factors such as

C. Guerrero-Bosagna • M.K. Skinner (✉)
Center for Reproductive Biology, School of Biological Sciences, Washington State University, Pullman, WA 99164-4236, USA
e-mail: skinner@wsu.edu

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environmental contaminants, stress, or dietary compounds early during fetal and postnatal development have a significant impact on human health (Guillette and Iguchi 2012). Many common human diseases have seen a dramatic increase in incidence in the past decade. Exposure to environmental factors are estimated to account for 40 % of deaths worldwide (Pimentel et al. 2007), which includes the majority of cancers being linked to environmental exposures. Regional environmental influences are an important component in noninfectious disease incidence (Wallace 2010). For example, regional variations exist in cancer incidence worldwide (Forouzanfar et al. 2011; Parkin 2004). Differences in lifestyles, exposure to dietary compounds, or environmental toxicants are the primary factors involved. Regions of the world with high consumption of salt, processed meat, and N-nitroso compounds are associated with increased risk of gastric cancer (Tsugane and Sasazuki 2007). Other noninfectious diseases are correlated with exposure to environmental toxicants. For example, human populations that are highly exposed to arsenic tend to present increased susceptibility to develop liver, bladder, skin, and lung cancer (Anetor et al. 2007).

One of the disease states that has emerged as a result of exposure to environmental factors is the increasing incidence of abnormalities of the male reproductive system (Giwercman and Giwercman 2011). Recent epidemiological trends indicate changes in the incidence of several pathologies of the male reproductive tract in humans in recent decades such as decreases in sperm count and quality (Sharpe 2010) and increases in testicular cancer (Skakkebaek et al. 2007) or suggested increases in cryptorchidism or hypospadias (Main et al. 2010). These trends have led authors to group these male reproductive conditions into the complex disease trait of testicular dysgenesis syndrome, which has been associated with the environmental exposures that humans have been subjected to in recent decades (Giwercman and Giwercman 2011; Skakkebaek et al. 2001). A number of examples exist of a direct correlation between environmental toxicants and effects on male reproductive health. Accidental in utero exposures of humans to the synthetic organic pollutants polychlorinated biphenyls and polychlorinated dibenzofurans in Taiwan was reported to produce a marked effect in semen quality and motility (Guo et al. 2000, 2004). Cases of massive agroworker pesticide-induced sterilization have been observed in California (1970s) (Whorton et al. 1979) and in Costa Rica (early 1960s to 1984) (Thrupp 1991) due to exposure to nematicide 1,2-Dibromo-3-chloropropane (DBCP). Exposure to naturally available estrogenic compounds has also been associated with reduced fertility in male animals. For example, the identification of phytoestrogens as having estrogenic or reproductive effects in animals started with observations from farmers in New Zealand who found that ewes would become infertile after eating clover (Adams 1981, 1990). The same effect was further reported in cattle (Adams 1995). Understanding the basic developmental biology of the male reproductive tract (e.g., testis) and mechanisms of action of these environmental factors is reviewed below.

Epigenetic and Transgenerational Effects of Environmental Exposures

Epigenetics is defined as molecular factors around the DNA that regulate genome activity independent of DNA sequence and that are mitotically stable (Skinner 2011; Skinner et al. 2010). The factors involved include DNA methylation, histone modification, chromatin structure, and noncoding RNAs. Environmental epigenetics involves the ability of environmental factors to alter epigenetic marks that then alter genome activity and cellular function (Skinner et al. 2010). Since the vast majority of environmental factors cannot influence or alter DNA sequence, epigenetics provides an efficient mechanism to mediate environmental impacts on biology (Skinner 2011). Many research groups have documented the epigenetic actions of environmental exposures. Environmental factors can directly influence epigenetic marks that generate phenotypic variation that includes the induction of disease such as subfertility and imprinting disorders (Inbar-Feigenberg et al. 2013). Epigenetic tools will help identify etiological factors causing specific molecular pathologies (Ogino et al. 2013). For example, environmental effects such as trauma, stress, or disorganized attachment can induce epigenetic changes in the brain to cause long-term effects on the regulation of the genome function to promote psychopathology, such as schizophrenia (Gonzalez-Pardo and Perez Alvarez 2013). Studies have shown that early-life environment and epigenetics have an important role in a variety of diseases, such as cardiovascular disease (Sun et al. 2013), allergies (North and Ellis 2011), and asthma (Karmaus et al. 2013). Several environmental factors have been described as causing epigenetic effects, including hypoxia (Yuen et al. 2013), phytochemicals (Guerrero-Bosagna and Skinner 2012), organic environmental toxicants (Manikkam et al. 2012a), inorganic compounds (Cheng et al. 2012), and nanosized materials (Stoccoro et al. 2012).

A number of environmental exposures have been shown to produce transgenerational effects on disease and phenotypic variation (Anway et al. 2005; Skinner et al. 2010). Epigenetic transgenerational inheritance processes involve key features such as the action of environmental toxicants on gestating females during the period of fetal gonadal sex determination resulting in generational phenotypes (Skinner et al. 2010). Since the gestating female (F0 generation), fetus (F1 generation), and fetal germline (F2 generation) are directly exposed, phenotypes in these generations are due to multigenerational exposures. Interestingly, the occurrence of phenotypes for three generations or more, following the initial F0 generation exposure, constitutes an epigenetic transgenerational inheritance phenomenon (Skinner 2011; Skinner et al. 2010). The role of germline in transmitting epigenomes is essential for this phenomenon and is becoming well established in several different organisms (Arico et al. 2011; Carone et al. 2010; Dunn and Bale 2011; Guerrero-Bosagna et al. 2010; Morgan and Bale 2011). During the initiation of development of the germline, a major DNA methylation erasure occurs followed by a reestablishment of DNA

methylation patterns (Lees-Murdock and Walsh 2008; Reik et al. 2001). DNA methylation erasure takes place during the migration of primordial germ cells to the genital ridge and gonad, and then remethylation is initiated during the first events of sex determination (Allegrucci et al. 2005; Durcova-Hills et al. 2006). This period in germ cell development and epigenetic programming represents a window of sensitivity to environmental factors, and when an altered epigenetic programming is induced, it can be perpetuated across generations (Anway et al. 2005; Skinner et al. 2010).

A number of different environmental toxicants have been shown to promote exposure-specific alterations in the F3 generation sperm epigenome (DNA methylation) (Manikkam et al. 2012a). These include dioxin (Bruner-Tran and Osteen 2011; Manikkam et al. 2012c), a mixture of plastic compounds [bisphenol A (BPA) and phthalates] (Manikkam et al. 2013), the pesticide methoxychlor (Anway et al. 2005), a mixture of pesticide and insecticide (permethrin and DEET) (Manikkam et al. 2012b), and a hydrocarbon mixture (JP8 jet fuel) (Tracey et al. 2013). In addition to environmental toxicants, nutritional compounds (Burdge et al. 2011; de Assis et al. 2012) and stress (Champagne 2008; Crews et al. 2012) can promote epigenetic transgenerational phenotypes.

Testis Development and Biology

The process of gonadal development is essential for sex determination and the establishment of the germline. Cell lineages and cell populations are established during early embryonic development; they then influence adult gonadal function, endocrine responses, and fertility. Understanding the fetal basis of adult onset testis defects and infertility requires an elucidation of the molecular and cellular events during gonadal sex determination. The adult testis is a complex organ that is composed of seminiferous tubules enclosed by a surrounding interstitium. The seminiferous tubules are the site of spermatogenesis where germ cells develop into spermatozoa in close interaction with Sertoli cells. The Sertoli cell is an important testicular somatic cell that controls the germ cell environment by the secretion and transport of nutrients and regulatory factors (Fawcett 1975; Sertoli 1865). Tight junctional complexes between the Sertoli cells contribute to the maintenance of a blood–testis barrier (Setchell and Waites 1975) and create a unique environment within the tubule (Waites and Gladwell 1982). Surrounding the basal surface of the Sertoli cells is a layer of peritubular myoid cells that function to contract the tubule. The peritubular cells surround and form the exterior wall of the seminiferous tubule. The interstitial space around the seminiferous tubules contain another somatic cell type, the Leydig cell, which is responsible for testosterone production. Leydig cells have a major influence on spermatogenesis through the actions of testosterone on both the seminiferous tubule and the pituitary. Although the Leydig cell has numerous secretory products (Skinner 1991), testosterone is the most significant secretory product of the cells. Interaction of all three somatic cells, Sertoli, peritubular, and Leydig, is important for the regulation of normal spermatogenic function in the

testis (Skinner 1991). The coordinated interactions of different testis cell populations are critical for the initial morphogenesis process through the adult stage of maintaining the process of spermatogenesis.

The process of fetal testis formation occurs late in fetal development (embryonic day 13, E13, in the rat). Initially this involves migration of primordial germ cells from the yolk sac to the hindgut and then from the hindgut to the genital ridge and gonad. After migration, germ cell differentiation in the gonad is dependent on gonadal sex determination and the induction of specific transcription factors (McLaren 1991; Takasaki et al. 2001). The gonad is bipotential after germ cell migration and can be distinguished morphologically from the adjoining mesonephros (E12 in rat) but cannot be identified as an ovary or a testis. A variety of genes such as *Sry* (sex determining region Y), *Sox9* (SRY box 9), *Sf1* (splicing factor 1), *Dmrt1* (double sex and mab-3 related transcription factor), and *Tcf21* (transcription factor 21) are involved in the transcriptional induction of Sertoli cell differentiation and testis development (Bhandari et al. 2012b; Clinton and Haines 2001; Drews 2000; Ikeda et al. 2001; McLaren 2000; Ostrer 2000; Parker et al. 2001; Raymond et al. 2000; Vaillant et al. 2001). Two morphological events occur early at embryonic day 13 (E13) during sex determination to alter the bipotential gonad. First, Sertoli cells, which are proposed to be the first cell in the testis to differentiate, aggregate around primordial germ cells (Jost et al. 1981; Magre and Jost 1980). Secondly, migration of mesenchymal cells occurs from the adjoining mesonephros and coelomic epithelium into the developing gonad to surround the Sertoli cell-germinal cell aggregates. It has been speculated that the migrating population of cells is preperitubular cells (Buehr et al. 1993; Merchant-Larios et al. 1993; Ricci et al. 1999). The mechanism for this migration signal is from the testis to promote cell migration (Clement et al. 2011) through observations that female mesonephros cells can also be stimulated to initiate cell migration after close interaction with a developing testis (McLaren 2000). In addition, using an organ culture system in which mesonephros and embryonic testis were separated by an embryonic ovary, mesonephros cells migrated through the ovary to the testis (Karl and Capel 1998). Another cell migration event required for cord formation involves endothelial cells from the coelomic epithelium to form the testis vasculature (Bott et al. 2008; Cool and Capel 2009; Cool et al. 2008). The cords develop neonatally into seminiferous cords and at the onset of puberty develop into seminiferous tubules.

Molecular Processes in Fetal Development

SRY is the testis-determining factor on the Y chromosome proposed by Jost that initiates the molecular events for Sertoli cell differentiation and male gonadal sex determination (McClelland et al. 2012; Parma and Radi 2012). The combined interactions between SRY and SOX9 are critical for male sex determination and precursor Sertoli cell differentiation (Kim and Capel 2006; Miyamoto et al. 2008; Ottolenghi et al. 2007). Upstream genes such as *Wt1* (Wilms tumor 1) precede *Sry*

(Gao et al. 2006; Kanai et al. 2005), but SRY initiates Sertoli cell differentiation, which subsequently involves an upregulation of SOX9 in Sertoli cells (Gao et al. 2006; Kidokoro et al. 2005; Sekido et al. 2004). SRY and SOX9 expression in Sertoli cells is associated with germ cell–Sertoli cell aggregation prior to cord formation (Moreno-Mendoza et al. 2003; Sekido et al. 2004). Abnormal SRY or SOX9 expression is associated with sex reversal and other disease states, including abnormal testis development (Barrionuevo et al. 2006; Bouma et al. 2005; Bullejos and Koopman 2005; Moreno-Mendoza et al. 2003; Nikolova and Vilain 2006). In regards to the regulation of the *Sry* promoter and inducing factors, very little is known outside the timing of the event in the genital ridge (Daneau et al. 2002; Hiramatsu et al. 2009; Nikolova and Vilain 2006). In regards to downstream genes to *Sry*, a large number of binding targets have recently been identified (Bhandari et al. 2012a). A downstream target of SRY is the basic helix loop factor TCF21 that promotes a secondary cascade of events associated with Sertoli cell differentiation (Bhandari et al. 2012b). Another downstream function of SRY/SOX9 is the production of prostaglandin D2 (Daneau et al. 2002; DiNapoli and Capel 2008; Malki et al. 2005; Wilhelm et al. 2005), but SOX9 appears to be the primary regulator of prostaglandin synthesis (Wallis et al. 2008; Wilhelm et al. 2007). Synergistic actions of SRY and SF1 have been shown on the *Sox9* promoter (Sekido and Lovell-Badge 2008). Recent SRY downstream *gene* candidates have been suggested (Bhandari et al. 2012a; Bradford et al. 2009), such as the *Cbln4* gene with no known function. Recently *Wdr5* (WD repeat domain) has been shown to be a downstream target of SRY (Xu et al. 2012) and NTF3 (neurotropin 3) (Clement et al. 2011) and the bHLH factor TCF21 (Bhandari et al. 2011). Interestingly, NTF3 was previously shown to act as a Sertoli-cell-produced chemotactic factor to promote mesonephros cell migration into the developing testis to promote cord formation (Cupp et al. 2003). The induction of fetal testis cord function is an anticipated initial downstream function for SRY (Cupp et al. 2003), while TCF21 is proposed to be involved in the induction of Sertoli cell differentiation (Bhandari et al. 2011, 2012b).

Environmental Exposures and Fetal Testis Development

Early life exposures to nutritional alterations or environmental compounds have been shown to cause later life adult onset disease (Manikkam et al. 2012a; Skinner et al. 2010). The fetal basis of adult onset disease is now well established and one of the primary mechanisms involved is epigenetics (Skinner 2011). The fetal exposure to an environmental insult at a critical window of development for an organism can shift the epigenetic programming that is mitotically stable to then promote altered gene expression and adult onset disease (Skinner 2011; Skinner et al. 2010). The critical window of exposure for the testis and subsequent adult onset testis disease is the gonadal sex determination period. This is when the somatic cells fate, germline cell fate and initial differentiation develops. The later life adult onset disease associated with these fetal exposures are spermatogenic cell apoptosis and defects

(Shukla et al. 2012), as well as male infertility (Anway et al. 2005, 2006). Fetal exposure to vinclozolin during male gonadal sex determination has been shown to promote later life testis spermatogenic cell defects (apoptosis) in 90 % of the males and in adult rats at 1 year of age a 30 % increase of male infertility (Anway et al. 2005, 2006). Vinclozolin is a commonly used agriculture fungicide which is an antiandrogenic endocrine disruptor. In addition to promoting adult onset testis disease in the F1 generation, the germline (sperm) epigenome becomes permanently programmed epigenetically to transmit the epigenetic alterations (epimutations) and disease phenotypes to subsequent generations (F1–F4) through epigenetic transgenerational inheritance of the disease phenotype (Anway et al. 2005, 2006; Skinner et al. 2010). Therefore, the *in vivo* exposure of a gestating female during the period of gonadal sex determination for the F1 generation fetus promotes adult onset testis disease in the F1 generation, as well as induces an epigenetic transgenerational inheritance of the testis disease phenotype to subsequent generations (Anway et al. 2005, 2006). Other authors have shown similar transgenerational effects on fertility after peritoneal exposure to bisphenol-A (Salian et al. 2009).

Epigenetic Alterations of Testis Cell Biology and Fertility

Epigenetic mechanisms are fundamental to ensuring normal gonadal development and spermatogenesis (Carrell 2012; Rajender et al. 2011; Skinner et al. 2010). One of the crucial processes that depends on epigenetic mechanisms is the exchange of histones for protamines, which results in the genome's becoming tightly compacted (heterochromatin) in the sperm and in inhibition of expression (Carrell 2012; Rajender et al. 2011). For this process to occur, hyperacetylation of histone H4 is needed (Sonnack et al. 2002). Recent experiments have shown that H4K12ac associates preferentially with regions near the transcription start site and in promoters that express transcripts stored in mature human sperm (Paradowska et al. 2012). Interestingly, decreased histone H4 acetylation in spermatids results in impaired spermatogenesis and decreased fertility (Sonnack et al. 2002). Additional histones such as H2AL1 and H2AL2 have also been described to mark pericentric regions in condensing spermatids and be involved in forming new nucleoprotein structures (Govin et al. 2007). Recently, several publications have highlighted the interaction between histone modifications and DNA methylation in several organism models (Du et al. 2012; Johnson et al. 2007; Ooi et al. 2007). This would also be the case for histone modifications during spermatogenesis. Observations suggest that the fertilized zygote inherits specific histones and histone-based chromatin organization from the sperm (Paradowska et al. 2012), but the potential random nature of this programming needs to be assessed. Histone binding and chromatin organization in the male germline would be a consequence of fine-scale base composition variation GC and CpG content (Vavouri and Lehner 2011). GC-rich regions in promoters would be prone to retain the nucleosomes and not exchange them for protamines, which happens in 4 % of the sperm genome (Vavouri and Lehner 2011). These regions with

nucleosome retention would prevent reprogramming of DNA methylation after fertilization (Vavouri and Lehner 2011). Interestingly, it was recently shown that infertile men display abnormalities in both histone modifications (H3K4me and H3K27me) and DNA methylation at imprinted and developmental loci (Hammoud et al. 2011, 2010; Houshdaran et al. 2007) in the sperm DNA. Reduced histone methylation in the *Brdt* (bromo domain testis-specific) promoter is associated with reduced BRDT expression in subfertile men (Steilmann et al. 2010). Studies have also shown that sperm from men with fertility problems have altered DNA methylation patterns in imprinted genes (Boissonnas et al. 2010; Kobayashi et al. 2007; Marques et al. 2004, 2008, 2010), which would generate imprinting abnormalities in the offspring when this sperm is used in assisted reproductive technologies (Kobayashi et al. 2007; Marques et al. 2004). Adult exposure to butyl-paraben has been shown to produce DNA methylation changes in the sperm (Park et al. 2012). Prenatal exposure to ethanol has also been shown to induce decreased spermatogenesis and DNA methylation changes in imprinted genes (Stouder et al. 2011). It is postulated that the methylenetetrahydrofolate reductase (Mthfr) gene would have a central role in idiopathic male infertility. Some Mthfr-deficient strains of mice have alterations in sperm DNA methylation in a number of sites (Chan et al. 2010). Also, Mthfr DNA hypermethylation in sperm is associated with idiopathic male infertility in humans (Wu et al. 2010). In addition to the importance of DNA methylation changes in germline development in imprinted and developmental loci, the DNA methylation in repeat elements such as B1 SINEs has been proposed as having a role in transcriptional regulation of testis-specific genes (Ichiyanagi et al. 2011). Genes involved in the pathway of PIWI-associated small RNAs (piRNAs), such as *Piwil2* (Piwi-like 2) and *Tdrd1* (tudor domain containing 1), are hypermethylated in the testicular tissue of males with different forms of fertility problems (Heyn et al. 2012).

Epigenetic modifications have also been reported in the somatic cells controlling the process of spermatogenesis, such as Sertoli and Leydig cells. In Sertoli cells *Rhox5* (reproductive homeobox 5) deletion produces subfertility, increases germ-cell apoptosis, and decreases sperm count and motility through two promoters repressed by DNA methylation (Shanker et al. 2008). Another interesting observation relates to epigenetic changes produced in the proximal promoter of the fatty acid amide hydrolase (Faah) gene (reduced DNA and histone H3 methylation) in response to estradiol in mouse Sertoli cell cultures (Grimaldi et al. 2012). Epigenetic changes have also been observed in Leydig cells after exposure to environmental contaminants. Changes in DNA methylation have been observed in mouse Leydig TM3 cell line cultures following exposure to either low or high doses of arsenic (Singh and DuMond 2007). Exposure of these cells to cadmium leads to reduced expression of DNA methyltransferase 1 (Singh et al. 2009). In utero exposure to di-(2-ethylhexyl) phthalate has been shown to produce postnatal alteration in demethylation in several nuclear receptor genes in Leydig cells, among them the estrogen receptor beta (ER-beta), Nr142 (thyroid receptor beta), peroxisome proliferator activated receptor alpha (PPAR-alpha), and mineralocorticoid receptor (MR) (Martinez-Arguelles et al. 2009). Interestingly, treatment of Leydig cells with luteinizing hormone causes cellular hypomethylation, suggesting that environmental exposures that alter

Table 5.1 Epigenetic modifications in testicular somatic or germ cells associated with infertility or poor semen parameters

Epigenetic modification	Tissue/cell type	Reference
DNA methylation promoting spermatogenic defects and infertility transgenerationally	Sperm	Anway et al. (2005), Guerrero-Bosagna et al. (2010)
DNA methylation change at *Mthfr*	Sperm	Wu et al. (2010)
DNA methylation change at Igf2-H19 locus	Sperm	Boissonnas et al. (2010), Stouder et al. (2011)
DNA methylation change at Mest and Igf2-H19 locus	Sperm	Marques et al. (2008, 2010), Poplinski et al. (2010)
DNA methylation changes at several imprinting loci	Sperm	Kobayashi et al. (2007)
DNA methylation changes in several imprinted and nonimprinted genes	Sperm	Houshdaran et al. (2007)
Histone and DNA methylation changes in developmental and imprinted genes	Sperm	Hammoud et al. (2010, 2011)
Histone methylation change	Sperm	Steilmann et al. (2010)
Histone acetylation change	Spermatids	Sonnack et al. (2002)
DNA methylation change at *Mthfr*	Testis biopsies	Khazamipour et al. (2009)
DNA and histone methylation change at *Faah*	Sertoli cell culture	Grimaldi et al. (2012)
DNA methylation changes at several nuclear receptor genes	Leydig cells	Martinez-Arguelles et al. (2009)
DNA methylation change at several loci	TM3 Leydig cell culture	Singh and DuMond (2007)

DNA methylation in testicular cells may influence hormone actions (Reddy and Reddy 1990). Epigenetic modifications in somatic testicular tissues or germ cells that are associated with infertility or poor semen parameters are shown in Table 5.1.

Conclusions

Increasing concerns about the decrease in fertility in men have developed over the past few decades. An explanation for this trend is the exposure of the human population to toxicants derived from industrial products or processes. Many of these contaminating agents are capable of altering epigenetic programming in organisms. These alterations are generally produced during early developmental stages and generate diseases in adults. A number of reproductive and metabolic diseases have been shown to have an epigenetic and developmental component. Interestingly, these environmentally induced disease states can become transgenerationally transmitted. Strong evidence has accumulated in recent years showing that environmental toxicants alter developmental and epigenetic processes to promote abnormal spermatogenesis in men. Although the molecular mechanisms await full elucidation, there is no longer any doubt that an important component of the disruption of

spermatogenic cell development is exposure to environmental toxicants. Therefore, future studies addressing fertility in humans should place special emphasis on the role of environmental epigenetics on testis development and spermatogenic-cell-associated disease.

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Chapter 6
Protamine Alterations in Human Spermatozoa

Meritxell Jodar and Rafael Oliva

Abstract Protamines are the major nuclear proteins in sperm cells, having a crucial role in the correct packaging of the paternal DNA. The fact that protamine haploinsufficiency in mice resulted in abnormal chromatin packaging and male infertility suggested that the protamines could also be important candidates in explaining some of the idiopathic male infertility cases in humans. The first clinical studies focused on analyzing protamines at the protein level. Various studies have found the presence of an altered amount of protamines in some infertile patients, in contrast to the normal situation in fertile individuals where the two protamines, protamine 1 and protamine 2, are both present in approximately equal quantities. Subsequently, the protamine genes were the subject of various mutational genetic screening studies in search of variants that could be associated with deregulation in the protamine expression observed. The results of these protamine mutational studies showed that the presence of high penetrant mutations is a very rare cause of male infertility. However, some variants and some haplotypes described may behave as risk factors for male infertility. More recently, the presence of RNA in the mature sperm cell has also been investigated. The present chapter will introduce the basic aspects of protamine evolution and function and review the various articles published to date on the relationship between the protamines studied at the DNA, RNA, and protein levels and male infertility.

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M. Jodar
Human Genetics Research Group, IDIBAPS, University of Barcelona, Casanova 143, 08036 Barcelona, Spain

R. Oliva (✉)
Biochemistry and Molecular Genetics Service, Clinic Hospital, Villarroel 170, 08036 Barcelona, Spain

Faculty of Medicine, University of Barcelona, and Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Spain
e-mail: roliva@ub.edu

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Introduction

The protamines are a family of small basic proteins that are synthesized in the last steps of spermatogenesis of many animals and plants and are the nuclear proteins that are most abundant in sperm cells (Ando et al. 1973; Balhorn 2007; Bloch 1969; Dahm 2005; Mezquita 1985; Miescher 1874; Oliva 2006; Oliva and Dixon 1991). Protamines are rich in arginine residues in their sequences (48 % in human protamines), and these positive charges allow the formation of a highly condensed complex with the paternal genomic DNA, which has a strong negative charge. In mammals, two types of protamines are known: protamine 1 (P1) and the family of protamine 2 proteins (P2). The P1 protamine is present in all species of mammals studied (Oliva 2006; Oliva and Dixon 1991). The protamine P2 family is only present in some mammalian species, including human and mouse (Balhorn et al. 1987; McKay et al. 1986). In humans the components of the P2 family are formed by HP2, HP3, and HP4 (Arkhis et al. 1991; Balhorn et al. 1987; McKay et al. 1986). Both protamines, apart from arginine, which is by far the major amino acid, are rich in cysteine, which can form disulfide bonds and zinc bridges, contributing to the stabilization of the nucleoprotamine (Balhorn et al. 1992; Bjorndahl and Kvist 2010). The normal P1/P2 ratio is roughly 1 in human sperm, and variations above or below 1 have been shown to be present in many infertile patients (Aoki et al. 2005b; Balhorn et al. 1988; Belokopytova et al. 1993; Carrell and Liu 2001; Chevaillier et al. 1987; de Mateo et al. 2009; de Yebra et al. 1993, 1998; Oliva and Mezquita 1986; Torregrosa et al. 2006). This chapter introduces first the basic aspects of the evolution of protamines, protamine genes, transcription and translation, and their potential function. Subsequent sections of this chapter cover alterations in protamine content, mutations and polymorphisms in protamine genes, and protamine transcript alterations in infertile patients. It also complements other recent reviews of the structure, function, and evolution of protamines and the identification of sperm nuclear proteins (Ausio et al. 1999; Baker et al. 2012; Balhorn 2007; Dacheux et al. 2012; de Mateo et al. 2013; Dorus et al. 2012; Eirin-Lopez and Ausio 2009; Kasinsky et al. 2011; Oliva 2006, 2012; Oliva and Ballesca 2012; Oliva and Castillo 2011; Oliva et al. 2009).

Evolution of Protamines

The proteins present in the sperm chromatin of the various species are a heterogeneous group as compared with the proteins associated with the somatic chromatin (histones). Three major groups of sperm small nuclear basic proteins have been established (Balhorn 2007; Oliva and Dixon 1991).

1. Histone-type proteins: these sperm proteins are very similar to somatic histones. The sperm chromatin associated with these types of proteins present a pattern of condensation very similar to that of somatic cells.
2. Protaminelike proteins: these represent an intermediate group between histone-type proteins and the protamine type. These proteins are rich in both lysine and arginine residues (however, larger than the protamine type) and are related to the histone 1 family (Eirin-Lopez et al. 2006)
3. Protamine-type proteins or true protamines: these are smaller proteins enriched in arginine residues. Usually they also contain cysteine residues (Lewis et al. 2003). These proteins compact the sperm chromatin into a semicrystalline state.

Various studies suggest that the protamine family evolved from a histone 1 ancestor into specialized sperm-specific histones, and subsequently into protaminelike proteins, and finally into true protamines (Ausio et al. 1999; Eirin-Lopez and Ausio 2009).

Histone-type proteins (or sperm-specific histones) have been identified with various sperm-specific variants in their sequence in several species, suggesting that they participate in the compaction of the sperm chromatin. The protaminelike proteins have in their sequences a higher content of arginine and lysine residues (35–50 %) as compared to histones and have been identified in the sperm of many species ranging from sponges to amphibians. Finally, protamines have been identified in more than 100 vertebrate species, are enriched in arginine residues (up to 70 %), and contain multiple serine and threonine residues that can be used as phosphorylation sites. Moreover, the protamines in the majority of eutherian mammals contain multiple cysteine residues in their sequences, allowing the formation of disulfide bridges that enable linking of the adjacent protamines and strongly stabilize the chromatin into a highly compact nucleoprotamine complex (Saowaros and Panyim 1979).

Protamines have evolved very rapidly (Queralt et al. 1995; Retief et al. 1993). However, the mechanisms and drive forces of such an evolution are not yet completely clear. Various studies have shown that protamine 1 presents an unusual form of purifying selection in different species of mammals because it was observed that protamine 1 maintains in all species studied a high number of arginine residues, despite the fact that there is no selection at any particular amino acid position (Rooney et al. 2000). But it is not only the maintenance of a high quantity of positive charges that could stabilize the nucleoprotamine complex. Although the number of nonsynonymous substitutions is elevated in protamine 1 evolution, the conservation of other basic amino acids such as lysine is not observed. Perhaps the higher hydrogen bonding of the arginine residues toward DNA could favor the selection of this amino acid over that of lysine (Kasinsky et al. 2011).

These facts suggested that there is a positive selection to maintain a high number of arginine residues, and it was postulated that this enrichment in arginine residues in protamines could have an important role during fertilization. This unusual purifying selection has also been identified in less intensity in nonmammalian vertebrates and in some invertebrates.

Several vertebrates have two different protamines. The study of their sequences suggested a common origin and indicated that protamine 2 derived from a duplication of protamine 1 precursor (Krawetz and Dixon 1988).

Protamine Genes

All mammals studied so far have protamines. Humans and some mammals, such as mouse, hamster, rat, and stallion, have one functional genomic copy encoding for each of the two types of protamine described (protamines 1 and 2). However, in other mammals, such as bull and boar, it has been shown that the protamine 2 gene is mutated and does not produce a functional protein (Maier et al. 1990). It was postulated that protamine 2 derived from a duplication of protamine 1, although protamine 2 diverged very fast. It was demonstrated that the expression of protamine 2 is not redundant because the disruption of the *PRM1* or *PRM2* gene resulted in male infertility, and it was observed that the sperm cells of both types of knockout mice had an incomplete chromatin condensation and increased levels of DNA damage (Cho et al. 2001, 2003).The two protamine genes in mammals (*PRM1* and *PRM2*) are characterized by the presence of two exons and one small intron (Fig. 6.1), while the protamine genes for birds and salmonid fish have only one exon and no introns.

The genes that encode for different protamines are commonly clustered together in a small region of genomes. In human and mouse the protamine gene cluster is present in chromosome 16 (Nelson and Krawetz 1995).

The members of the protamine gene cluster in humans and other mammals, such as mouse, rat, and bull, are the *PRM1*, *PRM2*, and *TNP2* (transition protamine 2). The *TNP2* gene encodes for a protein more basic than histones and less basic than protamines, and also participates in the repackaging of the sperm DNA. In addition, it has been identified in the protamine gene cluster an open reading frame (ORF) located between *PRM2* and *TNP2* that is called gene 4 or *PRM3*. The *PRM3* gene was mistakenly called protamine 3 initially, but it is now clear that the *PRM3* gene is not a protamine but instead encodes a very acidic protein with a large proportion of negative residues (polyglutamic motif) and a low quantity of arginines in its sequence. Thus, the *PRM3* protein is not related at all to protamines. A *PRM3* knockout male animal presents a low sperm motility, but its fertility capacity is not affected (Martin-Coello et al. 2011). In addition, the fact that *PRM3* is present in the cytoplasm of elongated sperm and not in mature spermatozoa further indicates that it is a gene completely unrelated to protamines.

Transcription and Translation of Protamines

The members of the *PRM1* (protamine 1), *PRM2* (protamine 2), and *TNP2* (Transition protein 2) gene cluster (protamine cluster) are regulated at both the

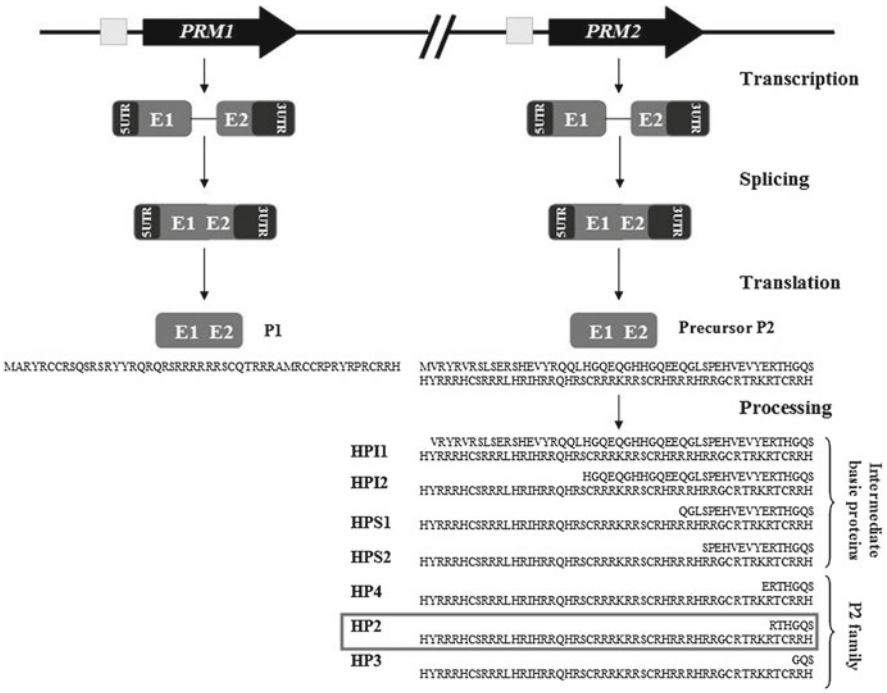

Fig. 6.1 *Transcription and translation of human protamine genes*. Schematic representation of human protamine genes and transcription and translation processes that give rise to mature proteins. While protamine 1 is translated as a mature protein, protamine 2 is translated as a precursor; after proteolytic processing it generates the mature protamine 2 family

transcription and translation levels, but these processes are uncoupled during spermatogenesis (Steger 1999). The protamine cluster is located in a DNA loop flanked by two cis-regulatory units involved in the attachment of DNA to the nuclear matrix (MARs) (Martins et al. 2004). The protamine cluster is believed to be first *potentiated* (potentiation is a mechanism that governs the selective opening of chromatin domains) in late pachytene spermatocytes and afterward transcribed in round spermatids, and these flanked MARs are required for the proper regulation of protamine genes (Martins and Krawetz 2007). This conformation enables the regulation of trans-regulatory transcription factors via the promoter region [TATA-box protein, cAMP response modulator (CREM), and Y-box proteins] (Queralt and Oliva 1993, 1995).

After transcription, protamine RNAs are stored in translationally repressed ribonucleoproteins (RNPs) and translated only in elongated spermatids (Oliva et al. 1988). This translational regulation is not entirely understood, but some regulatory sites in the 3'UTR region have been identified (Kleene 2003). This fact was demonstrated in a transgenic mouse lacking a 3'UTR of protamine 1 gene, which presents premature translation of protamine 1 transcripts and precocious condensation of nuclear DNA and abnormal sperm heads (Lee et al. 1995).

There is a marked difference between the syntheses of the two protamines. While protamine 1 is synthesized as a mature protein, protamine 2 is synthesized as a long precursor. In the last steps of spermiogenesis it is rapidly phosphorylated, and after phosphorylation the precursor is processed by proteolytic cleavage of the amino terminus in the various mature components of the protamine 2 family (HP2, HP3, and HP4), with HP2 being the most abundant (Arkhis et al. 1991; Balhorn et al. 1987). Various studies have also identified some intermediate basic proteins derived from the protamine 2 precursor (HPI1, HPI2, HPS1, and HPS2). These are defined as the putative precursors of the various mature forms (de Mateo et al. 2011b) (Fig. 6.1).

After their synthesis the protamines are quickly phosphorylated, predominantly in serine and threonine residues (Oliva and Dixon 1991). This proper phosphorylation may be required to allow a correct binding between protamines and the DNA. This hypothesis is supported by the results observed in *Camk4* knockout mice. It was described that the *Camk4* protein is responsible for the phosphorylation of protamine 2 in vitro. Mice lacking *Camk4* are infertile with impaired spermatogenesis due to the retention of *TNP2* in the DNA and the absence of protamine 2. Therefore, it was concluded that proper phosphorylation of protamine 2 is required in order to replace *TNP2* during spermatogenesis (Wu et al. 2000).

After the protamines bind to the DNA, an extensive dephosphorylation occurs. However, in some species, such as human, some residues remain phosphorylated: serine 8 and 10 in protamine 1 and serine 14 in protamine 2 (Chirat et al. 1993). Perhaps these marks also have an important role in sperm DNA packaging.

During the last steps of spermiogenesis and during the epididymal transit, after the binding of the protamines to the DNA and the phosphorylation and desphosphorylation processes, the sperm chromatin is further stabilized with the formation of intra- and intermolecular disulfide bridges (Vilfan et al. 2004). It was observed that the thiol groups that are not in disulfide bridges could bind metals such as zinc. This was observed especially in protamine 2 and could have an important role in the stabilization of the sperm chromatin (Bjorndahl and Kvist 2010). These covalent unions maintain and stabilize the toroidal structures that the protamine creates upon binding the sperm DNA (Hud et al. 1993). This situation is reversed once the sperm enters the egg, where thiol groups are reduced, the disulfide bridges break, and the protamines are removed.

Toroidal structures are present in the vast majority of sperm DNA (85 % in human sperm). Each toroidal structure contains approximately 50 kb of DNA, and it has been suggested that between each toroid there are nuclease-sensible regions that correspond to DNA regions that interact with the nuclear matrix in sperm nuclei (Ward 2010). The rest of the sperm DNA (15 % in humans) remains bound with histones forming nucleosome structures that could contain between 10 and 100 kb of DNA. Various studies have established that there is enrichment in the promoters of genes with important roles in the early embryo development, in miRNAs, and in imprinted regions in nucleosomal sperm DNA (Arpanahi et al. 2009; Hammoud et al. 2009).

Potential Function of the Protamines

Several functions have been proposed for the protamines (reviewed by Balhorn 2007; Oliva 2006; Oliva and Dixon 1990, 1991). The most obvious would be:

1. Generation of a condensed paternal genome with a more compact and hydrodynamic nucleus. The spermatozoa with the most hydrodynamic nucleus, being able to fertilize the oocyte first, would move faster. Therefore, the most condensed and hydrodynamic sperm would transmit the advantageous trait to future generations through an extremely marked "Darwinian" selection at the cellular level.
2. Protection of the paternal genetic message delivered by the spermatozoa by making it inaccessible to nucleases or mutagens potentially present in the internal or external media.
3. Competition and removal of transcription factors and other proteins from spermatids, resulting in a blank paternal genetic message devoid of epigenetic information, thereby allowing its reprogramming by the oocyte.
4. Involvement in the imprinting of the paternal genome during spermatogenesis. Also, protamines themselves could confer an epigenetic mark on some regions of the sperm genome affecting its reactivation or repression upon fertilization.

Some lines of evidence support all the foregoing potential functions for protamines. The phenomenon of sperm competition is consistent with the first hypothesis since the spermatozoa with the most hydrodynamic nucleus would move faster, making it possible for them to fertilize the oocyte first (Luke et al. 2012; Martin-Coello et al. 2009). Therefore, condensation capacity would be a strong evolutive drive.

Some clues in support of the second hypothesis (protection of the genome) are derived from knockout mice lacking protamine genes. In knockout mice presenting a disruption of one copy of the gene in one of two protamines (resulting in a decreased amount of protamine protein), male infertility, abnormal chromatin packaging, DNA damage, and altered sperm morphology occur. These results demonstrate that the two protamine genes (*PRM1* and *PRM2*) are needed in order to achieve good fertilization success (Cho et al. 2001). An additional transgenic mouse with protamine 2-deficient sperms also has a reduced amount of protamine 1, abnormal sperm morphology, aberrant chromatin condensation, and developmental embryo failure following intracytoplasmic sperm injection (ICSI) treatment (Cho et al. 2003). These studies reveal that an altered amount of protamines leads to an incomplete chromatin condensation (as derived from the observation of the abnormal morphology in the sperm cells), and that this abnormal condensation results in a high susceptibility to DNA damage. Therefore, the proper and intact transmission of male DNA to the next generation is disrupted. In addition, some support for the second hypothesis comes from the observations derived from assisted reproduction linking defects in protamination with injured sperm DNA, which is compatible with fertilization of the oocyte but precludes subsequent embryo development (Aoki et al. 2005a; Balhorn et al. 1988; Belokopytova et al. 1993; Carrell and Liu 2001; Chevaillier et al. 1987; de Mateo et al. 2009; de Yebra et al. 1993, 1998; Oliva and Mezquita 1986; Torregrosa et al. 2006).

Support for the third hypothesis stems from its extremely high affinity toward DNA, which would have the potential to compete and displace any other chromatin-associated proteins such as histones and transcription factors (Oliva et al. 1987, 1990; Oliva and Dixon 1990). Finally, a set of clues supporting the fourth hypothesis lies in the observation that the genomic gene distribution in the protamine and histone condensed regions in human sperm is not random (Arpanahi et al. 2009; Azpiazu et al. unpublished observations; Hammoud et al. 2009), suggesting an epigenetic potential function for these proteins. In addition, it has been shown that infertile patients have an altered distribution of genes in the nucleohistone and nucleoprotamine domains (Arpanahi et al. 2009; Hammoud et al. 2011; Oliva and Ballesca 2012).

Alterations in Protamine Protein Content

Various groups have studied the relative proportion of the two protamines in the mature sperm of different species that express both protamines. In humans the normal ratio observed is 1:1. However, in other mammals the protamine 2 content was found to range between 0 in the sperm of ungulates, bull and boar, and 80 % in the sperm of some primate species (Corzett et al. 2002). In addition, approximately 20 years ago various groups established a correlation between the presence of an abnormal P1/P2 ratio and the presence of human male infertility (Balhorn et al. 1988; Belokopytova et al. 1993; de Yebra et al. 1993). However, at that time the protocols available for the analysis of protamines were slow and tedious. Subsequently, the method was optimized to allow easier and faster extraction and analysis of human protamines (de Yebra and Oliva 1993) (Fig. 6.2).

Several groups have also found a high association between the presence of altered seminal parameters (sperm concentration, motility, and morphology) and the presence of an altered protamine ratio (Bach et al. 1990; Lescoat et al. 1988; Mengual et al. 2003). The altered protamine ratio can be increased or decreased in infertility patients. It has been described that some patients with a high protamine ratio show an increase in protamine 2 precursors as detected by western analysis (de Yebra et al. 1998). In addition, a positive correlation has been found between the presence of an altered protamine ratio or the presence of protamine 2 precursors and the presence of DNA damage as assessed using a variety of different methods such as SCSA, TUNEL, or COMET (Aoki et al. 2005b; Castillo et al. 2011; Torregrosa et al. 2006). These observations support the hypothesis that protamines are involved in the protection of sperm DNA.

There is also evidence that correlates the presence of DNA fragmentation and low ICSI or in vitro fertilization (IVF) rates (Evenson and Wixon 2005; Tamburrino et al. 2012). Several groups have studied the protamine ratio in infertile patients undergoing IVF or ICSI treatments and found a negative correlation between the presence of an altered protamine content and the sperm penetration score, fertilization rates, and pregnancy rates (Aoki et al. 2005a, 2006c; Carrell and Liu 2001; de Mateo et al. 2009; Khara et al. 1997; Nasr-Esfahani et al. 2004; Simon et al. 2011) (Table 6.1).

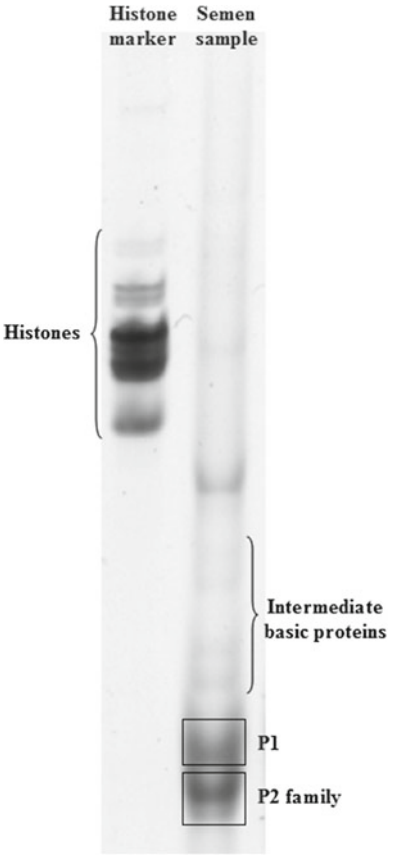

Fig. 6.2 *Analysis of human protamine protein ratio*. Following sperm nuclear protein extraction, the protamines are directly separated and analyzed in acid-urea polyacrylamide gels. The stained gels obtained are then scanned and the intensity of the bands quantified. The P1/P2 ratio is calculated using the direct quotient of the intensities of the P1 and P2 bands (see *boxes*) (unpublished results)

The majority of the studies done so far have focused on clinical infertility. Recently, a prospective study in the general population reported a higher-than-expected range in the protamine ratio. This result suggests that the relation between the presence of an altered protamine ratio and male infertility and its potential use as a clinical biomarker may not be so clear (Nanassy et al. 2011). Of potential clinical relevance, the sperm selection methods used in assisted reproduction techniques have the potential to improve sperm chromatin quality (de Mateo et al. 2011b).

As explained previously in section "Transcription and Translation of Protamines", the sperm DNA of fertile men is organized by protamines, except for a small part (5–15 %) that remains bound to histones. The differential distribution of histones in infertile patients with abnormal seminal parameters indicates that a higher quantity of DNA remains bound to histones (5–32 %). Also, patients with a normal quantity of histones bound to DNA presented a different distribution of histone patterns (Hammoud et al. 2011). These results suggest that an abnormal protamination could change the distribution pattern of histones and could affect early embryo development. In addition to this potential epigenetic distribution of the sperm chromatin in

Table 6.1 Studies measuring protamine P1/P2 protein ratio in infertile patients

Study	P1/P2 ratio	Associated phenotype
Castillo et al. (2011)	Decreased P1/P2	Increased DNA damage
Simon et al. (2011)	Decreased P1/P2	Lower fertilization rates, poorer embryo quality, reduced pregnancy rates
De Mateo et al. (2009)	Decreased P1/P2 Decreased Pre-P2/P2	Poor pregnancy outcome (IVF and ICSI treatment)
Aoki et al. (2006c)	Decreased P1/P2 and increased P1/P2	Low IVF score
	Decreased P1/P2	Low pregnancy ratio
Torregrossa et al. (2006)	Decreased P1/P2 and increased Pre-P2	Increase DNA damage
Aoki et al. (2005a)	Decreased P1/P2 and increased P1/P2	Reduced semen quality and sperm penetration ability (IVF)
Aoki et al. (2005b)	Decreased P1/P2	DNA damage
Nasr-Esfahani et al. (2004)	Altered P1/P2	Reduced fertilization rate and embryo quality (ICSI)
Mengual et al. (2003)	Increased P1/P2	Asthenozoospermic and oligozoospermic patients
Carrell and Liu (2001)	No P2	Reduced sperm penetration ability, sperm morphology, and motility
Khara et al. (1997)	Decreased P1/P2 and increased P1/P2	Fertilization index <50 %
Bach et al. (1990)	Altered P1/P2	Altered seminal parameters
Lescoat et al. (1988)	Altered P1/P2	Altered seminal parameters

ICSI intracytoplasmatic sperm injection, *IVF* in vitro fertilization

histone- versus protamine-associated regions, it is also important to consider the rest of the associated proteomic changes that can determine many as yet unknown additional epigenetic changes (de Mateo et al. 2007, 2011a ; Martinez-Heredia et al. 2008; Oliva and Ballesca 2012).

Polymorphisms and Mutations in Protamine Genes

Several factors have been postulated and studied as possible causes of protamine deregulation. Many different groups have performed mutational studies in infertile patients in search of mutations in protamine genes that could explain the abnormalities found in protamine expression. However, the various mutational studies done so far in the protamine genes suggest that pathogenic variants in these genes are a rare cause of male infertility.

Considering the various studies in which the coding region of the protamine genes were examined by direct sequencing of PCR-amplified DNA (Aoki et al. 2006a; Iguchi et al. 2006; Imken et al. 2009; Jodar et al. 2011; Ravel et al. 2007; Tanaka et al. 2003; Tuttelmann et al. 2010; Venkatesh et al. 2011), approximately 1,431 infertile patients and 887 controls were analyzed. In these 2,318 subjects,

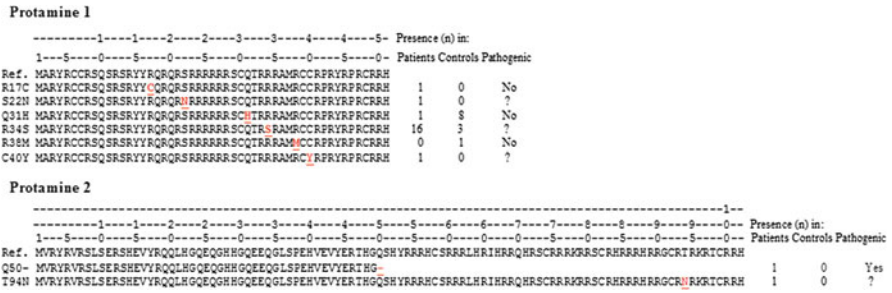

Fig. 6.3 *Missense variants reported in protamine genes*; alignment of amino acid sequences corresponding to missense variants identified in protamine genes. On the right-hand side of the alignment is indicated the number of samples in which the variants were identified. Also, the potential presence or absence of a pathogenic effect is indicated

only 6 different missense variants in the protamine 1 and two in the protamine 2 genes were identified (Fig. 6.3).

Of these eight variants, six were identified only in infertile patients; however, only the variant Q50* (Gluatmine-50 changes to a stop codon) in protamine 2, which was identified in only one azoospermic patient in a heterozygous state (Tanaka et al. 2003), seemed to be a cause of infertility in this patient. These results have been substantiated by studies in transgenic mouse, where the haploinsufficiency of protamines caused a male infertility (Cho et al. 2003).

The most frequent missense mutation identified in protamine 1 is a change of one arginine, quite well conserved in position 34, to a serine residue (R34S). This variant was always found in the heterozygous state. Some studies initially reported it as a pathogenic variant (Iguchi et al. 2006). However, other studies have shown that it is a common polymorphism in some populations (Kichine et al. 2008). A metaanalysis performed by Jodar et al. (2011) indicated that the presence of this polymorphism in protamine 1 (R34S) did not result in an increased risk of infertility (Gazquez et al. 2008; Jodar et al. 2011). This relatively low incidence of pathogenic mutations in the coding regions of the protamine genes in infertile men suggests the possibility of a strong negative selection against any potential pathogenic mutations that might have appeared at some point in evolution. It also indicates that the present sequence is necessary for the functioning of the gene and that some sequence variability is tolerated.

It is known that spermatogenesis is a highly regulated process at the transcriptional level with specific stages of transcriptional control. Many of the transcription factors responsible for this regulation are also expressed in other cell types, but it was described that, in testicles, these factors could regulate the transcription of special and unique transcripts or new splice variants (Freiman 2009). In fact, in testes the use of specific promoters and alternative splicing is prevalent, although the mechanisms of these phenomena are not yet known. Recently, a new form of transcriptional control in spermatogenesis was described based on changes in the 3'UTR regions of many transcripts, where the transcripts with a truncated 3'UTR region are specifically selected (Liu et al. 2007). This suggested the importance of

further studying these regions (promoter and UTR regions). Therefore, the latest mutational studies have also focused on the noncoding 5'and 3' regions since variants in these regions could also explain the detected variations in protein expression. Several variants in the promoter and UTR regions in the protamine genes have been identified. However, so far, it has not been possible to associate any of them with infertility as pathogenic variants. It is interesting to note that two variants have been found in the noncoding region of the protamine 1 gene. The first one is a polymorphism in the promoter of the *PRM1* gene (c.-191 C>A), which correlates with altered sperm morphology in the Spanish population (Gazquez et al. 2008; Jodar et al. 2011). The other one is a variant identified in the 5'UTR region of the *PRM1* gene, which in two studies was found only in infertile patients, suggesting an association between the presence of this variant and male infertility (Hammoud et al. 2007; Imken et al. 2009; Ravel et al. 2007). However, a subsequent study indicated that this variant seemed to be a normal polymorphism found in the sub-Saharan African population (Kichine et al. 2008).

The proximity of the two protamine genes and the presence of common polymorphisms in both genes made it possible to define the haplotypes present in the protamine genes in two different studies. The first one, by Tuttelmann et al. (2010), found that three common polymorphisms (1 in the coding region of *PRM1* and 2 in the intron region of *PRM2*) were present in significant linkage disequilibrium and could allow for the definition of six different haplotypes. Three of the haplotypes defined were the most common ones present in the population studied. The homozygous subjects with the most abundant haplotype in homozygosity presented a higher sperm count and concentration, while the presence of this haplotype in heterozygosity or its absence showed a significant decrease with these parameters. The authors hypothesized that perhaps there was a positive selection of this common haplotype because its presence was correlated with an increased amount of sperm cells, and a high concentration of sperm cells usually increases the chances of reproductive success.

The promoter and UTR regions of both protamine genes were included in the second study (Jodar et al. 2011). A total of three additional common but novel polymorphisms in the promoter region of the protamines (1 in *PRM1* and 2 in *PRM2*) were found in linkage disequilibrium with the three polymorphisms reported in the first study (Tuttelmann et al. 2010). With these six common polymorphisms, nine different haplotypes could be identified. Four of them were present in 97 % of the population studied, although no associations between the presence of one particular haplotype and the seminal parameters studied, in this case sperm morphology, were found. However, it was found that the presence of the five less abundant haplotypes was significantly higher in infertile patient than in fertile controls. It will be interesting to establish the haplotypes present in the entire protamine gene cluster including *PRM3* and the transition protein 2 (*PRM1-PRM2-*[*PRM3*]*-TNP2*) and its possible association with seminal parameters or fertility status. There is also substantial interest in the potential identification of genetic polymorphisms that may alter the function or the expression of the proteins and that could be either in linkage disequilibrium with a mutation on part of the same gene or haplotype. An important

genomewide study was performed in azoospermic and severe oligozoospermic patients (Aston and Carrell 2009). However, in this study no variants in the protamine genes were found to be associated with spermatogenic failure.

Alterations in Protamine mRNAs

Alterations in the presence of protamine RNAs have been identified in mature ejaculated spermatozoa (Wykes et al. 1997; Depa-Martynow et al. 2012). Additionally, some studies have found a significant correlation between the presence of an altered protamine protein ratio and the presence of an altered mRNA protamine ratio in mature sperm cells (Aoki et al. 2006b; Depa-Martynow et al. 2012).

The deregulation of the protamine protein ratio may due to an altered transcription or to an altered regulation of the translation of the protamine transcripts. The second possibility is supported by the finding that patients who presented a low protamine protein ratio due to low quantities of P1 protein had a higher level of *PRM1* transcripts, indicating the presence of a high retention of *PRM1* mRNA (Aoki et al. 2006b). On the other hand, in patients with a high protamine protein ratio due to a low amount of the P2 protein, it could be due to a low level of the *PRM2* transcript, as has been observed, or to an altered posttranslational regulation because it was shown that an alteration in the cleavage of the *PRM2* precursors could result in the accumulation of these precursors and low levels of P2 protein (de Yebra et al. 1998).

Table 6.2 summarizes the various studies that have found a significant association between the presence of an altered amount of protamine transcripts and the different seminal parameters in infertile patients. The initial studies analyzed the protamine transcripts in testicular RNA. However the latest studies measured the RNA present in mature ejaculated spermatozoa.

It can be observed that infertile patients usually have an altered protamine transcript ratio or altered amounts of these transcripts (Steger et al. 2001, 2008). The altered protamine transcript patterns show a strong correlation with the various seminal parameters such as sperm concentration and motility (Jodar et al. 2012; Kempisty et al. 2007; Lambard et al. 2004) and also with sperm fertilization capacity and embryo quality (Depa-Martynow et al. 2007; Mitchell et al. 2005). These results open up the possibility of future clinical use of these transcripts as biomarkers of fertility status. Similar observations have also been reported in model species, and the use of differential transcripts have been proposed as a method to identify good semen producers (Ganguly et al. 2012).

Concluding Remarks

Despite more than a century of research since protamines were first described, various issues must be resolved to allow a full understanding of their function and its implication in male infertility. The mechanisms and the proteins involved in the

Table 6.2 Studies in infertile patients where protamine transcripts have been quantified using real-time PCR

Study	Phenotype studied	Tissue studied	mRNA *PRM1*	mRNA *PRM2*	mRNA *PRM1/PRM2*
Ganguly et al. (2012)	Good versus poor quality semen producer	Bovine sperm	Decreased in poor producers		
Jodar et al. (2012)	Infertile patients versus fertile controls	Human sperm	Decreased in patients	Decreased patients	
Steger et al. (2008)	Infertile patients versus fertile controls	Human testis			Aberrant
		Human sperm			Aberrant
Depa-martynow et al. (2007)	IVF-treated couples fertilization versus no fertilization	Human sperm	Decreased in cases of no fertilization	Decreased in cases of no fertilization	
Kempisty et al. (2007)	Asthenozoospermic men versus normozoospermic men	Human sperm	Decreased in cases of asthenozoospermia	Decreased in cases of asthenozoospermia	
Mitchel et al. (2005)	TESE treated couples fertilization versus no fertilization	Human testis	Decreased in cases of no fertilization		
Lambard et al. (2004)	High versus low motility	Human sperm	Increased in cases of low motility		
Steger et al. (2003)	Infertile patients versus fertile controls	Human testis	Decreased in infertile patients		Aberrant
Steger et al. (2001)	Infertile patients versus fertile controls	Human round spermatid			Aberrant

IVF in vitro fertilization

nucleohistone–nucleoprotamine transition are just now beginning to be uncovered (Chocu et al. 2012; de Vries et al. 2012; Govin et al. 2012; Oliva 2012; Oliva and Castillo 2011; Rousseaux and Khochbin 2012). The additional proteins, besides protamines and histones, that are present in the sperm cell and their potential epigenetic contribution to the embryo are also being uncovered (Amaral et al. 2013; Baker et al. 2012, 2013; Dacheux et al. 2012; de Mateo et al. 2011a, b; Dorus et al. 2012; Oliva and Ballesca 2012; Oliva et al. 2009; Wang et al. 2012). From a practical perspective it will be interesting to clarify the mechanism by which polymorphisms present in the protamine genes in infertile patients may act as risk factors affecting sperm fertilizing capacity (Jodar et al. 2012). From etiological and clinical perspectives it will be important to determine what causes altered levels of protamines to appear at either the protein or RNA level (Castillo et al. 2011; Jodar et al. 2011). In addition to providing an answer to these questions, it will also be important to investigate the relation between the presence of DNA breaks, alterations in protamines, epigenetic changes in spermatozoa, and infertility (Oliva and Ballesca 2012; Simon et al. 2011). This aspect is important because of the potential transmission of an injured or altered genome to future generations. Finally, it will be interesting to determine the potential relationship between environmental or exogenous factors, such as the presence of polluting agents, thermal stress, or radiation, and alterations to protamines and sperm chromatin structure. It is foreseeable that the present genomic, transcriptomic, proteomic, and metabolomic tools, as well as systems biology, will further contribute to clarifying the factors involved in the normal remodeling of the sperm nucleus and in the identification of the pathogenic mechanisms involved in infertility.

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Chapter 7
Sperm DNA Fragmentation and Base Oxidation

Sheena E.M. Lewis

Abstract Sperm DNA damage has been shown to be a valuable diagnostic and prognostic biomarker for male infertility and assisted reproductive treatment (ART) outcome. It is linked to every fertility checkpoint from reduced fertilization rates, lower embryo quality and pregnancy rates to higher rates of spontaneous miscarriage and childhood diseases. It is more robust than conventional semen parameters.

The aim of this chapter is to provide an overview of current laboratory tests and relationships between sperm DNA damage and clinical outcomes. The conclusion is that sperm DNA damage is an important indicator of semen quality, and its routine use in the fertility clinic would improve ART success rates.

Keywords Sperm DNA damage • Art outcomes • Male infertility diagnosis

Introduction

Infertility affects approximately 15 % of couples of reproductive age (Cates et al. 1985; Hull et al. 1985; Kols and Nguyen 1997; Rutstein and Shah 2004), with male infertility contributing nearly 50 % of all cases (Irvine 1998; Niederberger et al. 2007; Vela et al. 2009; WHO 2010). As a result of population ageing and adverse lifestyle changes, infertility continues to increase, but with only marginal improvement in pregnancy and birth rates after assisted reproductive treatment (ART), in the developed world (Dupas and Christine-Maitre 2008; HFEA 2008; Povey and Stocks 2010; Ferraretti et al. 2012). In the last 30 years ART has become increasingly utilized with the number of cycles (de Mouzon et al. 2010) increasing by up

S.E.M. Lewis (✉)
Centre for Public Health, Queen's University Belfast, Room 0017, Ground Floor, Belfast, Northern Ireland, UK

Institute of Pathology, Grosvenor Road, Belfast, Northern Ireland BT12 6BJ, UK
e-mail: s.e.lewis@qub.ac.uk

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to 7 % per year in Europe. Still, pregnancy and live birth rates remain disappointingly low (average 27–33 %) (HFEA 2008; Ferraretti et al. 2012). One reason for this is that little has been done to resolve the causes and potential therapies for male infertility at the molecular level. Furthermore, there are currently no routine pharmaceutical therapies for male infertility.

Sperm DNA damage is a substantial indicator of ill health at the cellular level. It has been identified as a major contributor to male infertility as well as outcomes following ART, including impaired embryo development, miscarriage and birth defects in offspring (Freour et al. 2010; Koskimies et al. 2010; Bungum et al. 2011; Ebner et al. 2011; Gu et al. 2011; Simon et al. 2011; Zribi et al. 2011). Childhood health-related issues, such as childhood cancers, have also been linked to sperm DNA damage resulting from oxidative stress caused to sperm by smoking (Fraga et al. 1996; Ji et al. 1997). Since using sperm with damaged DNA for assisted conception means risking the long-term health and wellbeing of children conceived by ART, it is simply 'best practice' to test sperm DNA before using them clinically.

Current Semen Tests: Benefits and Limitations

Conventional semen analysis remains the gold standard for the initial investigation of male infertility. However, despite being the universal battery of tests, semen analysis is today considered of only limited value in predicting a couple's chance of pregnancy with ART. The World Health Organization (WHO) provides guidance for semen analysis through measures of concentration, motility and morphology (WHO 2010). Sperm motility is probably the most useful of these parameters since it is a real-time indicator of sperm metabolism (see later). Sperm concentration and morphology have minimal relation to ART success. In addition, both motility and morphology measurements are susceptible to inter- and intra-laboratory variation, and their wide ranges reflect the fact that sperm are amongst the most heterogeneous of all human cells (Jörgensen et al. 1997).

In 2001, Guzick's group (Guzick et al. 2001) examined semen from a large cohort of fertile and infertile men and reported a significant overlap in the semen profiles of the two groups. They concluded that sperm morphology, motility and concentration reference values were a blunt instrument in assessing male reproductive potential. This was subsequently confirmed by reports that the WHO (1999) reference values were not clinically predictive (Nallella et al. 2006; Van der Steeg et al. 2011). Since a very small proportion of sperm get to the site of fertilisation in vivo (Williams et al. 1992), expectations that information about the wider ranging properties of a complete ejaculate is unrealistic. In summary, a semen analysis is only useful in identifying those men with very few or no sperm.

For any test to be useful diagnostically or prognostically, it must have a threshold value which provides adequate discriminatory power in a clinical situation. Routine semen analysis does not meet these standards [Lefièvre et al. 2007; Guzick et al. 2001; reviewed by Lewis (2007) and Barratt et al. (2011)], so improved assays are needed.

Unfortunately, the success of intra-cytoplasmic sperm injection (ICSI) has allowed those in the field of infertility to become complacent, choosing to bypass rather than address the problem of male infertility. ICSI has reduced the significance and perceived need for sperm quality tests; ICSI requires only one sperm – even if morphologically abnormal and immotile – for the procedure to be around 25 % successful in most European clinics. ICSI is now the most widely used means of fertilisation in ART, but should the infertility speciality be satisfied with this modest level of success? Will adoption of a better test than semen analysis improve results?

Tests Currently Available to Assess Sperm DNA Damage

Since sperm have few repair mechanisms, DNA damage is ubiquitous in human sperm, even within donor populations (Simon et al. 2010). However, what is important clinically is the level of damage that adversely impacts ART outcomes.

The tests most often used today are the comet assay, SCSA, the terminal transferase dUTP nick end labelling (TUNEL) assay and the sperm chromatin dispersion (SCD or halo) test.

Comet Assay

The comet assay is a single-cell gel electrophoretic test that quantifies broken strands of DNA in individual sperm. As the mass of DNA fragments streams out from the head of unbroken DNA, it resembles a comet tail, hence the name of the assay. The comet is sensitive, repeatable and capable of detecting both high and low levels of damage in sperm (Irvine et al. 2000; Trisini et al. 2004; Aitken and De Iuliis 2007). A major advantage of this assay is that it requires only 5,000 sperm and so is suitable for the assessment of small samples left over from clinical use or for samples where only a few sperm are available. The comet assay can measure both single- and double-strand breaks and, with an additional step, can detect oxidised bases (Simon et al. 2010). This is important because we do not yet know which types of DNA damage are most deleterious to male fertility. A further advantage of the comet assay is that, unlike other tests which detect primarily breaks in histone-associated chromatin, it has a broader use in detecting breaks in both protamine- and histone-bound chromatin equally.

Clinical thresholds for diagnosis of male infertility and prediction of success with in vitro fertilisation (IVF) (Simon et al. 2010, 2011, 2013) have now been established by studies including over 500 couples. Unlike other sperm DNA fragmentation tests that give a DNA fragmentation index (DFI), which is the proportion of sperm in an ejaculate with some damage, the comet can detect damage in all individual sperm, even from fertile donors. The threshold values from the comet assay are measures of the actual damage in individual sperm above which spontaneous conception or success with IVF is less likely (Simon et al. 2010, 2013).

Analysis of repeatability was performed using the S_r^2, repeatability variance of the within-laboratory variances for single DNA damage measurements. It was 3.7 % but decreased to 2.6 % and 2.2 % for duplicates and triplicates respectively [ISO 5725:1994(E) guidelines for determination of repeatability of a standard measurement method, as described in Simon et al. (2013)]. In light of these results, analysis of just 50 of the 5,000 sperm included in the assay was sufficient to provide a measurement of DNA damage in the total sperm population with a coefficient of variation lower than 4 %.

In a recent study, the effects of male infertility alone on ART were evaluated by excluding all couples presenting with female factors or without detectable fertility problems from either partner (idiopathic infertility) (Simon et al. 2011). This study design allowed clinical thresholds for male infertility (25 %), success with IVF (25–50 %) or the need for ICSI (over 50 %) to be identified.

Most recently, live birth data were reported for the first time using the comet assay. Couples whose pregnancy resulted in a live birth had significantly lower sperm DNA fragmentation than those couples who did not achieve a live birth following IVF treatment (Simon et al. 2013). With the benefits of comet assay sensitivity, 80 % previously unexplained couples now have a diagnosis in the form of sperm DNA damage (Simon et al. 2013). In this latest study, high levels of sperm DNA damage were also associated with markedly lower live birth rates following IVF in 80 % couples with idiopathic infertility.

The usefulness of progressive sperm motility compared with DNA damage as predictive tools for in vitro fertilization rates has also been reported using the comet assay (Simon et al. 2011). Progressive motility is the only semen parameter that correlates with sperm DNA damage. This may be explained as a real-time functional test of sperm vitality. However, while fertilization rates are directly dependent upon both sperm progressive motility and DNA fragmentation, the latter is a stronger test, with an odds ratio of 24.18 (5.21–154.51) to determine fertilization outcome compared with 4.81 (1.89–12.65) for progressive motility (Simon et al. 2011).

Sperm Chromatin Structure Assay

The SCSA is a fluorescence cell sorter test which measures the susceptibility of sperm DNA to denature after exposure to acid conditions.

Neat semen is diluted with a pH 1.2 buffer for 30 s, and then the sperm are stained with acridine orange (AO) (Darzynkiewicz et al. 1975). Both the 30 s, low-pH-induced opening of the DNA strands at sites of DNA breaks and the biochemical interaction between AO and DNA/chromatin are precisely repeatable. This is proven by comparing cytogram scatter plots with 1,024 channels for both X (red) and Y (green) fluorescence values in repeat measures of individual semen samples (Evenson et al. 1991). The software SCSAsoft computes the raw red versus green fluorescence data as red/red + green fluorescence (Evenson et al. 2002).

This produces a vertical dot pattern for non-denatured DNA and a horizontal dot pattern for sperm with fragmented DNA. The SCSAsoft frequency histogram of DFI allows a precision determination of percentage DFI. Following repeated studies (Evenson et al. 1999; Spano et al. 2000; Evenson and Wixon 2006a, b; Bungum et al. 2007) an internationally accepted statistical threshold for natural and intrauterine insemination (IUI) conception of approximately 25 % DFI was adopted. The SCSA has robust statistical power, but it is unsuitable for samples with low counts. In addition, it measures only single-stranded fragments and has demonstrated associations between native, but not prepared, sperm and ART outcomes.

Sperm Chromatin Dispersion (Halo) Test

The halo test is a simple and inexpensive assay, available to fertility labs in kit form. Unlike all the other tests, it measures relaxed intact DNA associated with only peripheral histones rather than the damaged DNA in sperm. The test is convenient in that it does not rely on either colour or fluorescence intensity and is simple to analyse in a routine laboratory with light microscopy. One limitation of the assay is that its low-density nucleoids are relatively faint, with less contrasting images. To date, correlations have been observed between DNA damage and other sperm parameters, although few correlations between sperm DNA damage and ART outcomes have been established with the halo test, even in large ($n = 600$) studies. However, Meseguer et al. (2009) reported that sperm DNA damage as measured by halo has a negative impact on pregnancy.

TUNEL Assay

The TUNEL assay detects 'nicks' (free ends of DNA) by incorporating fluorescently stained nucleotides. This allows the detection of single- and double-stranded damage. The cells can be assessed either microscopically or by flow cytometric (FCM) analysis. This gives the assay the flexibility to be used for small numbers with microscopic analysis and in small laboratories which do not have dedicated and expensive FCM facilities. However, it can also be analysed by FCM, giving it the advantage of robust numbers with reduced time and labour. A disadvantage of the assay is its many protocols, which makes comparison between laboratories almost impossible and explains its many clinical thresholds. Recently, Aitken's group (Mitchell et al. 2010) improved the TUNEL assay by including a preliminary step of DDT to relax the whole chromatin structure and allow access to all nicks. They also added a viability stain so that DNA damage is measured only in live sperm. This has eliminated a previous inaccuracy of measuring damage (often at high levels) in dead cells. Robust clinical thresholds have yet to be established.

Novel Tests for Oxidised Bases

DNA damage tests usually measure strand breaks. While this provides data on the final stage of damage, these tests give little information about how the damage came about. Knowledge about the DNA adducts present in human sperm will also provide information about earlier stage DNA damage and thereby enhance the prognostic value of our current tests. DNA damage in sperm is primarily from oxidative stress (OS) (Aitken et al. 2010). A low physiological level of reactive oxygen species (ROS) is considered necessary to maintain normal sperm function, but ROS levels above physiological norms may cause deteriorating function or reduced survival (Aitken et al. 1989). The sperm most susceptible to OS are those that survived incomplete or abortive apoptosis in the testis and sperm that underwent flawed chromatin remodelling during spermiogenesis (Aitken and De Iuliis 2007). A number of biological and environmental factors that create DNA adduct formation in sperm are associated with impaired embryonic development and the health of the offspring (Adler 2000; Anderson 2001).

The measurement of sperm DNA modifications such as 8-hydroxy-2-deoxyguanosine (Lee et al. 2009; Makker et al. 2009; Gharagozloo and Aitken 2011; Thomson et al. 2011) and xenobiotic adduct formation (Zenes 2000) including benzo[a]pyrene (Park et al. 2008), are the latest area of research. Already, these two lesions have been reported as significant in male infertility and childhood health (Anderson 2001; Lee et al. 2009). The characterisation of OS markers can result from a number of infertility aetiologies suggesting that OS is a major mediator of DNA damage in the male germ line (De Iuliis et al. 2009). The incidence of these markers together with the detection of others, such as advanced glycation end products, may confirm specific pathologies such as diabetes (Agbaje et al. 2008).

Sperm DNA Damage in Male Infertility Diagnosis

Infertile males have greater sperm DNA fragmentation compared to those in the general population or men with recently proven fertility (Schulte et al. 2010). Two independent, population-based studies, one from the USA (Evenson et al. 1999) and one from Denmark (Spano et al. 2000), have shown that sperm DNA damage is a useful marker in the prediction of fertility in males from couples of unknown fertility. Both of these studies have shown that the chance of spontaneous conception declines at sperm DNA damage (DNA fragmentation index, DFI; this parameter relates to SCSA tests only) values above 20 % and approaches zero for readings over 30–40 %. This means that although low sperm DNA damage (<20 %) does not guarantee normal male fertility, higher levels of damage suggest more substantial male infertility. Furthermore, the SCSA data indicate that for men who have been

classified as normal by a semen analysis the risk of infertility starts to increase at DFI levels above 20 % [odds ratio (OR) 5.1, 95 % confidence interval (CI): 1.2–23]. The threshold becomes even lower (10 %) if the man's semen has a subnormal semen analysis as well (Giwercman et al. 2010). In another study (Simon et al. 2011), this one using the comet assay, there was also a strong correlation between sperm DNA fragmentation and the fertility status of men, with 95 % of fertile donors having DNA fragmentation below 25 % and 98 % (mean DNA damage per sperm) of infertile men having DNA fragmentation values above 25 %. The prognostic value of sperm DNA fragmentation in relation to infertility showed an OR for infertility of 120 (95 % CI: 13–2700) in men with DNA damage above 25 % (Simon et al. 2011). Thirdly, a comparison between male infertility patients and sperm donors using a flow cytometric TUNEL assay gave 19.25 % as the cut-off value with no donors but 65 % patients having DNA damage above this level (Sharma et al. 2010). Thus there is robust evidence from all the DNA fragmentation tests that the chance of spontaneous pregnancy is reduced when DNA damage is high.

Sperm DNA Damage and Assisted Reproduction

Success rates for IUI are similar to those for spontaneous pregnancies, indicating a reduction in the chances of pregnancy with sperm DNA damage values above 20 %, according to the SCSA (Bungum et al. 2007). If a test for oxidised bases is employed (8-hydroxy-2`-deoxyguanosine; 8-OHdG), the results are even more sensitive, with a lower threshold value of 11.5 % (Thomson et al. 2011). Lewis' group recently reported that 80 % of couples with unexplained infertility, and therefore those couples likely to be offered IUI as a first treatment, although they have significant sperm DNA damage (Simon et al. 2013).

For IVF, Zini and Sigman (2009) published a meta-analysis showing an increased chance of pregnancy (OR: 1.7; 95 % CI: 1.3–2.2) in cases where the proportion of DNA damaged sperm was below the threshold values for SCSA or TUNEL. As a result of these data, sperm DNA testing is now employed routinely throughout southern Sweden. Support for these data is given in two studies using the comet assay (Simon et al. 2010, 2013), both published after Zini and Sigman's (2009) meta-analysis with an OR of 76 (95 % CI: 8.7–1700) for clinical pregnancy if the mean DNA fragmentation per sperm was below 52 % (Simon et al. 2011). The latest study using the comet assay showed that couples with low levels of sperm DNA fragmentation (<25 %) have a live birth rate of 33 % following IVF treatment. In contrast, couples with high levels of sperm DNA fragmentation (greater than 50 %) had a much lower live birth rate of 13 % following IVF treatment. Thirty-nine percent of couples with idiopathic infertility have high (greater than 50 %) sperm DNA damage. Sperm DNA damage was also associated with lower live birth rates following IVF in couples with idiopathic infertility than in couples with detectable causes.

When considering the reports to date, this author is of the opinion that our expectations of sperm DNA testing tend to be excessive. How can a single parameter (from only one of the two gametes) provide an absolute criterion for fertility or infertility? A successful ART outcome will depend on many other traits of sperm quality and function, as well as the influences of the oocyte, uterine receptivity and maternal immune system competence.

Implications of DNA Damage for the Health of Future Generations

Animal studies provide compelling evidence that the induction of DNA damage in the male germ line can induce miscarriage and morbidity in offspring (Fernández-Gonzalez et al. 2008). A higher risk of morbidity in the offspring is presented by smoking, and again paternal smoking induces sperm DNA damage (Fraga et al. 1996) as well as childhood cancers in the offspring (Ji et al. 1997; Lee et al. 2009). Paternal age is also linked to a high incidence of DNA damage in human sperm (Singh et al. 2003; Schmid et al. 2007; Varshini et al. 2012). Paternal age is also linked to a higher incidence of epilepsy, schizophrenia, autism, and bipolar disease (Sipos et al. 2004; Reichenberg et al. 2006; Aitken and De Iuliis 2007; Frans et al. 2008). It is also linked to an increased risk of cancer in the offspring (Hemminki et al. 1999; Johnson et al. 2011) and congenital anomalies (Green et al. 2010). This suggests that adverse paternal effects on the offspring health are passed on by DNA damaged sperm. This may be even more important in men seeking infertility treatment. In a recent meta-analysis (Wen et al. 2012) it was reassuring to note that no difference was found between the risks associated with IVF and those associated with ICSI. However, in contrast, a recent analysis of pregnancies in South Australia revealed a significantly enhanced chance of birth defects in ICSI compared with IVF children (Davies et al. 2012).

Potential of Antioxidant Therapy

If OS is involved in the aetiology of DNA damage, then antioxidant therapy should be part of the cure (Greco et al. 2005). Men who have been diagnosed with oxidative sperm DNA damage by one of the tests described earlier might be helped by taking antioxidants before ART is begun. A recent review paper (Gharagozloo and Aitken 2011) summarised 20 clinical trials of ART outcomes following antioxidant use over the last decade. All the trials showed a reduction in sperm OS, and some also reported improvement in clinical outcomes such as pregnancy. Clinicians routinely recommend the use of antioxidant(s), as was recently reported (Lanzafame et al. 2009; Zini et al. 2009; Ross et al. 2010; Showell et al. 2011; Gharagozloo and Aitken 2011). Regimes of concentration, constituents or duration of therapy have not been carefully considered, but these dietary supplements are probably not unsafe, although their benefits may be limited. In contrast, higher doses and long

durations of administration as well as the use of synthetic or chemically modified versions of antioxidants should be avoided. One example of danger to health was reported in the large cancer prevention SELECT clinical trials (long-term use of vitamin E at 400 IU/d) where a significant rise in prostate cancer among 35,533 healthy men was found (Klein et al. 2011). Future research and clinical studies should address these issues as a matter of urgency.

Why Does ICSI Work with Poor Sperm?

Sperm DNA damage has not been found to be predictive for ICSI treatment (Zini 2011), with one exception (Bungum et al. 2007). However, in this study, couples were not randomised for IVF or ICSI, so the impact of other factors contributing to the choice of treatment cannot be excluded. A number of reasons have been put forward to explain the finding that poor sperm DNA does not appear to adversely affect ICSI outcomes. Firstly, unlike IVF, up to 30 % of women (with subfertile partners) having ICSI have no detectable problems. They may be fertile and their oocytes may have more capacity to repair DNA damage, even if the injected sperm is of poor quality. This is supported by the findings of Meseguer et al. (2011) where high-quality oocytes from donors offset the negative effect of sperm DNA damage on pregnancy.

Secondly, a recent major study (Dumoulin et al. 2010) showed that even the birth weight of IVF babies can be markedly influenced by minor differences in culture conditions. In contrast to IVF, ICSI sperm are injected into the optimal environment of the ooplasm within a few hours of ejaculation. This may protect them from laboratory-induced damage.

Thirdly, it is well documented that sperm from up to 40 % of infertile men have high levels of ROS (Henkel 2011; Aitken et al. 2012), and their antioxidant content is also significantly lower than in fertile men (Lewis et al. 1995). During the IVF process, oocytes can be exposed to an overnight oxidative assault from 0.5 million spermatozoa releasing ROS. This may well impair the oocyte's functional ability, including its capacity to repair sperm DNA fragmentation after fertilization.

Finally, as mentioned earlier, evidence is emerging that embryos with high sperm DNA damage are associated with early pregnancy loss, as reviewed by Zini et al. (2008) using 11 studies composed of 808 IVF and 741 ICSI cycles, so ICSI success rates are sometimes affected adversely by sperm DNA damage, but at a later stage. In fact, high levels of sperm DNA damage are associated with increased risk of pregnancy loss (OR: 2.5; 95 % CI: 1.5–4.0), regardless of the in vitro technique applied, as reviewed by Robinson et al. (2012).

Conclusion

Thus, as a matter of best practice, to improve ART outcomes, sperm DNA damage testing should become part of routine semen analysis (Fig. 7.1).

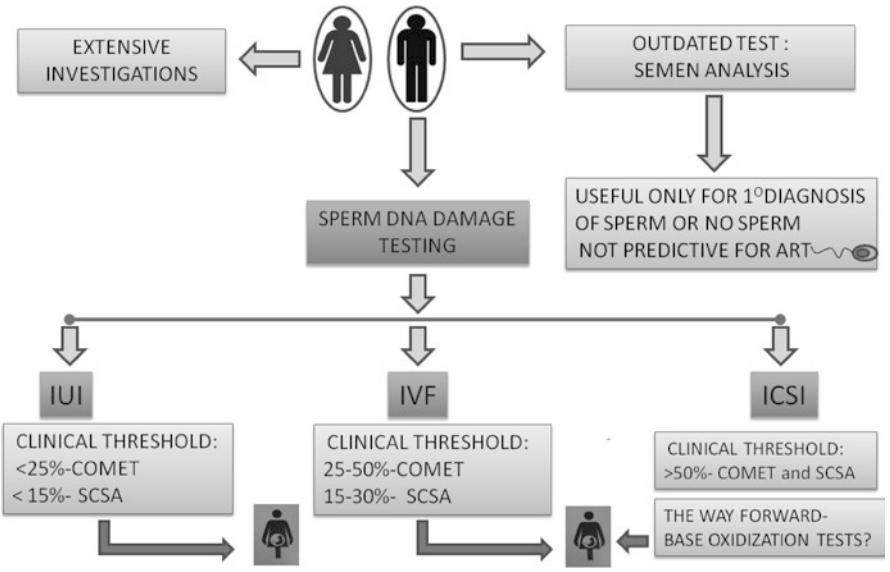

Fig. 7.1 Summary of clinical use of sperm DNA damage testing

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Chapter 8
Iatrogenic Genetic Damage of Spermatozoa

Cristian O'Flaherty

Abstract Various factors that negatively influence male fertility can affect sperm morphology and physiology. Many studies on humans and animals suggest that both radiation and chemotherapy alter the sperm chromatin, inducing significant damage to sperm DNA, and decrease the level of protamination, thereby altering DNA compaction. Spermatozoa from cancer survivors are affected by chemotherapy even years after the end of treatment. We are exposed to various toxicants present in the environment (e.g., products of air pollution, pesticides, and plasticizers) whose impact on human male reproduction has not yet been established.

This chapter aims to update our knowledge on how the sperm chromatin structure is modified by external agents and to describe the different strategies available to better study this complex structure in infertile men.

Keywords Sperm chromatin • Chemotherapy • Radiation • Sperm DNA damage • Male infertility • Protamination • Thiol oxidation

Introduction

Sperm Chromatin Structure

The ultimate goal of any given spermatozoon is to deliver the paternal genetic information into a mature oocyte during fertilization. To accomplish this essential task for any species, a round cell spermatogonium must go through several divisions and

C. O'Flaherty (✉)
Division of Urology, Department of Surgery, McGill University and the Research Institute-McGill University Health Centre, Royal Victoria Hospital, room H6.46, 687 Avenue des Pins Ouest, Montréal, Québec H3A 1A1, Canada
e-mail: cristian.oflaherty@mcgill.ca

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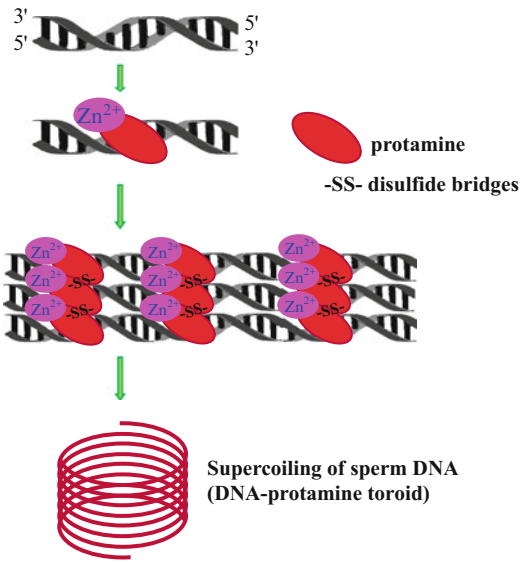

Fig. 8.1 Organization of sperm chromatin structure. The intimate interaction between the DNA strands and protamine and the formation of disulfide bridges (−SS-) among protamines and the incorporation of zinc (Zn^{2+}) make for a tightly compacted structure

transformations in the testis to become a fully formed spermatozoon (Kerr et al. 2006). Then the spermatozoon leaves the testis to start a journey through the epididymis, a long, single, and highly convoluted tube, to complete its maturation, acquiring some elements needed for fertilization and the ability to move (Robaire et al. 2006). Finally, it must reside in the female reproductive tract, specifically the oviduct, to achieve fertilizing ability, be able to recognize the oocyte, and undergo the acrosome reaction needed to go through the zona pellucida that surrounds the oocyte. This process is called capacitation and involves a series of biochemical and morphological changes that prepare the spermatozoon for fertilization (Yanagimachi 1994; de Lamirande and O'Flaherty 2008, 2012).

To accomplish this long journey through the testicular, epididymal, and female tract environments, the sperm chromatin is transformed into a complex structure, involving the association of DNA with basic proteins called protamines and other elements, forming a toroid structure; this transformation aims to avoid potential damage to the genomic material (Fig. 8.1). During spermatogenesis, histones are replaced by protamines, allowing a tighter compaction of the sperm DNA compared to somatic cells (Aoki and Carrell 2003; Govin et al. 2004). Histones are replaced first by transitional protein (Meistrich et al. 2003) and then by protamines 1 and 2 (P1 and P2) in human spermatids (Aoki and Carrell. 2003; Govin et al. 2004) during spermiogenesis (Kerr et al. 2006). In vitro studies suggest that hyperacetylation, an epigenetic modification of histones, allows the replacement of histones by protamines (Oliva and Mezquita 1982; Oliva et al. 1987, 1990). A cycle of phosphorylation-dephosphorylation occurs in protamines before binding to DNA and during nucleosome maturation (Marushige and Marushige 1978; Papoutsopoulou et al. 1999). Protamines have a high number of positively charged residues, allowing the formation of a highly condensed complex with the sperm DNA that has strong negative charge (Retief et al. 1993; Wouters-Tyrou et al. 1998; Lewis et al. 2003; de Mateo et al. 2011; Martinez-Heredia et al. 2006).

Despite this massive protein exchange, promoting an 85–95 % association of sperm DNA with protamines, 5–15 % remains associated with histones (Gatewood et al. 1987; Zalensky et al. 2002; Balhorn 2007). Several histone isoforms (e.g., H2A, H2B, H3, and H4) and isoform variants are present in human spermatozoa, with histone H 2B being the predominant isoform (Gatewood et al. 1990a); the increased levels of histones or histone variants are associated with abnormal DNA compaction and DNA damage in astenozoospermic infertile men (Zini et al. 2008). Similarly, the change in the P1/P2 ratio due to an increase in P2, together with increased levels of the pre-P2, is associated with sperm DNA fragmentation in infertile men (de Yebra et al. 1998; Torregrosa et al. 2006), and a low P1/P2 ratio has been associated with low pregnancy rates (de Mateo et al. 2008).

The stabilization of sperm chromatin is accomplished, in part, by the addition of zinc (Zn^{2+}) to the sperm nucleus at the initiation of nuclear compaction (Gatewood et al. 1990a; Bjorndahl and Kvist 2009; Kerr et al. 2006). This micronutrient is important for fertility as Zn^{2+} deficiency induces arrest at spermiogenesis, a decrease in germ cell proliferation, and impairment of sperm motility in different species including humans (Yamaguchi et al. 2009; Croxford et al. 2011). Zn^{2+} contributes to the stabilization of sperm chromatin by binding to free thiol (−SH) groups and forming Zn^{2+} bridges among protamines (Gatewood et al. 1987, 1990a; Bianchi et al. 1992; Bench et al. 2000). The stabilization of sperm chromatin is completed by the formation of disulfide (−SS-) bridges among protamines during epididymal maturation (Bedford et al. 1973; Marushige and Marushige 1975; Seligman and Shalgi 1991); in normal human spermatozoa, less than 1.5 % of cysteines are found as reactive –SH (Rousseaux and Rousseaux-Prevost 1995). Increased (Zini et al. 2001) or decreased (Seligman et al. 1994; O'Flaherty et al. 2008; Ramos et al. 2008) levels of free –SH has been observed in infertile men, indicating that under- or over-oxidation of –SH is associated with abnormal sperm function. This alteration is due to the improper oxidation of –SH groups in most sperm proteins, including protamines, indicating abnormal epididymal maturation (Bedford et al. 1973; Bedford and Calvin 1974). Our current understanding of the players and the intrinsic mechanisms that induce sperm chromatin condensation during spermiogenesis is poor. It is possible that this task is performed by the nuclear isoform of glutathione peroxidase (nGPX4), which is necessary for protamine thiol cross-linking during sperm maturation (Pfeifer et al. 2001). Mice lacking the nuclear isoform of glutathione peroxidase 4 (nGPX4) have spermatozoa with increased DNA decondensation; however, these animals are viable and fertile (Conrad et al. 2005). More research is needed to reveal the other players in the sperm DNA condensation mechanisms to understand better how the sperm chromatin is shaped during sperm maturation.

Alterations of Sperm Chromatin Structure

The sperm chromatin can be altered and therefore is susceptible to damage at different stages of sperm production and maturation. Starting in the testis, it could be affected by apoptosis during spermatocytogenesis or during chromatin remodeling at the

time of spermiogenesis (Kerr et al. 2006). Approximately 50 % of germ cells that enter into meiosis become apoptotic and are removed by Sertoli cells. Sometimes, this process is not as efficient as required and some defective germ cells continue developing and can be found in the ejaculate (Sakkas et al. 1999; Sakkas and Alvarez 2010). It is then possible that spermatozoa carrying apoptotic markers such as Fas, caspase activities, p53, and annexin-V can found in semen (Glander and Schaller 1999; Sakkas et al. 2002; Cayli et al. 2004; Said et al. 2006; Mahfouz et al. 2009). Although there are correlations between apoptotic markers and poor semen quality (Glander and Schaller 1999; Sakkas et al. 2002; Cayli et al. 2004; Said et al. 2006; Mahfouz et al. 2009), some infertile men have spermatozoa with normal morphology and good motility, and it is impossible to discriminate between affected and healthy sperm cells (Lee et al. 2010). Many strategies are being investigated to overcome this problem, but they will not be discussed further in this chapter.

As mentioned earlier, the sperm chromatin remodeling during spermiogenesis is another stage where this structure is susceptible to damage. The replacement of histones by protamines requires nuclease activity that creates DNA nicks to provide relief from torsional tension. This helps to achieve the necessary chromatin arrangement during histone replacement in spermatids in many species, including humans (McPherson and Longo 1992, 1993; Marcon and Boissonneault 2004). A deregulation of this process may promote abnormal chromatin packaging or DNA fragmentation, which can be detected in the ejaculated spermatozoa.

After spermiation, spermatozoa enter into the epididymis to acquire the ability to move and fertilizing ability (Yanagimachi 1994; Robaire et al. 2006). During the journey through the epididymis, which varies among species but in humans is 5–6 days, spermatozoa can be damaged and can show significant DNA fragmentation (Ollero et al. 2001; Greco et al. 2005). This is the result of reactive oxygen species (ROS) action or ROS-modified metabolites; for instance, it is known that hydrogen peroxide produces DNA fragmentation in human spermatozoa (Aitken et al. 1998) and that the radical hydroxyl can attack DNA bases, promoting oxidation and an increase in 8-oxoguanosine (8-oxoG) among other DNA metabolites. Another consequence of ROS attack is the production of abasic sites, which destabilize the double helix and can result in strand breaks (Nakamura et al. 2000).

Drugs and other chemicals can affect human sperm chromatin (Fossa et al. 1997; Tempest et al. 2007; O'Flaherty et al. 2010, 2012). Common lesions found associated with agents are the presence of interstrand cross-linking and chemical adducts. These are highly toxic DNA lesions that prevent translation and replication by inhibiting DNA strand separation (Hurley 2002; Deans and West 2011). This property is convenient when the goal is to eliminate cancerous cells; however, chemotherapy severely affects germ cells (Petersen and Hansen 1999; Gandini et al. 2006). Animal studies suggest that spermatogonia (Marcon et al. 2010, 2011) and Sertoli cells (Meistrich et al. 1982; Marcon et al. 2008) are deeply affected by chemotherapeutic agents.

DNA methylation and histone modifications (e.g., phosphorylation, methylation, and acetylation) are epigenetic changes that occur during spermatogenesis in the spermatozoa; they are important in assuring the development of the future embryo.

Thus, any changes in the programming epigenetic modifications during spermatogenesis by different causes (e.g., drugs, diseases) may promote male infertility (Trasler 2009; Hammoud et al. 2011).

Evaluation of Sperm Chromatin Structure

Different assays have been developed to determine sperm DNA or other components of the sperm chromatin; interestingly, each of them measures a certain type of damage, thus having limited use in characterizing the entire sperm chromatin structure. This is important to mention because only a multi-assay approach will allow a proper characterization of this complex structure; however, this type of analysis is limited by the amount of spermatozoa in a given sample (O'Flaherty et al. 2008).

Assays to Determine Sperm DNA Damage

Acridine Orange/Sperm Chromatin Structure Assay (SCSA)

Acridine orange (AO) is a metachromatic probe that binds to single or double strands of DNA, giving a red or green fluorescence, respectively, when it is excited at 470–490 nm (Evenson and Wixon 2005). This property is useful for identifying spermatozoa with denaturated (single-stranded) DNA. Sperm DNA can then be analyzed by fluorescence microscopy or flow cytometry (Evenson et al. 1980; Kosower et al. 1992).

This assay determines the susceptibility of sperm DNA to acid denaturation (pH 1.20); the low pH induces the opening of the sperm DNA strands at the sites of DNA breaks. Then sperm are incubated with AO and the red and green fluorescence is acquired using a flow cytometer (Ballachey et al. 1988; Evenson et al. 2002).

The SCSA is one of the most well-tested assays for studying the sperm chromatin structure and has been used by various laboratories worldwide. Three main sperm populations can be distinguished after analysis of the acquired data: (1) sperm with no DNA damage, (2) sperm with moderate or high DNA damage, and (3) sperm with high AO DNA stainability. Based on these sperm populations, data are expressed as the mean DNA fragmentation index (DFI), as the standard deviation of DFI (SD DFI), as a percentage of DFI (%DFI, corresponding to the percentage of cells outside the main population), and as a percentage of spermatozoa with high green fluorescence or high DNA stainability (%HDS), as an indication of sperm DNA compaction (Evenson and Wixon 2005). The DFI and HDS are the most widely used SCSA parameters for characterizing the sperm chromatin, but the mean DFI and SD DFI are also powerful parameters to determine the presence of sperm DNA damage. For instance, significant sperm DNA fragmentation can be found with the DFI, mean DFI, and SD DFI parameters in semen samples from

infertile men, whereas the mean DFI indicated significant DNA damage in samples from Hodgkin's lymphoma patients with a DFI value similar to that of healthy controls (O'Flaherty et al. 2008).

Comet Assay

The single-cell gel electrophoresis assay, or comet assay, is a common tool in male reproductive toxicology for studying DNA fragmentation in spermatozoa in humans and in animal models (McKelvey-Martin et al. 1997; Haines et al. 1998, 2002; Codrington et al. 2004; O'Flaherty et at. 2008). This assay is based on the electrophoretic migration of DNA fragments from the core of chromatin after a sperm suspension is treated with a buffer with neutral or alkaline pH. These fragments originate from single- and double-strand breaks of sperm DNA. Then the slides are stained with a DNA labeling dye (e.g., propidium iodide, SBYR green), and individual pictures of spermatozoa are taken using a fluorescence microscope. The extension of the sperm DNA damage can be determined using a software that provides the following parameters: percentages of the DNA in the head or the tail of the comet, the tail length, and the tail extent moment (O'Flaherty et al. 2008). It is a very reliable assay to determine sperm DNA damage in humans, particularly in severe olizoospermic samples from infertile men (O'Flaherty et al. 2008) or cancer survivors (O'Flaherty et al. 2010).

TUNEL Assay

Another way to detect single- and double-strand DNA breaks is the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Sergerie et al. 2005). The 3'-OH openings of single- and double-strand breaks are labeled by the addition of FITC-labeled deoxyuridine triphosphate nucleotides in a reaction catalyzed by deoxynucleotidyl transferase (Schmid et al. 1994; Telford et al. 1994). Labeled spermatozoa are then analyzed by fluorescence microscopy or flow cytometry (Gandini et al. 2000; Muratori et al. 2000, 2003; O'Flaherty et al. 2008). Both the microscopy- and flow-cytometry-based methods have been extensively used to determine sperm DNA damage in human spermatozoa (Gandini et al. 2000; Muratori et al. 2000, 2003; Weng et al. 2002; Marchiani et al. 2007; O'Flaherty et al. 2008).

Sperm DNA Oxidation Determination

The oxidation of sperm DNA generates the formation of 8-hydroxy-2′ deoxyguanosine (8-OHdG) promoting single- and double-strand DNA breaks; this damage is associated with oxidative stress, which is a major component in the pathophysiology of male infertility (Gagnon et al. 1991; de Lamirande and Gagnon 1995; Agarwal et al. 2006;

Aitken and Baker 2006; Tremellen 2008; Aitken and Curry 2011; Gong et al. 2012). Therefore, the determination of the 8-OHdG levels in spermatozoa should be an important part of the semiology in infertile men. Immunocytochemistry- and flow-cytometry-based techniques using antibodies or binding proteins that recognize 8-OHdG were used to determine sperm DNA oxidation in spermatozoa from different species, including humans (Chabory et al. 2009; Aitken et al. 2010; Paul et al. 2011). Recently, Cambi et al. (2013) developed a flow-cytometry-based method using an anti-8-OHdG moiety antibody that overcomes the problem of false positives sometimes given by methods using binding proteins (Cambi et al. 2013). In this study, the researchers found negative correlations between levels of 8-OH-G and sperm parameters in a cohort of 94 infertile men, thus demonstrating the clinical relevance of using the detection of 8-OHdG in combination with standard semen analysis for the diagnosis of male infertility.

Sperm Chromatin Dispersion Test

The sperm chromatin dispersion (SCD) assay was developed to determine sperm DNA damage for the diagnosis of male infertility (Fernandez et al. 2003). This is a microscope-based assay that detects sperm DNA fragmentation in samples that were previously acid-denatured. The treated sperm are then stained with a probe that binds to DNA (e.g., DAPI), and pictures are taken to quantify the dispersion area of the DNA using image analyzer software (Fernandez et al. 2003, 2005). The SCD detected significantly more sperm DNA fragmentation in infertile men with varicocele than in fertile controls, thus showing its potential for use in the clinic. It also has the potential advantage of being combined with other assays that could detect other abnormalities in the sperm chromatin (e.g., aneuploidy, DNA oxidation, DNA methylation) (Gosalvez et al. 2011); although this strategy is very appealing, more work must be done to confirm whether these combinatory techniques are possible for studying sperm chromatin structure in humans.

Assays to Evaluate Sperm DNA Compaction

Monobromobimame (mBBr) Thiol Group Labeling

As mentioned earlier, sperm DNA is tightly compacted due to specific interactions among components of the sperm chromatin. The –SH groups present in protamines are important elements in the maintenance of chromatin closely compacted in sperm nuclei (Bedford et al. 1973; Bedford and Calvin 1974). The amount of –SH groups can be determined by labeling them with the fluorescent probe mBBr (Kosower and Kosower 1987). It is important to mention that –SH groups are present not only in protamines within the sperm nucleus but also in the tail; thus it is essential to separate the head from the tail by sonication before labeling the spermatozoa with mBBr (O'Flaherty et al.

2008). The intensity of the mBBr labeling in the sperm head is then determined by flow cytometry (Zubkova and Robaire 2006; O'Flaherty et al. 2008). Parallel samples previously treated with dithiothreitol (DTT) are necessary to determine the percentage of free –SH ((fluorescence of sample/fluorescence of sample with DTT)*100). A high fluorescence intensity value corresponds to a high percentage of free –SH present in the sample and therefore an indication of less sperm DNA compaction.

Chromomycin A3 Labeling

Chromomycin A3 (CMA3) is a fluorochrome that specifically binds to guanosine-cytosine-rich sequences where the protamines are prone to bind; it was observed that CMA3 competes with sperm protamines for binding to the minor groove of DNA (Bianchi et al. 1993; Bizzaro et al. 1998). Thus, an increased CMA3 labeling is an indication of lower protamination in spermatozoa and lower DNA compaction (Sakkas et al. 1995; Bianchi et al. 1996; Lolis et al. 1996; Manicardi et al. 1998; Esterhuizen et al. 2002; O'Flaherty et al. 2008). CMA3 labeling can be determined by either microscopy or flow cytometry, and although some studies have associated increased CMA3 labeling with a high percentage of abnormal spermatozoa (Hammadeh et al. 2001; Franco et al. 2012), others have reported the presence of high levels of CMA3 labeling in morphologically normal spermatozoa (Bianchi et al. 1996; O'Flaherty et al. 2008). This discrepancy could be based on the method used to determine CMA3 labeling (e.g., microscopy or flow cytometry) or teratozoospermia (e.g., strict criteria).

Aniline Blue and Toluidine Blue Assays

The aniline blue (AB) and the toluidine blue (TB) assays are based on the property of these dyes to bind to different components of the sperm chromatin and can be performed with light microscopy, thus with minimum costs at a clinical settings. The AB assay detects histones, which are rich in lysine, and binds to them at low pH (Auger et al. 1990). The AB assay showed a significant correlation between high content of histone, abnormal sperm chromatin, and male infertility. However, the correlation of AB results with other sperm parameters is inconsistent and controversial (Foresta et al. 1992).

TB is a basic planar nuclear dye useful for staining the sperm chromatin. It binds to DNA phosphate residues of sperm DNA in nuclei with loosely packed chromatin or impaired DNA, providing a metachromatic shift due to dimerization of the dye molecules from light blue to purple–violet color (Erenpreiss et al. 2001, 2004; Marchesi et al. 2010; Tsarev and Erenpreiss 2011). Recently, it was suggested that the TB assay could be a complementary tool in semen analysis to diagnose male infertility (Tsarev et al. 2009).

Both AB and TB are simple and inexpensive assays that can be performed with a light microscope and using previously prepared and stored smears. The main

disadvantage is that, as a microscope-based assay, the number of spermatozoa to be counted is limited compared to assays based on flow cytometry.

High DNA Stainability

Another parameter that can be obtained with the AO/SCSA is the percentage of spermatozoa with high DNA stainability (HDS), which is associated with low sperm DNA compaction (Evenson et al. 1999; Evenson and Wixon 2005). Accessibility to the double-stranded DNA by AO is increased when sperm DNA is not well compacted, and thus these spermatozoa will show an increased AO green labeling. This sperm population can be visualized on top of a green versus red fluorescence scatter plot (Fig. 8.2). It is noteworthy that those spermatozoa showing high HDS values do not have increased DNA damage and are rather considered to be immature cells due to the high amount of histones retained (Evenson and Wixon 2005).

The assays described previously were used by several laboratories to study human sperm chromatin with the goal of associating their outcome with regular semen analysis (Zini et al. 1999; Spano et al. 2000; Virro et al. 2004; Evenson and Wixon 2005, 2006; Makhlouf and Niederberger 2006; Payne et al. 2005). Different results were obtained in these studies, and there is still controversy as to which is the best approach to evaluate human sperm chromatin. A multiassay approach appears to be more useful for characterizing the sperm chromatin structure in a given semen sample than an individual test (O'Flaherty et al. 2008, 2012).

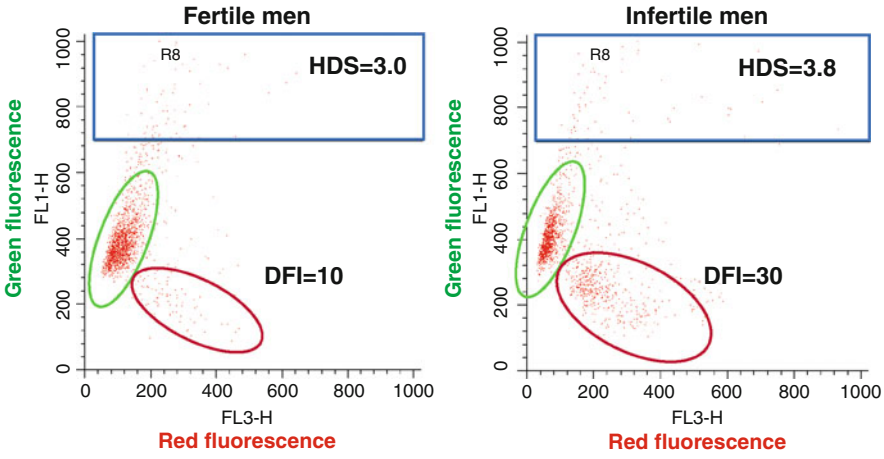

Fig. 8.2 Sperm chromatin structure assay scatter plot from fertile and infertile men. Three main sperm populations can be distinguished: (1) main population with normal DNA (*green oval*), (2) spermatozoa from outside the main population with moderate to high DNA fragmentation (*red oval*), and (3) spermatozoa with normal DNA showing high DNA stainability (*blue box*). DFI = DNA fragmentation index, HDS = high DNA stainability

Sperm Chromatin and Cancer Treatments

There is growing evidence showing the detrimental effects that chemo- and radiation therapy have on male reproduction and, particularly, in the sperm chromatin structure (Fossa et al. 1997; Thomson et al. 2002; Tempest et al. 2007, 2009; O'Flaherty et al. 2008, 2010, 2012; Kenney et al. 2012). The field of oncology has advanced with the design of chemotherapeutic agents and new drug combinations that target cancerous cells with minimal toxic effects on normal cells; but although there are high survival rates over 5-years (80–96 % of cases) in young adults for some malignancies such as testicular cancer and Hodgkin's lymphoma (Huddart and Birtle 2005; Kopp et al. 2006; Theis et al. 2006; Aben et al. 2012), cancer survivors must suffer another burden, which is the possibility of infertility (Petersen et al. 1994, 1998; Petersen and Hansen 1999; Aben et al. 2012). Moreover, depending on the type of cancer and treatment, childhood cancer survivors may have severe oligozoospermia or azoospermia in adult life (Romerius et al. 2011).

As mentioned earlier, chemotherapeutic agents promote a variety of damage to the sperm chromatin; depending on the cancer treatment, differential changes in components of the sperm chromatin structure may be observed. Patients successfully treated for testicular cancer or Hodgkin's lymphoma have spermatozoa with normal DNA compaction but with significantly elevated levels of sperm DNA damage 24 months after the end of chemotherapy (Tempest et al. 2007; O'Flaherty et al. 2010, 2012). These findings are an indication that germs cells might have been irreversibly affected due to the treatment, although some components of the sperm chromatin return to normal in new generations of spermatozoa (O'Flaherty et al. 2010, 2012). It is important to mention that there is individual variability among patients in terms of how fast chromatin integrity will be restored. For instance, patients with advanced testicular cancer or Hodgkin's lymphoma showed high sperm DNA damage, determined by the alkaline comet assay, over a period of 2 years after the end of chemotherapy (O'Flaherty et al. 2010). Concurrently, levels of HDS (SCSA) were higher in cancer patients compared to those of healthy donors, suggesting that poor DNA compaction was still present in sperm from cancer patients during this period of time (O'Flaherty et al. 2012). It is possible that insufficient Zn^{2+} bridges were formed to stabilize the sperm chromatin (Bjorndahl and Kvist 2009), making this structure still susceptible to acid denaturation in spermatozoa from cancer patients. However, the level of protamination (as measured by CMA3 labeling) and of free –SH in sperm of cancer patients was similar to that in sperm of healthy donors at 18 months after chemotherapy (O'Flaherty et al. 2012). Overall, these data indicate that some components of the sperm chromatin are repaired over time after chemotherapy; however, there is still significant sperm DNA damage and low compaction even in normozoospermic samples from cancer survivors. These findings stress the need to use a complementary approach to evaluate sperm chromatin quality; a single assay may not be sufficient to determine whether spermatozoa from cancer survivors have good chromatin quality (O'Flaherty et al. 2008, 2012).

Sperm Chromatin and Environmental Toxicants

Increasing evidence supports the hypothesis that the exposure to environmental toxicants, including pesticides and air pollution (products from engine combustion and waste incineration), is a cause of high sperm DNA fragmentation (Oh et al. 2005; Rubes et al. 2005). Nonoccupational exposure to pesticides or their metabolites present in the environment, such as 3-phenoxybenzoic acid (3-PBA), the pyrethroid metabolite with the highest detected rate in the general population, is worrisome (Kimata et al. 2009; Couture et al. 2009; Sams and Jones 2012; Fortes et al. 2013). High levels of sperm DNA fragmentation were associated with high levels of urinary 3-PBA in infertile men (Ji et al. 2011). Similar associations were found also with other metabolites of the pesticide chlorpyrifos present in the urine of infertile men (Meeker et al. 2004).

Animal studies suggest that pesticides affect the sperm chromatin structure, promoting a variety of alterations; diazinon, an organophosphorus pesticide (OP), promotes increased DFI (sperm DNA fragmentation) and of CMA3 labeling (low protamination) values along with phopshorylation of protamines in spermatozoa recovered from treated male mice after 8 days following the end of treatment (Piña-Guzman et al. 2005). This study suggests that late spermatids are affected by diazinon, resulting in alteration of the sperm chromatin including increased DNA decondensation, low protamination, and DNA damage. Similar toxic effects were observed in humans; spermatozoa treated with different OPs showed increased levels of DNA fragmentation (Salazar-Arredondo et al. 2008). It is noteworthy that the toxicity of OPs is not equal among them, and in some cases the oxon metabolite is ten times more toxic than the original compound (Salazar-Arredondo et al. 2008).

Air pollutants were associated with low sperm chromatin quality at a level that can be associated with male infertility (Rubes et al. 2005). In this study, men exposed to high levels of air pollution have normal semen analysis (Rubes et al. 2005). This evidence, along with that from other studies in different cohorts of men (i.e., infertile men, cancer patients) showing low or no correlation between sperm chromatin assays and semen analysis (Evenson et al. 1999; Spano et al. 2000; Virro et al. 2004; Rubes et al. 2005; Payne et al. 2005; O'Flaherty et al. 2008), supports the need for a complementary analysis of sperm chromatin quality to better characterize the health of human spermatozoa.

Toxicants that are increasingly looking like potential detrimental compounds for male reproduction are plasticizers and bisphenols, compounds used in the plastic industry. Since the discovery of the properties of these compounds, humans have been progressively more exposed to these compounds, which can leak from plastic containers into food, water, and other liquids. Urinary levels of different phthalate (plasticizer) metabolites suggest that exposure to these compounds is much higher and common that previously suspected (Blount et al. 2000) and they might be associated with sperm abnormalities including poor chromatin quality (Pant et al. 2008). Although other studies suggest low or no association between phthalate metabolites and impairment of sperm quality in humans, there is a need for further research with

appropriate epidemiological studies (Meeker 2010) to rule out the possibility that these compounds are responsible for alterations of sperm chromatin quality and other sperm parameters that might explain cases of male infertility.

Conclusions and Future Directions

Based on basic and clinical studies, it is now evident that the human sperm chromatin can be altered by different agents or conditions, some of them still difficult to control. Damaged chromatin may impact negatively on the development of the embryo, inducing miscarriages. Therefore, it is necessary to develop tools to better analyze sperm chromatin quality to assure that the paternal genome is not altered, especially at the time of sperm selection for assisted reproductive technologies (ARTs). Although many tests exist for evaluating individual aspects of the sperm chromatin, there is still a limitation in characterizing its structure using a more global approach. The combination of two or more assays would make it possible to overcome this difficulty. More research is needed to improve the tools that we have today to evaluate this vital element of the spermatozoon. Moreover, large-scale epidemiological studies are necessary to help understand the extent of the exposure to drugs and environmental toxicants. The combination of knowledge harvested by basic and clinical research and data generated by epidemiological studies will serve to design better strategies to detect and possibly isolate sperm samples carrying significant sperm chromatin damage, which will help obtain semen samples that are safe for use in ARTs.

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Chapter 9
Sperm Cryopreservation: Effects on Chromatin Structure

Donatella Paoli, Francesco Lombardo, Andrea Lenzi, and Loredana Gandini

Abstract Cryopreservation is a technique that can keep sperm alive indefinitely, enabling the conservation of male fertility. It involves the cooling of semen samples and their storage at −196°C in liquid nitrogen. At this temperature all metabolic processes are arrested. Sperm cryopreservation is of fundamental importance for patients undergoing medical or surgical treatments that could induce sterility, such as cancer patients about to undergo genotoxic chemotherapy or radiotherapy, as it offers these patients not only the hope of future fertility but also psychological support in dealing with the various stages of the treatment protocols.

Despite its importance for assisted reproduction technology (ART) and its success in terms of babies born, this procedure can cause cell damage and impaired sperm function. Various studies have evaluated the impact of cryopreservation on chromatin structure, albeit with contradictory results. Some, but not all, authors found significant sperm DNA damage after cryopreservation. However, studies attempting to explain the mechanisms involved in the aetiology of cryopreservation-induced DNA damage are still limited. Some reported an increase in sperm with activated caspases after cryopreservation, while others found an increase in the percentage of oxidative DNA damage. There is still little – and contradictory – information on the mechanism of the generation of DNA fragmentation after cryopreservation. More studies are needed to establish the true importance of such damage, especially to improve the results of ART.

Keywords Semen cryopreservation • Sperm DNA damage • Male fertility preservation

D. Paoli (✉) • F. Lombardo • A. Lenzi • L. Gandini
Department of Experimental Medicine, University Laboratory of Seminology – Sperm Bank, University of Rome "La Sapienza", Rome, Italy
e-mail: donatella.paoli@uniroma1.it; francesco.lombardo@uniroma1.it; andrea.lenzi@uniroma1.it; loredana.gandini@uniroma1.it

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Sperm Cryopreservation

Cryopreservation is a technique that can keep sperm alive indefinitely, enabling the conservation of male fertility. It is of fundamental importance for patients undergoing medical or surgical treatments that could induce sterility, such as cancer patients about to undergo genotoxic chemotherapy or radiotherapy. Testicular cancer and Hodgkin's and non-Hodgkin's diseases are the most common conditions in patients cryopreserving their sperm, as they mainly affect males of childbearing age. Despite their serious disease, sperm cryopreservation offers these patients not only the hope of future fertility but also psychological support in dealing with the various stages of the treatment protocols (Saito et al. 2005).

Advances in cancer treatments and increasingly sophisticated assisted reproduction technologies (ARTs) have opened up new possibilities for infertile men; cryopreservation is thus also indicated even for severely damaged sperm that would have had no reproductive capacity in the pre-intracytoplasmic sperm injection (ICSI) era. Given the potential importance of reproduction for these patients, who are often young and childless, it is essential that this option be recommended by specialists and offered as quickly and efficiently as possible. Cancer patients must thus be informed of this possibility before undergoing any treatment that might have an irreversible effect on their ability to father a child, and cryopreservation must be performed before beginning any such treatment.

Sperm Cryobiology

The ability to store cells indefinitely in a state of suspended animation was a pivotal event in reproductive medicine. In the last 60 years, the cryobiology of gametes, testicular tissue, embryos and, recently, ovarian tissue has been studied in parallel to the development of ART. The first attempt at freezing semen dates back to 1776, when abbot Lazzaro Spallanzani reported that sperm could be stored by cooling in snow. In 1800, Paolo Mantegazza came up with the idea of the sperm bank, to ensure the continued lineage of soldiers going off to war. Discussion of cryopreservation began when Rostand (1946), and Polge, in 1949, discovered that glycerol could act as a cryoprotectant, while in 1953 Bunge and Sherman reported three pregnancies in humans after insemination with sperm that had been treated with glycerol and frozen in dry ice. The discovery that glycerol protected sperm cells from freezing damage enabled human semen to be stored in dry ice at −78°C (Polge et al. 1949; Bunge and Sherman 1953; Bunge et al. 1954). However, later studies by Sherman (1963) demonstrated that sperm stored in liquid nitrogen at –196 °C could be kept even longer and, moreover, conserved flagellar movement after thawing. In fact, molecular movements, and hence the biochemical processes of cell metabolism, stop at extremely low temperatures, recommencing once the cell has thawed.

Cell life depends on the simultaneous interactions of various chemical reactions, kept in balance through homoeostatic control mechanisms. Long-term storage is

thus only possible by minimising these reactions by lowering the temperature until life is cryogenically suspended. In liquid nitrogen at −196°C, no chemical reaction can take place as there is insufficient thermal energy at this temperature. In fact, below −130°C, water exists only in a crystalline or vitreous state, in which its viscosity is so high as to limit its diffusion. The chemical and physical phenomena that arise during cooling of cells and tissues affect the viability of the system, reducing enzyme activity and active transport mechanisms, changing membrane conformation and causing trans-membrane ion loss.

However, the main problem the cell must overcome during cooling is the transition of water to ice. Water plays an important role in cell life, acting as an intra- and extracellular carrier of hydrophilic structures such as proteins, metabolites and ions. During freeze/thaw phase changes, the concentration of solutes in intra- and extracellular aqueous solutions varies considerably, thus giving rise to the greatest biological problem encountered with freezing.

The solutes in the aqueous medium in which the cells are suspended take its freezing point down to –10 to −15°C, i.e. below that of pure water (0°C). At these low temperatures the water in the extracellular environment freezes, increasing the concentration of the solutes. This generates osmotic pressure, causing solvent to flow through the plasma membrane, from inside to outside the cell. This extracellular need for water leads to a reduction in cell volume and then to dehydration, a process that is essential to protect cells from the formation of intracellular ice, which can cause them to die. However, this intense dehydration process can reach a point of no return (at approximately 40 % of the original cell volume), at which the cell suffers permanent damage.

The extent of dehydration depends mainly on the cooling rate. If the cell is cooled very quickly, dehydration may not be complete and intracellular ice crystals can form. In this case, there is no osmosis or volume change, but upon thawing there may be mechanical damage to the membrane and subcellular organelles. In contrast, if the cell is cooled very slowly, then extracellular water freezes before intracellular water due to the protective effect of the cell membrane. The extracellular environment thus becomes hypertonic, causing water to flow from inside to outside the cell, resulting in excessive dehydration.

During thawing, the reverse takes place. The cells are resuspended in the newly thawed hypertonic solution, which becomes progressively dilute as it thaws. This causes water to be drawn back into the cells (reverse osmosis), restoring the intracellular content and cell volume. In this phase, there is a risk that intracellular ice crystals might form (recrystallisation). For this reason, thawing must be fast, to avoid the formation of larger ice crystals. Thawing damage is thus mainly caused by the flow of water, which causes changes to the cell membrane, the formation of ice crystals, inducing mechanical damage to the membrane and subcellular organelles, and exposure to hypertonic solutions, which can cause fatal damage. Membrane damage can be measured by the degree of unsaturated fatty acid peroxidation, which seems to increase during the freeze–thaw cycle, probably due to the release of free radicals (Alvarez and Storey 1992). Some authors have in fact shown an increase in superoxide ions during thawing in bovine sperm (Chatterjee and Gagnon 2001).

Although there is a correlation between lipid peroxidation of the plasma and mitochondrial membrane and loss of motility, other factors are probably responsible for post-thawing membrane damage and reduced motility (Bell et al. 1993). The optimal warming rate depends on the cooling rate in a given system (Mazur 1984). Rapid thawing leads to greater post-thawing viability. Freeze-thawing was most successful when rapid vapour freezing was followed by thawing at 37°C, demonstrating a significant interaction between the freezing method and the thawing temperature (Verheyen et al. 1993). Thawing protocols nowadays do in fact use a temperature of 37°C; higher temperatures would enable more rapid thawing but are not used to avoid cell damage such as lipid peroxidation. In fact, such reactions have a steep temperature coefficient (Alvarez et al. 1987), and so the risk of peroxidation damage is thought to be greater if thawing takes place at 40°C rather than at a lower temperature.

Cell survival after freezing/thawing thus depends on the ability to minimise the formation of intracellular ice crystals. It should therefore be optimised by establishing a cooling rate that enables the cell to remain in equilibrium with the extracellular solution. An intermediate rate is needed: fast enough not to cause excessive solute concentration but slow enough to avoid intracellular crystallisation.

The ability of a cell to survive freezing also depends on its shape, size, water content, permeability and membrane lipid composition. Human sperm can tolerate a series of temperature variations and are fairly resistant to damage caused by rapid cooling due to the unsaturated fatty acids in the lipid bilayer (65–70 % phospholipids – side chains of docosahexaenoic acid), which result in high membrane fluidity (Clarke et al. 2003), to their small cell size, and to the compact cellular organisation of the head. Their low water content (around 50 %) may also make them more resistant than other cells to freezing damage. Nevertheless, motility is generally reduced by 30–50 % upon thawing.

Cryoprotectant media containing low molecular weight compounds that modify the cell environment are used to obviate these problems. By replacing the water content, they keep the extracellular environment in the liquid phase even when the temperature drops below the freezing point. These substances have been defined as ‘any additive which can be provided to cells before freezing and yields a higher post-thaw survival than can be obtained in its absence’ (Karow 1974). Although the chemical composition of these cryoprotectants differs, they are all highly water soluble and have a concentration-dependent toxicity. They work directly on the cell membrane through electrostatic interactions, lowering the freezing point of the solution and modifying the intra-and extracellular environments as their displacement of the water reduces both the formation of ice crystals and the quantity of salt and solutes in the liquid phase. There are two main classes of cryoprotectant:

1. Permeating cryoprotectants penetrate the cell membrane. This class includes dimethyl sulfoxide (DMSO), glycerol, 1,2 propanediol (PROH) and ethylene glycol. These hydrophilic substances have a molecular weight of less than 400 and cross the membrane very easily, creating an osmotic gradient and causing water to leave the cell by lowering the freezing point even further.

2. Non-permeating cryoprotectants not cross the cell membrane. These include sucrose, fructose, glucose, dextrose, starch, lipoproteins and polyvinylpyrrolidone (PVP). These large molecules, with a molecular weight of greater than 1,000, increase the concentration of extracellular solutes, thereby generating an osmotic gradient that causes water to leave the cell, leading to dehydration before freezing takes place.

Glycerol is the most commonly used cryoprotectant for human sperm. It acts on the membrane structure, the permeability and stability of the lipid bilayer, the association of surface proteins and cell metabolism (Fabbri et al. 2004). However, Sherman (1990) showed that the use of glycerol alone could damage the plasma membrane, the inner acrosomal membrane, the nucleus and the mitochondrial cristae. Other substances such as DMSO and PROH were used subsequently, but with little success, due to their harmful effects on human sperm.

Almost all cryoprotectant media contain glycerol, to protect against thermal shock; sugars, which provide the sperm with energy and optimise osmolarity and hydrogen ion concentration; egg yolk, which improves the fluidity of the cytoplasmic membrane, provides structural and functional protection and, through its lipoprotein content, safeguards sperm integrity; and antibiotics, to protect against any microorganisms that might be present. Cryopreservation is carried out in nitrogen vapour with one of two techniques, leading to either slow or fast freezing.

Cryopreservation Methods

Rapid freezing was first proposed by Sherman (1990). It does not require automatic equipment. The most common technique is vertical freezing, carried out in cryogenic containers of a suitable size. Semen samples are diluted with cryoprotectant medium and left to equilibrate at 37°C for 10 min. The suspension is aspirated with a vacuum pump into 300- or 500-μL straws. The straws are sealed and placed in nitrogen vapour for 8 min and then immersed in liquid nitrogen at −196°C. Nitrogen vapour contains a thermal gradient depending on the distance from the surface of the liquid nitrogen and the volume of underlying liquid. The straws are placed 15–20 cm above the liquid (Fabbri et al. 2004) and are then slowly lowered to reach the surface before being raised once more. This gives a fast freezing rate (approximately 20°C/min). Following this phase, the straws are plunged into liquid nitrogen.

Slow freezing was proposed by Behrman and Sawada (1966). It uses automatic equipment that takes cells from room temperature to the storage temperature at a controlled rate. The sample is first diluted with cryoprotectant and then taken from room temperature to 5°C at a rate of 0.5–1°C/min (Mahadevan and Trounson 1984). The temperature is then lowered to −80°C at 1–10°C/min, and finally the sample is plunged into liquid nitrogen (Thachil and Jewett 1981). However, this method can cause ice crystals to form if the freezing rate is too fast or too slow.

In both of these methods, the cells are exposed to cryoprotectant before freezing to protect them from cooling damage. This stage is called conditioning. The efficacy of cryoprotectant is a function of how long the cryoprotectant and the cells have to interact and the temperature at which this exposure takes place. For this reason, cryoprotectant medium is added drop by drop, mixed gently to avoid osmotic stress and the mixture incubated at 37°C for 10–15 min to allow the cells and medium to equilibrate. Another important point is that the thawing techniques must enable the cells to recover their normal biological activities, minimising fast temperature changes as much as possible. For this reason, the straws are extracted very slowly to enable a thermal equilibrium between the cells and the external environment to be reached. Different protocols can be used: (1): straws are kept at room temperature for 10 min and then incubated at 37°C for 10 min; (2): straws are placed in a thermostatic bath at 37°C for 10 min; (3): straws are thawed at room temperature (22°C) for 15 min.

Effect of Cryopreservation on Sperm DNA Integrity

Damage can take place not only during freezing but also during thawing. On thawing, ice crystals can cause mechanical damage to the subcellular organelles. Various studies have shown that the damage induced by cryopreservation can affect the integrity of the plasma membrane (Arav et al. 1996; Zeron et al. 1999), which contains phospholipids and cholesterol (Giraud et al. 2000).

Human and rabbit sperm are less sensitive to cryopreservation than sperm from other animals, such as rams and bulls, due to the different lipid composition of the sperm plasma membrane (Bailey et al. 2000). In bull and ram sperm, the membrane contains less cholesterol and a higher ratio of unsaturated and saturated fatty acids than found in human sperm. Nevertheless, during cryopreservation of human sperm the cooling process can cause a membrane lipid phase change as well as functional damage to the intramembrane proteins responsible for ion transport (Oehninger et al. 2000). The sperm plasma membrane contains a glycocalyx of oligosaccharide chains bound to intramembrane proteins and lipids. Cryopreservation can have adverse effects on the composition of carbohydrates in the glycocalyx, thus interfering with the function of intramembrane proteins (Benoff 1997) and with other physiological functions such as immunological protection (Cross and Overstreet 1987) and acrosome reaction (Lassalle and Testart 1994).

Cryopreservation also has adverse effects on sperm motility and speed due to membrane swelling and acrosome degeneration. Fatty acids, which are abundant in the sperm membrane (Halliwell and Gutterridge 1984), are also vulnerable to peroxidation; this can damage the plasma membrane and cause a loss of intracellular enzymes and inhibit oxidative phosphorylation (White 1993). The mitochondrial membrane is in fact susceptible to damage at low temperatures, and a change in membrane fluidity can affect its potential and cause the release of reactive oxygen species (ROSs) (Said et al. 2010).

Given that the antioxidant activity of sperm is also reduced by cryopreservation, it is clear that sperm are susceptible to ROS damage (Lasso et al. 1994). This damage may involve single- or double-stranded DNA breakage. Various studies have examined how ROSs form during cryopreservation and thawing. Mazzilli et al. (1995) studied 45 subjects for the presence of ROSs in sperm selected by swim-up before and after cryopreservation in liquid nitrogen. In 42.2 % of subjects there was already evidence of ROS production prior to cryopreservation, which increased after thawing. In 20 % of subjects there was no ROS production prior to cryopreservation, but ROSs were detected after freezing/thawing. The study suggested that cryopreservation procedures could induce or increase ROS production in some semen samples. In ROS-producing subjects, the recovery of sperm motility and viability was significantly lower than in ROS-free subjects. This was probably due to damage by oxidative stress leading to lipid peroxidation of the sperm membrane.

In light of this, several studies have examined the role of in vitro antioxidant supplementation to protect sperm DNA from oxidative damage. These found that if genistein (Martinez-Soto et al. 2010), resveratrol and ascorbic acid (Branco et al. 2010) were added to the semen sample during cryopreservation, there was no reduction in oxidative DNA damage, while vitamin E (Taylor et al. 2009), ascorbate and catalase (Li et al. 2010) seemed to improve motility and reduce ROS levels, although they did not improve chromatin viability and integrity. High ROS concentrations and the loss of antioxidants can also trigger apoptosis (Wang et al. 2003).

Thawing can also cause sperm DNA damage. In fact, some studies have shown that DNA fragmentation increases during the first 4 h after thawing. For this reason, sperm should be used rapidly after thawing (Gosalvez et al. 2009).

The various studies of DNA integrity after cryopreservation and thawing produced conflicting results. Some, but not all, authors found significant sperm DNA damage after cryopreservation. Spanò et al. (1999) evaluated sperm chromatin damage in 19 normozoospermic subjects using sperm chromatin structure assay (SCSA). This technique exploits the properties of the metachromatic fluorescent dye acridine orange (AO) to reveal the susceptibility of double-stranded DNA to denaturation induced by acid stress. Each sample was divided into three aliquots: the first aliquot was evaluated without further treatment, the second underwent swim-up and the third was stored according to standard cryopreservation techniques in liquid nitrogen at −196°C. An aliquot of the cryopreserved sample was also processed by swim-up after thawing. Each aliquot was then evaluated by light microscopy, fluorescence microscopy for evaluation of the AO test, and flow cytometry for evaluation of SCSA. This study showed that the nuclear integrity of both fresh and cryopreserved sperm populations selected by swim-up was higher than that of the unselected sperm population. The authors suggested that cryopreservation, correctly carried out, is not able to damage spermatozoa per se but can enhance defects already present in the sperm population.

This result is consistent with the findings of Oehninger et al. (2000), who reported that the severity of sperm damage after cryopreservation was higher in infertile men, and the extent of the damage was correlated with the degree of oligoasthenoteratozoospermia (OAT). Poor-quality semen samples are more susceptible to

DNA damage and cell death after cryopreservation than semen samples with normal parameters (Said et al. 2010).

Donnelly et al. (2001a) studied the effects of cryopreservation on chromatin integrity by comet assay in 17 fertile and 40 infertile men. Each sample was divided into four aliquots: fresh semen, cryopreserved semen and sperm prepared by density gradient from fresh and cryopreserved semen. In fertile controls, there were no significant differences in DNA damage between the unprocessed semen sample and sperm separated by discontinuous Percoll gradient. The sperm of these subjects was therefore resistant to damage from freezing. However, for the infertile subjects, there was a significant (24 %) reduction in chromatin integrity after freezing/thawing of semen samples, reaching 40 % in selected sperm. There is in fact less chromatin condensation in poor-quality sperm, making their DNA potentially susceptible to nuclease and polymerase action (Bianchi et al. 1993) or to fragmentation (Gorczyca et al. 1993). Infertile men are well known to have a higher percentage of sperm with fragmented DNA than fertile men (Sun et al. 1997; Lopes et al. 1998). The authors also found a significant reduction in normal forms after cryopreservation in both semen samples and selected sperm from both fertile and infertile subjects but did not find any correlation between morphology and chromatin integrity. As in previous studies (Hammadeh et al. 1999), this study found that the cryopreservation of sperm from fertile and infertile subjects affected sperm morphology, while sperm nucleus from semen samples from fertile men was more resistant to damage. In other words, the genotype and phenotype of human sperm were not equally affected by cryopreservation. In contrast, subsequent studies (Kalthur et al. 2008) showed that morphologically abnormal sperm were more sensitive to DNA damage induced by cryopreservation in liquid nitrogen. These authors evaluated chromatin integrity pre- and post-cryopreservation in 20 normozoospermic and 24 teratozoospermic semen samples using the comet assay and AO test. Prior to freezing, 17.5 % of sperm from normozoospermic and 24.9 % from teratozoospermic samples were denatured; post-thawing, this percentage was three times higher in teratozoospermic samples than in normozoospermic samples.

These results can be explained by the fact that abnormal sperm, as demonstrated by several studies, are a major source of free radicals. The cryopreservation-induced ROS level may thus be higher in abnormal than in morphologically normal sperm. The importance of oxygen radicals in the cryopreservation process is confirmed by various studies that show that sperm in seminal plasma seem to be more resistant to the shock of cryopreservation than gradient-selected sperm. This could be due to the presence in the seminal plasma of antioxidant enzymes such as superoxide dismutase and catalase and scavengers such as albumin and taurine, which remove ROSs (Donnelly et al. 2001a)

Confirmation of this hypothesis comes from another study by Donnelly et al. (2001b) carried out on 40 infertile patients. Sperm frozen unprepared in seminal fluid appeared more resistant to freezing damage than frozen prepared sperm. Freezing sperm in seminal plasma improves post-thaw motility and DNA integrity. Furthermore, De Paula et al. (2006) demonstrated by TUNEL assay an increase in post-cryopreservation apoptotic fragmentation in 47 oligozoospermic and 30

normozoospermic subjects. According to these authors, cryopreservation induced fragmentation independently of sperm concentration, as the increase was similar in both groups. Other studies by Ngamwuttiwong and Kunathikom (2007) found a decrease in sperm chromatin integrity on AO test in 20 men from infertile couples after 6 months of sperm cryopreservation.

Various studies in the literature have evaluated the impact of cryopreservation on chromatin structure, albeit with contradictory results; this may be due to small caseloads, different freezing procedures, different methods for assessment of DNA integrity or different semen preparation techniques (Di Santo et al. 2012).

However, studies attempting to explain the mechanisms involved in the aetiology of cryopreservation-induced DNA damage are still limited. Some reported an increase in sperm with activated caspases after cryopreservation, while others found an increase in the percentage of oxidative DNA damage. Studies in animal models showed that cryopreservation induced an increase in apoptotic events, such as modification of mitochondrial membrane potential, activation of caspases and externalisation of phosphatidylserine. Duru et al. (2001) reported an increase in the percentage of sperm with membrane translocation of phosphatidylserine after cryopreservation in both fertile and infertile patients, but this was not associated with impaired sperm integrity. Paasch et al. (2004) achieved the same results, demonstrating in 11 pools of cryopreserved semen samples and 9 pools of fresh semen samples that cryopreservation was significantly associated with the activation of caspase 3, 8 and 9 and impaired mitochondrial membrane potential, but not with any impairment of DNA integrity evaluated by TUNEL.

Although caspase activation following cryopreservation and thawing is fairly uniform, this mechanism differs in annexin V-positive and negative sperm. These interactions suggest that cryopreservation and thawing trigger the caspase activation through mechanisms possibly linked to membrane translocation of phosphatidylserine. However, more recent studies suggested that DNA fragmentation could be associated with increased cryopreservation-induced oxidative stress rather than caspase activation and apoptosis (Thomson et al. 2009). These authors compared the percentage of sperm with fragmented DNA, the percentage of 8-oxo-7,8-dihydro-2′deoxyguanosine (8OHdG) and the percentage of positive caspases in semen samples from infertile patients. There was an increase in the percentage of DNA fragmentation, oxidative damage and caspase activation after cryopreservation. In fresh semen, there was a correlation between caspase-positive cells and DNA fragmentation and between 8OHdG and DNA fragmentation due to the simultaneous nature of the two pathways unified by ROS production by abnormal sperm. In contrast, there was a negative correlation between caspase-positive cells and DNA fragmentation after thawing, suggesting that cryopreservation could induce DNA fragmentation independently of caspase activation, and that this process probably takes place in non-apoptotic cells.

Here, too, ROSs seem to play an important role in the pathophysiology of DNA damage in human sperm. However, other studies found no correlation between DNA fragmentation and oxidation. Zribi et al. (2010) evaluated sperm DNA fragmentation by TUNEL and oxidative damage by flow cytometric analysis of 8OHdG

Table 9.1 Literature data: results on effects of cryopreservation on DNA integrity

Year	Reference	Method	Effect of cryopreservation on DNA integrity
1999	Spanò et al.	SCSA, AO	Human spermatozoa in normozoospermic semen samples, both before and after swim-up and after cryopreservation, exhibited a general improvement in all SCSA-related parameters when compared with unselected populations. Cryopreservation can enhance defects already present in a sperm population
2001a	Donnelly et al.	Comet	DNA from fresh semen samples and prepared spermatozoa separated from fertile men was found to be unaffected by cryopreservation; in contrast, there was a significant reduction in chromatin integrity after freezing/thawing of semen samples and selected sperm from infertile subjects
2001b	Donnelly et al.	Comet	In infertile men, unprepared sperm in seminal fluid appeared more resistant to freezing damage than prepared sperm. The DNA of spermatozoa prepared either by Percoll density centrifugation or swim-up procedure and frozen in seminal plasma from the same ejaculate also appeared to be more resistant to freezing
2001	Duru et al.	TUNEL	No significant effect on DNA fragmentation in fertile and infertile samples after freezing/thawing
2004	Paasch et al.	TUNEL	No difference in DNA fragmentation between pools of cryopreserved and fresh semen samples
2006	De Paula et al.	TUNEL	Sperm from normozoospermic and oligozoospermic patients presented a similar increase in double-strand DNA fragmentation following cryopreservation
2007	Ngamwuttiwong and Kunathikom	SCSA	Decrease in sperm chromatin integrity after 6 months' cryopreservation of sperm from infertile patients
2008	Kalthur et al.	Comet, AO	DNA damage was higher in teratospermic than normospermic samples; morphologically abnormal sperm were more sensitive to cryopreservation-induced DNA damage
2009	Thomson et al.	TUNEL	Cryopreservation induced an increase in DNA fragmentation and oxidative damage in sperm from infertile patients
2010	Zribi et al.	TUNEL	DNA fragmentation was significantly higher in subjects with abnormal semen than in those with normal semen before cryopreservation

in 15 semen samples pre- and post-cryopreservation. The increased fragmentation induced by cryopreservation was associated with a slight increase in the percentage of sperm with oxidative damage. This suggested that the effect of cryopreservation and thawing follows different pathways, such as amplification of defects already present in the sperm cell, which could activate apoptotic mechanisms, or DNA repair enzyme defects (Table 9.1).

Although cryopreservation can reduce motility and cause plasma membrane, acrosomal and chromatin damage, it is still an important tool in the treatment of infertility, and its successful use has increased considerably since the development of more sophisticated ART techniques. In the past, cryopreserved semen was used for intrauterine insemination, but the pregnancy rate was low, probably due to the negative effects of freezing/thawing on sperm kinetics. The introduction of ICSI has overcome this problem, giving patients a reasonable chance of conceiving. In fact, ICSI has similar success rates with both fresh and cryopreserved sperm. Various studies of fresh or frozen semen from OAT patients found no difference in conception rate, embryo development or embryo quality following ICSI, while other studies found a difference in the conception rate only in asthenozoospermic semen samples. Despite this, the implantation, pregnancy and spontaneous abortion rates were similar for fresh and frozen semen, indicating that once an egg has been fertilised, implantation and pregnancy progress as with eggs fertilised with sperm from oligo- and normozoospermic patients (Kuczyski et al. 2001; Borges et al. 2007).

However, little research has been conducted on the rate of use of cryopreserved semen in relation to the outcome of ART. The usefulness and success of cryopreservation can be evaluated by the number of babies born and the number of men who have succeeded in becoming fathers with cryopreserved sperm, the main objective of patients choosing to bank their semen. Various studies report pregnancy rates of from 33 % up to 72 % (mean 54 %) with cryopreserved sperm (Van Casteren et al. 2008). Given these comforting reports, and the little – and contradictory – information on the mechanism of the generation of DNA fragmentation after cryopreservation, more studies are needed to establish the true importance of such damage, especially to improve the results of ART.

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Chapter 10
Sperm Selection: Effect on Sperm DNA Quality

Lodovico Parmegiani, Graçiela Estela Cognigni, and Marco Filicori

Abstract The selection of spermatozoa without DNA fragmentation and chromosomal diseases prior to assisted reproductive techniques helps to optimize the outcome of the treatment; in particular, sperm selection prior to in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) is crucial. In fact, although ICSI has been successfully and safely applied worldwide for almost 20 years, at the present time we have no real knowledge regarding the hypothetical long-term side effects on ICSI adults, given the increased likelihood of spermatozoa with defective nuclear content fertilizing oocytes.

In the case of DNA damage, the basal sperm DNA fragmentation rate can be significantly reduced by some sperm processing procedures that improve the percentage of spermatozoa with normal chromatin structure by filtering out DNA-damaged spermatozoa. After this first step, new advances in micromanipulation can be performed to choose the "ideal" mature spermatozoa for ICSI, reducing potential damage to the gametes. In fact, it is possible to prevent fertilization by DNA-damaged and chromosomal-unbalanced spermatozoa by selecting ICSI sperm by maturation markers such as hyaluronic acid or other zona pellucida receptors. Furthermore, novel noninvasive imaging techniques can be valid tools for helping in the morphological selection of ICSI spermatozoa.

Keywords Hyaluronic acid • Intracytoplasmic sperm injection • Intracytoplasmic morphologically selected sperm injection • Sperm motility • Physiological ICSI • Physiological IMSI

L. Parmegiani (✉) • G.E. Cognigni • M. Filicori
GynePro Medical Centers, Reproductive Medicine Unit,
Via T. Cremona, 8-40137 Bologna, Italy
e-mail: l.parmegiani@gynepro.it

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Introduction

Sperm DNA quality plays a major role in male fertility. Many couples failing to achieve pregnancy may be affected by male genomic factors, including sperm DNA damage (Siffroi et al. 1997; Joly-Helas et al. 2007; Kim et al. 2011; Sakkas and Alvarez 2010). It has been demonstrated that when the male partner has a high rate of DNA-damaged spermatozoa, the couple has a high probability of waiting a long time before conceiving (Loft et al. 2003) and should be considered of low potential for natural fertility (Spanò et al. 2000; Zini et al. 2001). Furthermore, if these couples finally achieve pregnancy, they have a higher risk of miscarriage (Spanò et al. 2000). Finally, it should also be pointed out that multiple studies have demonstrated that sperm DNA damage increases with advancing male age (Humm and Sakkas 2013).

For these reasons, the selection of spermatozoa without DNA fragmentation and chromosomal diseases prior to assisted reproductive (AR) techniques helps to optimize the outcome of the treatment, in particular, in the case of in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (Ebner et al. 2012). In fact, ICSI of aneuploid spermatozoa seems to be the cause of the vast majority of genetic deviations in ICSI newborns (Bonduelle et al. 2002), and the insemination of DNA-damaged spermatozoa seems to be involved in an increased abortion rate (Zini et al. 2008). Furthermore, the injection of DNA-damaged spermatozoa also seems to have long-term side effects in adult animals, such as aberrant growth, premature aging, abnormal behavior, and mesenchymal tumors (Fernandez-Gonzalez et al. 2008). In addition, it should be pointed out that the widespread use of ICSI increases not only the chances of injecting spermatozoa that are defective in their genetic constitution (Sakkas et al. 1997; Marchesi and Feng 2007) but also for centrosome integrity (Schatten and Sun 2009), phospholipase C zeta content (Heytens et al. 2009), and DNA methylation (Navarro-Costa et al. 2010).

For all these reasons sperm selection prior to in vitro insemination by IVF and ICSI is crucial. This selection should be performed keeping to a minimum any additional risk that might increase sperm damage in the laboratory, and – at least for those processes that might be influenced by the embryologist – should be performed in a safe and preferably physiological manner (Parmegiani et al. 2010a, b, c).

In the case of DNA damage, before IVF–ICSI, the basal sperm DNA fragmentation rate can be significantly reduced by some sperm processing procedures such as swim-up (Spanò et al. 2000; Parmegiani et al. 2010a), density gradient (Sakkas et al. 2000), selection by motility without centrifugation (Ebner et al. 2011), and magnetic cell sorting (MACS) with annexin V (Rawe et al. 2010).

These semen selection procedures improve the percentage of spermatozoa with a normal chromatin structure by filtering out DNA-damaged spermatozoa. After this first step, new advances in micromanipulation can be performed to chose the "ideal" mature spermatozoa for ICSI by the restoration of natural fertilization checkpoints such as sperm-hyaluronic acid binding (Parmegiani et al. 2010a) or sperm-zona pellucida binding (Paes de Almeida Ferreira Braga et al. 2009) or by using high magnification microscopy, i.e., intracytoplasmic morphologically selected sperm injection (IMSI) (Bartoov et al. 2003).

Sperm DNA Damage

DNA fragmentation can be induced by numerous factors, such as apoptosis, oxygen radicals, radiation and chemotherapy, or environmental toxicants. It has been hypothesized that DNA damage in sperm may occur during production or transport by the testes (Sakkas and Alvarez 2010). During spermatogenesis, specific screening mechanisms in the testes mark individual spermatozoa with apoptotic markers, which cause phagocytosis of these cells (Billig et al. 1996). Furthermore, testicular endogenous nuclease activity, assisting protamination, increases the percentage of DNA-damaged spermatozoa (McPherson and Longo 1993). Since testicular spermatozoa show lower levels of DNA damage than epididymal or ejaculated sperm, it could be hypothesized that sperm DNA fragmentation is even more frequently generated during sperm transport through the epididymis (Ollero et al. 2001; Greco et al. 2005). In this scenario, if intratesticular and intraepididymal mechanisms for the removal of defective spermatozoa do not function properly, these gametes will end up in the ejaculate. In fact, sperm DNA fragmentation is high in oligoasthenoteratozoospermic men and correlates with failed fertilization, impaired preimplantation development, and altered pregnancy outcome (Evenson et al. 1999; Carrell et al. 2003; Seli et al. 2004; Borini et al. 2006; Velez de la Calle et al. 2008; Zini et al. 2008). Sperm DNA integrity, and in particular DNA fragmentation, is commonly checked in assisted reproduction (AR) laboratories by specific tests:

- Sperm chromatin structure assay (SCSA) (Evenson et al. 2002),
- TdT-mediated-dUTP nick-end labeling (TUNEL) (Gorczyca et al. 1993)
- Sperm chromatin dispersion (SCD) test (Fernandez et al. 2003),
- Comet assay (Hughes et al. 1996; Enciso et al. 2009).

These assays are based on two different approaches – directly detecting DNA damage (direct tests, e.g., TUNEL) or measuring DNA fragmentation after a rather mild denaturation process (indirect tests, e.g., SCSA, SCD). Both direct and indirect assays seem to be reliable in predicting the levels of DNA fragmentation (Chohan et al. 2006; Zhang et al. 2010). DNA breaks can be single- or double-stranded; single defects are probably easier to repair compared with double breaks (Sakkas and Alvarez 2010), which may generate reciprocal translocations (Richardson and Jasin 2000).

DNA Damage and the Outcome of Assisted Reproduction Techniques

Animal models can be useful in investigating the effect DNA-damaged spermatozoa (Ahmadi and Ng 1999a, b; Fernandez-Gonzales et al. 2008; Upadhya et al. 2010). It is now known that DNA-damaged spermatozoa have the ability to fertilize an

Table 10.1 Effect of sperm DNA damage on IVF/ICSI outcome[a]

Author	Patients	Assay	Negatively affected outcome
Morris et al. (2002)	60	Comet	Nothing
Bungum et al. (2004, 2007, 2008)	1,296	SCSA	Nothing
Gandini et al. (2004)	34	SCSA	Nothing
Pregl Breznik et al. (2013)	133	SCD	Nothing (ICSI)
Lopes et al. (1998)	131	TUNEL	Fertilization
Huang et al. (2005)	303	TUNEL	Fertilization
Payne et al. (2005)	100	SCSA	Fertilization
Sun et al. (1997)	143	TUNEL	Fertilization, embryo quality
Virant-Klun et al. (2002)	183	AO	Fertilization, embryo quality
Benchaib et al. (2003)	104	TUNEL	Fertilization, pregnancy
Muriel et al. (2006)	85	SCD	Fertilization, embryo quality
Simon et al. (2010)	360	Comet	Fertilization, embryo quality
Pregl Breznik et al. (2013)	133	SCD	Fertilization, embryo quality (IVF)
Tomlinson et al. (2001)	140	NT	Pregnancy
Tomsu et al. (2002)	40	Comet	Embryo quality, pregnancy
Larson-Cook et al. (2003)	89	SCSA	Pregnancy
Henkel et al. (2004)	249	TUNEL	Pregnancy
Seli et al. (2004)	49	TUNEL	Embryo quality
Virro et al. (2004)	249	SCSA	Embryo quality, pregnancy
Borini et al. (2006)	132	TUNEL	Pregnancy, pregnancy loss
Frydman et al. (2008)	117	TUNEL	Pregnancy, pregnancy loss
Lin et al. (2008)	223	SCSA	Pregnancy loss
Avendaño et al. (2010)	36	TUNEL	Embryo quality, pregnancy
Speyer et al. (2010)	347	SCSA	Pregnancy
Meseguer et al. (2011)	210	SCD	Pregnancy

AO acridin orange staining, *NT* in situ nick translation, *SCD* sperm chromatin dispersion test, *SCSA* sperm chromatin structure assay, *TUNEL* TdT-mediated-dUTP nick-end labeling

[a]Slightly modified from Ebner et al. (2012)

oocyte, but embryo development seems to be related to the degree of DNA damage (Upadhya et al. 2010; Ahmadi and Ng 1999a, b). Furthermore, it can be hypothesized that the oocyte can repair the sperm DNA damage. However, this repairing mechanism seems to be efficient only if sperm DNA damage is less than 8 %; damage beyond this level could result in a low rate of embryonic development and high early pregnancy loss (Ahmadi and Ng 1999a).

In humans, the correlation between sperm DNA damage and low fertility is evident in natural conception and when performing a "low-technology" AR procedure such as intrauterine insemination (IUI). One prospective study revealed that above a threshold of 12 % sperm having strand breaks, no clinical pregnancy was achieved when performing IUI (Duran et al. 2002). Others studies on IUI confirmed that the best results in terms of pregnancy rate are obtainable when sperm DNA fragmentation index (DFI) is lower than 27–30 % (Bungum et al. 2004, 2007).

When analyzing in vitro insemination techniques, IVF and, particularly, ICSI, the correlation between sperm DNA damage and clinical outcome is more controversial (Table 10.1). Some studies – five original papers (Bungum et al. 2004, 2007, 2008;

Gandini et al. 2004; Lin et al. 2008) and two metaanalyses (Li et al. 2006; Collins et al. 2008) -found no correlation between sperm DNA fragmentation in the neat semen and fertilization rate, embryo quality, or clinical pregnancy. Other studies demonstrated that ICSI allowed a better clinical outcome than IVF in the case of high sperm DNA fragmentation rate (Bungum et al. 2004, 2007; Huang et al. 2005). Furthermore DNA damage is inversely correlated with IVF fertilization rates (Sun et al. 1997; Høst et al. 2000a, b; Huang et al. 2005; Borini et al. 2006). Simon et al. observed that increased sperm DNA fragmentation resulted in lower fertilization rates, poorer embryo quality, and reduced pregnancy rates after IVF and ICSI (2011) and in reduced live birth rates in IVF, but not in ICSI (Simon et al. 2013).

In a very recent study on sibling oocytes, Pregl Breznik et al. (2013) found that sperm DNA damage was inversely related to fertilization and embryo quality in IVF, but the DFI had no relation to these parameters in the case of ICSI. According to these findings, ICSI should be the method of choice when the DFI is near or exceeds the threshold value of 30 % (Bungum et al. 2004, 2008).

DNA Damage and IVF/ICSI; Fertilization, Embryo Quality-Development Rate

Some authors have found a negative correlation between sperm DNA fragmentation and fertilization rate (Lopes et al. 1998; Benchaib et al. 2003; Huang et al. 2005; Borini et al. 2006) and zygote formation (Muriel et al. 2006) in both IVF and ICSI. However, this seems to be balanced in ICSI by a good subsequent development of embryos (Payne et al. 2005). Other studies reported an inverse correlation between DFI and embryo quality (Tomsu et al. 2002; Virant-Klun et al. 2002; Avendaño et al. 2010; Seli et al. 2004; Virro et al. 2004; Muriel et al. 2006).

DNA Damage and IVF/ICSI; Pregnancy-Implantation Rate

Meseguer et al. (2011) measured the effect of the DFI on subsequent pregnancy independent of the fertilization procedure (IVF or ICSI) or sperm origin (ejaculate or testicular) and calculated that for every 10 % increase in the DFI, the probability of not achieving pregnancy increased by 1.31. Avendaño et al. (2010) found a 3.5 times higher likelihood of pregnancy when the DFI was less than or equal to 17.6 % after sperm treatment. The DFI threshold in raw semen is still controversial: in a 2003 study, no clinical pregnancy was noted in the case of a DFI greater than or equal to 27 % (Larson-Cook et al. 2003), whereas Henkel et al. (2004) stated that the probability of pregnancy is higher when DFI is less than 36.5 %. Correspondingly, Spanò et al. (2000) observed that under a threshold of 40 % DFI, the pregnancy rate was significantly higher than in patients with a higher DFI. Similarly, a prospective analysis on 360 couples (Simon et al. 2010) showed significantly lower pregnancy rates above a threshold of 44 % DFI.

In terms of implantation, ICSI with a DFI greater than or equal to 19 % was associated with a lower implantation rate (Speyer et al. 2010). It has been hypothesized that "healthy/young" oocytes may compensate for the potential adverse effects on pregnancy and implantation of high sperm DNA fragmentation, but this has been refuted by some studies (Borini et al. 2006; Frydman et al. 2008). Nevertheless, some refinements of ICSI that allow us to minimize the risk of injection of DNA damaged spermatozoa seem to improve the clinical outcome, especially in the case of oocytes of older women (Souza Setti et al. 2013); this may suggest that sperm DNA damage plays a critical role in the case of advanced age of the female partner.

DNA Damage and IVF/ICSI Miscarriage Rate

It has been hypothesized that, although DNA-damaged spermatozoa can fertilize an oocyte, they are often associated with failed pregnancy or pregnancy loss (Henkel et al. 2004). Borini et al. (2006) observed a higher miscarriage rate in ICSI patients with a DFI greater than 10 %. Kennedy et al. (2011) reported a significant correlation between DFI and spontaneous abortion; furthermore, they observed that couples having triplet pregnancies had a lower DFI than those with pregnancy loss. Conversely, Bellever et al. (2010) found no correlation between a high DFI and spontaneous abortion. In a recent large-scale metaanalysis (1,549 cycles: 640 pregnancies and 122 pregnancy losses) Zini et al. (2008) concluded that sperm DNA damage is associated with a significantly increased risk of pregnancy loss after both IVF and ICSI (2008).

Remarks

All these studies seem to indicate a correlation between DNA damage and outcome of AR technologies, including IVF and ICSI. Since the outcome of AR treatments depends on many more factors than just sperm quality (above all female factors, but also environmental factors, patient selection, choice of appropriate AR treatment, and others), this makes it extremely difficult to compare the studies and to find a general agreement on the role of sperm DNA damage. Furthermore, it should be pointed out that sperm DNA fragmentation is not a static seminal parameter and the timing for assessing the DNA damage is critical: the in vitro aging and spermatozoa or exposure to nonphysiological conditions before DNA testing have a detrimental effect on DNA (Santiso et al. 2012). Unfortunately, information on the timing of DNA testing and DNA fragmentation dynamics is very often absent in the literature. In conclusion, the various factors influencing treatment outcome, the timing in performing the DNA fragmentation test, and the use of different DNA tests contribute to jeopardizing the reliability of the majority of the studies in the literature.

Sperm Selection for Assisted Reproduction Techniques: Effect on Sperm DNA Quality

DNA fragmentation rate in the raw semen can be reduced by sperm processing procedures, which improve the percentage of spermatozoa with a normal chromatin structure by filtering out apoptotic spermatozoa. In the case of in vitro insemination techniques (IVF/ICSI) following sperm preparation, high-magnification microscopy and selection by some sperm markers are valid tools in selecting spermatozoa with a reduced risk of being DNA-damaged.

Relationship Between Sperm Motility and DNA Integrity

It is accepted that oligozoospermia (Høst et al. 1999; Burrello et al. 2004), teratozoospermia (Høst et al. 1999; Muratori et al. 2003), and asthenozoospermia (Irvine et al. 2000; Giwercman et al. 2003; Varghese et al. 2009; Mahfouz et al. 2010) are associated with a higher percentage of sperm DNA aberrations.

Conversely, it seems that good sperm motility is related to sperm DNA integrity (Van den Bergh et al. 1998; Ramos and Wetzels 2001; Avendaño and Oehninger 2011). This has been highlighted by Ebner and colleagues (2011), who showed that fast progressive spermatozoa have intact DNA. This is probably due to the fact that not only nuclear but also mitochondrial DNA can be affected by DNA fragmentation (Alvarez 2005); in the latter case, the spermatozoa may have alterations in the production of ATP, which is the "fuel" for sperm motility. This hypothesis is confirmed by various studies. Kasai et al. (2002) observed a direct association between motility, mitochondrial volume, and membrane potential within the sperm midpiece; mutations or deletions within mitochondrial DNA were shown to be associated with reduced sperm motility (Ozmen et al. 2007); and Speyer et al. (2010) noted a positive correlation between DFI and sperm midpiece.

Sperm Preparation Techniques Before IVF/ICSI

In the case of DNA damage, prior to IVF–ICSI, basal sperm DNA fragmentation rate can be significantly reduced by some sperm processing procedures. These allow us to select motile spermatozoa, filtering out the low-motile apoptotic ones. During sperm preparation special care should be taken because it should be kept in mind that these procedures themselves might cause DNA damage (Twigg et al. 1998; Donelly et al. 2001; Dalzell et al. 2004; Santiso et al. 2012; Toro et al. 2009). First of all, the sperm must be processed immediately after liquefaction in order to avoid in vitro aging and degeneration of nuclear proteins (Sakkas and Alvarez 2010); then

Table 10.2 Sperm processing techniques and DNA fragmentation index (DFI) reduction[a]

	Patients	%DFI raw	% DFI after	% reduction
Swim-up				
Zini et al. (2000a)	22	10.1	4.8	52.5
Younglai et al. (2001)	29	2.4	1.1	54.2
Jackson et al. (2010)	30	17.8	8.3	53.4
Parmegiani et al. (2010a)	20	16.5	11.0	33.3
Density gradient centrifugation				
Zini et al. (2000a)	22	10.1	13.6	+34.6
Gandini et al. (2004)	34	12.0	5.5	54.2
Morrell et al. (2004)	9	20.9	12.8	38.7
Jackson et al. (2010)	30	17.8	7.1	60.1
Simon et al. (2010)	180	51.7	36.8	28.8
Ebner et al. (2011)	37	15.8	14.2	10.1
Castillo et al. (2011)	66	46.0	33.1	28.0
Magnetic-activated cell sorting (MACS)				
Said et al. (2006)	35	14.4	9.7	32.6
Hyaluronic acid binding				
Razavi et al. (2010)	77	32.9	30.9	6.1
Zeta method				
Razavi et al. (2010)	77	32.9	18.2	44.7
Zech selector				
Ebner et al. (2011)	37	15.8	0.4	97.5

[a]Slightly modified from Ebner et al. (2012)

the procedure must be performed so as to avoid spermatozoa exposure to stressful conditions, such as, for example, too high/low temperature and repeated high-speed centrifugation.

Although some articles reported no significant reduction in basal DFI following sperm preparation (Zini et al. 2000a; Gandini et al. 2004; Stevanato et al. 2008; Ebner et al. 2011; Boe-Hansen et al. 2005; Zini et al. 2000b), the vast majority of studies in this field support the fact that the following techniques improve the yield of DNA-intact male gametes:

- "Swim-up" (Zini et al. 2000a; Younglai et al. 2001; Jackson et al. 2010; Marchesi et al. 2010),
- Density gradient centrifugation (Donnelly et al. 2000, 2001; Morrell et al. 2004; Ahmad et al. 2007; Jackson et al. 2010; Marchesi et al. 2010; Castillo et al. 2011),
- Sperm selection based on membrane charge (Chan et al. 2006; Razawi et al. 2010),
- Cell sorting using a membrane-based electrophoretic filtration system (Fleming et al. 2008)
- Magnetic-activated cell sorting (Said et al. 2006; Lee et al. 2010; Polak de Fried and Denaday 2010; Rawe et al. 2010),
- Selection by motility without centrifugation (Ebner et al. 2011).

These studies are summarized in Table 10.2.

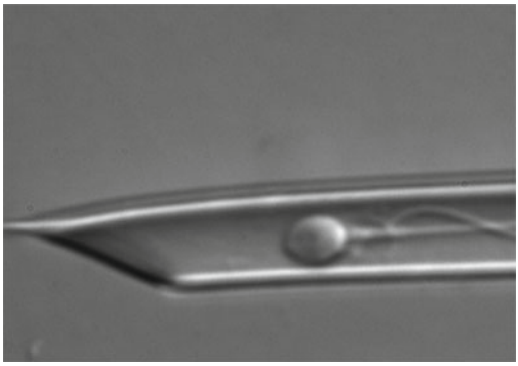

Fig. 10.1 Spermatozoon at high magnification (>6300×) for IMSI

Intracytoplasmic Morphologically Selected Sperm Injection (IMSI)

The introduction of ICSI (Palermo et al. 1992; Van Steirteghem et al. 1993) made possible pregnancies also in couples affected by severe male factor, in which the male partner had low sperm motility, poor zona binding, poor acrosome reaction, or abnormal sperm morphology. The observation of sperm morphology (Kruger et al. 1986, 1988) at the conventional magnification used for sperm evaluation (400×) has controversial prognostic value in ICSI cycle outcomes (Svalander et al. 1996; De Vos et al. 2003; French et al. 2010) and does not seem to influence embryo development or morphology (French et al. 2010; De Vos et al. 2003). However, at this magnification, sperm dimension and shape are not reliable attributes for predicting chromatin integrity or the presence of numerical chromosomal aberrations (Celik-Ozenci et al. 2004).

When using an unstained, real-time, high-magnification examination of spermatozoa called motile sperm organelle morphology examination (MSOME), it is possible to overcome the limitations of conventional magnification (Bartoov et al. 1994, 2001, 2002). MSOME is performed using an inverted light microscope equipped with high-power Nomarski optic enhanced by digital imaging to achieve a magnification of up to 6300× (Fig. 10.1). "The ICSI procedure using MSOME criteria is defined as IMSI: intracytoplasmic morphologically selected sperm injection" (Bartoov et al. 2003). Some authors have demonstrated that ICSI outcomes can be significantly improved by the exclusive microinjection of spermatozoa with a strictly defined, morphologically normal nucleus, especially in couples with previous ICSI failures (Bartoov et al. 2003; Berkovitz et al. 2005; Hazout et al. 2006; Antinori et al. 2008; Franco et al. 2008; Souza Setti et al. 2010), with severe male factor (Balaban et al. 2011; Souza Setti et al. 2011), or with advanced maternal age (Souza Setti et al. 2013). Conversely, the injection of spermatozoa with abnormal sperm head or with nuclear vacuoles negatively affects embryo development (Vanderzwalmen et al. 2008; Cassuto et al. 2009) and ICSI outcome (Berkovitz et al. 2006a, b;

Cassuto et al. 2009; Nadalini et al. 2009). The positive effect of the injection of spermatozoa without nuclear vacuoles is probably related to their significantly better mitochondrial function and chromatin status, and reduced aneuploidy rate as compared with vacuolized spermatozoa (Garolla et al. 2008; Boitrelle, et al. 2011). Furthermore, spermatozoa free of nuclear morphological malformations were found to be significantly associated with a lower incidence of aneuploidy in derived embryos (Figueira et al. 2011). However, in a few studies ICSI had the same performance as IMSI in terms of fertilization and early embryo development (Nadalini et al. 2009; Mauri et al. 2010; De Vos et al. 2013);

It should be pointed out that the large majority of articles published on IMSI were based on weak study designs or performed on a small number of patients. However, in a recent strict prospective sibling-oocyte study comparing ICSI and IMSI, performed on 350 treatments, De Vos and colleagues (2013) observed comparable results in terms of fertilization, embryo quality, and clinical outcome between the two groups. They concluded that a routine application of IMSI in unselected AR technology patients cannot be advocated. Furthermore, it is true that IMSI is undoubtedly a time-consuming procedure; selecting a normal spermatozoon according to MSOME criteria may require 60–120 min (Antinori et al. 2008). In addition, the process of searching for spermatozoa at high magnification may itself damage sperm cytoplasm: after 2 h on the microscope's heated stage, sperm nucleus vacuolization significantly increases (Peer et al. 2007). Despite these observations, IMSI can be considered a valid tool for safe, noninvasive selection of spermatozoa that have a reduced risk of DNA damage (Garolla et al. 2008; Ebner et al. 2012).

Restoration of Natural Fertilization Checkpoints: Hyaluronic Acid and Zona Pellucida Sperm Binding

In humans during the natural fertilization process, spermatozoa must pass two barriers before fusing with the oocyte membrane. The first is the cumulus oophorus–corona radiata complex, made up of cells and an extracellular matrix of polymerized hyaluronic acid (HA) and proteins. The second is the zona pellucida (ZP), a thick elastic coat of glycoproteins located right next to the oocyte (Yanagimachi 1994). In addition, in the testes during spermiogenesis, still immature spermatozoa (elongated spermatids) undergo cytoplasmic extrusion and plasma membrane remodeling to prepare themselves for oocyte fertilization (Kovanci et al. 2001). This sperm membrane remodeling determines the formation of the HA and ZP receptors. In human spermatozoa, two specific proteins are related to maturity, DNA integrity, chromosomal aneuploidy frequency, and fertilizing potential: the heat shock protein HspA2 chaperone – involved in meiosis – and creatine kinase (CK) – abundant in the sperm cytoplasm (Cayli et al. 2003). Mature spermatozoa have a high expression of HspA2 (Huszar et al. 2000) and a low expression of CK (Cayli et al. 2003). In contrast, spermatozoa with arrested maturity have a low HspA2 and a high CK expression. Low levels of HspA2 can cause meiotic defects and chromosomal

aneuploidies, whereas high retention of CK and other cytoplasmic enzymes can cause lipid peroxidation and, consequently, DNA fragmentation and abnormal sperm morphology (Huszar and Vigue 1993; Cayli et al. 2003).

Due to a lack of or partial membrane remodeling, immature spermatozoa have deficiency in the ZP and HA binding sites, and for this reason they are not able to fertilize oocytes naturally.

Hyaluronic Acid Binding

Since in natural fertilization human oocytes are surrounded by HA, this glycoprotein has a pivotal role in the mechanism of sperm selection. In fact, only mature spermatozoa that have extruded their specific receptors to bind to HA are able to reach and fertilize the oocyte. The role of HA as a physiological selector is also well recognized in vitro. In fact, spermatozoa that can bind to HA in vitro have completed their plasma membrane remodeling, cytoplasmic extrusion, and nuclear maturation (Cayli et al. 2003; Huszar et al. 2003, 2007).

Based on this selective ability of HA in binding only to mature sperm (Huszar et al. 2003; Prinosilova et al. 2009), a very simple diagnostic tool for assessing sperm maturity and function, the so-called sperm-hyaluronan-binding assay (HBA), has been developed as a commercial kit (Cayli et al. 2003; Huszar et al. 2003). HBA may be useful in predicting sperm-fertilizing ability in IVF or ICSI. In fact, HBA was found to be correlated with morphology (Ye et al. 2006; Tarozzi et al. 2009) but to a less significant extent than the association between sperm morphology and fertilization rate in IVF (Ye et al. 2006). At present, HBA does not seem to provide information for identifying patients with poor or absent fertilization (Ye et al. 2006; Nijs et al. 2010) or a threshold value for outcome in conventional IVF/ICSI treatment (Nijs et al. 2010; Kovacs et al. 2011). On the other hand, HBA seems to be significantly correlated with good embryo quality, miscarriage rate, and ongoing pregnancy rate after ICSI (Nijs et al. 2010) and with fertilization rate after IVF (Pregl Breznik et al. 2013). Recently, Worrilow et al. (2010, 2013) demonstrated that in patients in whom less than 65 % of total spermatozoa were bound to HA (HBA $\leq$ 65 %), a modified ICSI procedure with HA-bound spermatozoa – physiological HA-ICSI (see next paragraph) – produced a significant improvement in clinical outcome compared with conventional ICSI.

Physiological HA-ICSI

The selection of mature spermatozoa by HA represents a straightforward refinement of the ICSI procedure (Parmegiani et al. 2010a, b). It is known that HA-containing products have no negative effects on postinjection zygote development and can be metabolized by the oocyte, and HA-bound spermatozoa can be easily recovered using an injecting pipette (Balaban et al. 2003; Barak et al. 2001; Van den Bergh et al. 2009). A “homemade” HA–sperm selection system can be

produced in any IVF lab; however, at the present time two ready-to-use systems specially designed for sperm–HA binding selection are currently available: (1) PICSI, a plastic culture dish with microdots of HA hydrogel attached to the bottom of the dish, and (2) SpermSlow, a viscous medium containing HA that can be used instead of polyvinylpyrrolidone (PVP), the viscous medium commonly used to slow spermatozoa prior to ICSI.

This new approach to ICSI with HA-bound spermatozoa, when using a HA-viscous medium or HA-culture dishes, has been called physiological ICSI (Parmegiani et al. 2010a). It has been demonstrated that the injection of HA-bound spermatozoa improves embryo quality and development by favoring selection of spermatozoa with normal nucleus and intact DNA (Parmegiani et al. 2010a). Furthermore, improved implantation rates were observed when injecting HA-bound sperm as compared to PVP sperm (Parmegiani et al. 2010b). Nasr-Esfahani et al. (2008) found a higher fertilization rate when injecting oocytes with HA-selected spermatozoa using home-made HA–ICSI dishes. An improvement in fertilization rate and embryo quality and a reduction in the number of miscarriages were found by Worrilow et al. (2007) with PICSI versus conventional ICSI. In subsequent studies, the same authors confirmed that PICSI significantly improved clinical results in ICSI, particularly in patients with an HBA score less than or equal to 65 % (Worrilow et al. 2010, 2013). In contrast, some authors found no differences in ICSI outcome parameters when injecting HA-bound sperm compared with PVP sperm (Mènèzo and Nicollet 2004; Sanchez et al. 2005; Mènèzo et al. 2010; Van den Berg et al. 2009).

At the very least, in none of the studies did physiological HA-ICSI cause a detrimental effect on ICSI outcome parameters. If larger, multicenter, prospective-randomized studies confirm the suggested beneficial effects on ICSI outcome, HA should be considered the first choice for physiologic sperm selection prior to ICSI because of its capacity to reduce genetic complications and for its total lack of toxicity (Parmegiani et al. 2010c). In addition, the use of HA-ICSI avoids PVP, which seems to be toxic and to generate sperm DNA fragmentation (Salian et al. 2012). Since both the sperm–HA binding selection systems (PICSI and SpermSlow) are easily available, efficient, and approved for human IVF use, AR centers can choose the one best suited to their needs (Parmegiani et al. 2012a).

Zona-Bound Spermatozoa

As well as binding for HA, the spermatozoa–ZP binding process also plays a crucial role in the natural selection of spermatozoa. Immature spermatozoa show a low density of ZP binding sites as well as HA receptors (Huszar et al. 2003). Sperm binding to ZP exhibit attributes similar to those of HA-bound sperm, including minimal DNA fragmentation, normal shape, and low frequency of chromosomal aneuploidies (Yagci et al. 2010). Furthermore, the same sperm membrane protein seems to be involved, firstly, in hyaluronidase activity and, subsequently, in ZP binding (Hunnicutt et al. 1996). The spermatozoa–ZP binding test can be performed by culturing spermatozoa for a short time with immature metaphase I oocytes; in this

way, ZP-bound spermatozoa can be recovered and used for ICSI (Paes de Almeida Ferreira Braga et al. 2009). An increased embryo quality was observed when ZP-bound ICSI was performed compared with conventional ICSI (Paes de Almeida Ferreira Braga et al. 2009; Liu et al. 2011). In a small study, Black et al. (2010) observed a slightly better trend in pregnancy and implantation comparing 39 ZP-ICSI versus 39 conventional ICSI. At the present time, there is little information regarding all the factors involved in sperm-ZP binding and its mechanism, but the spermatozoa–ZP binding test could be an efficient method for identifying competent spermatozoa for ICSI. ZP selection could then be coupled with HA selection in order to replicate the natural path of the spermatozoa toward the oocyte.

Conclusions

Several factors contribute to the outcome of AR treatments, sperm DNA quality being just one factor. Although the role of sperm DNA damage in the clinical outcome of high-technology AR procedures like ICSI could still be considered debatable, it is undoubtedly wise to use all precautions to limit any potential long-term side effects of these techniques (Ebner et al. 2012).

At present, in the literature, there are still few data on the selection of the ideal sperm for ICSI, perhaps because for many years it was not possible to identify definitively a DNA-intact spermatozoon (Nijs et al. 2009). A potentially worrying aspect of the ICSI of DNA-damaged spermatozoa has been suggested by studies performed on animals that showed not only a negative effect on pregnancy and birth, but also later side effects on the health of adult animals such as aberrant growth, premature aging, abnormal behavior, and mesenchymal tumors (Fernandez-Gonzales et al. 2008). Fortunately, in humans, the risk of injecting DNA-damaged spermatozoa seems to be minimized by conventional sperm preparation techniques prior to ICSI (Zini et al. 2000a; Younglai et al. 2001; Donnelly et al. 2000, 2001; Morrell et al. 2004, Ahmad et al. 2007; Jackson et al. 2010; Marchesi et al. 2010; Castillo et al. 2011), and follow-up studies on ICSI children have demonstrated the basic safety of this revolutionary technique (Van Steirteghem et al. 2002; Leunens et al. 2008; Belva et al. 2011; Woldringh et al. 2011), although a slight increase in chromosome aberration seems to be caused by the injection of aneuploid spermatozoa (Bonduelle et al. 2002).

Given the need to reduce all potential long-term risks on ICSI adults, the search for the ideal spermatozoa is a stimulating challenge for embryologists. A final precise sperm selection is performed at the micromanipulator via IMSI, HA, or ZP sperm binding to minimize the chance of injecting DNA-damaged sperm. There is evidence that HA-bound mature spermatozoa show better morphology than immature sperm, both at conventional (Tygerberg criteria) and at high magnification (MSOME criteria) (Prinosilova et al. 2009; Nasr-Esfahani et al. 2008; Oliveira et al. 2009; Parmegiani et al. 2010a). Furthermore, HA-bound sperm show a low DFI and a reduced incidence of aneuploidies (Nasr-Esfahani et al. 2008; Parmegiani et al. 2010a; Jakab et al. 2005; Sanchez et al. 2005; Yagci et al. 2010). HA-bound mature

spermatozoa showed no cytoplasmic retentions, persistent histones, or DNA fragmentation (Huszar et al. 2007). It should be hypothesized that ZP-bound spermatozoa have characteristics similar to those of HA-bound sperm (Huszar et al. 2007; Yagci et al. 2010; Hunnicutt et al. 1996). In addition, sperm–HA binding seems to be related to DNA methylation, which is important for correct chromatin packaging; in fact, in patients with a total lack of HA binding this feature is restored with supplementation of a methylation effector (Junca et al. 2012). Since during normal spermiogenesis and plasma membrane remodeling, along with formation of the ZP binding site, formation of the HA binding site also occurs, this should form the basis of ideal ICSI sperm selection (Huszar et al. 2007; Ebner et al. 2012).

Regarding sperm morphology, modern technology allows us to obtain very high magnification, and the introduction of IMSI should be considered an exciting chapter in IVF history, whether or not its clinical usefulness is confirmed with studies on a large scale. IMSI technology has enabled exploration of the relationship between the morphology of spermatozoa and their DNA constitution, revealing that vacuole-free spermatozoa have a lower rate of DNA-fragmentation (Franco Jr. et al. 2008), MSOME abnormal spermatozoa show a high level of DNA fragmentation (Wilding et al. 2011), and, in particular, spermatozoa with large vacuoles are characterized by aneuploidies and chromatin condensation defects (Perdrix et al. 2011).

IMSI's lengthy procedure can be circumvented by preselecting HA sperm merging high-magnification microscopy with HA-sperm selection. This procedure has been called physiological IMSI (Parmegiani et al 2012b). In fact, HA helps to select a subpopulation of spermatozoa with normal nuclei according to MSOME criteria (Parmegiani et al. 2010a).

In conclusion, most of the alternative possible refinements of the ICSI procedure described in this chapter are easily reproducible and straightforward; for this reason, they could be offered to ICSI patients not only to optimize clinical results but, at the very least, to prevent fertilization by DNA-damaged and chromosomally unbalanced spermatozoa.

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Chapter 11
Effect of Antioxidants on Sperm Genetic Damage

Yves Menezo, Don Evenson, Marc Cohen, and Brian Dale

Abstract According to worldwide statistics, between one in four and one in five couples have fertility problems. These problems are equally distributed between males and females. Modern lifestyle has obviously increased these problems: endocrine-disrupting chemicals, such as plastic polymer catalysts, alkylphenols, phthalates and so on, and cosmetic additives seem to be strongly involved in this fertility problem. Many of these compounds increase oxidative stress (OS) and thus impair spermatogenesis. The oocyte has only a finite capacity, decreasing with maternal age, to repair sperm-borne decays. To decrease this DNA repair burden, reducing the sperm DNA damages linked to OS is tempting. Antioxidant vitamins are often given haphazardly; they are not very efficient and potentially detrimental. A detailed analysis of the sperm nucleus is mandatory (DNA fragmentation or lack of nuclear condensation) prior to any treatment. Here we discuss new concepts in OS and the corresponding therapeutic approaches.

Keywords Oxidative stress • Gametes DNA damages • Fertility • Antioxidants • Homocysteine

Y. Menezo (✉)
London Fertility Associates, 104 Harley Street, London, UK

Laboratoire CLEMENT, 17 Avenue d' Eylau, Paris 75116, France
e-mail: yves.menezo@gmail.com

D. Evenson
South Dakota State University, Brookings, South Dakota, USA

M. Cohen
Procrelys, Maison médicale Ambroise Paré 28, avenue Rockefeller, Lyon 69008, France

B. Dale
London Fertility Associates, 104 Harley Street, London, UK

Centre for Assisted Fertilization, Via Tasso 480, Naples, Italy

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Introduction

According to the 2002 National Survey of Family Growth by the Centers for Disease Control and Prevention (CDC), infertility affects approximately 12 % of the reproductive-age population. In the USA, this includes 7.3 million women and their partners. But by the World Health Organizations (WHO) (2004) estimation, infertility could now affect one couple in five in Western countries to one in four in the world-wide population [infecundity, infertility, and childlessness in developing countries (Demographic and Health Surveys, DHS, Comparative reports No. 9 ORC Macro and WHO 2004)]. Male reproductive failure is thought to be the cause of 50–70 % of infertility cases in Western countries: isolated males account for one-half of these and are a contributor in the other half (Krausz 2011). This, together with lifestyle variations, has led to a delay in first delivery in couples from around 25 years of age in the previous generation to over 30 years of age. Over the past half century, routine classic semen analysis has focused only on the morphology, number, and motility of sperm cells. The advent and large-scale development of assisted reproductive technology (ART) and especially intracytoplasmic sperm injection (ICSI), which bypass a natural "selection," have caused an upheaval. A better appreciation of sperm DNA integrity, especially due to the pioneering work of Evenson et al. (1980), has changed the scientific and therapeutic approach to male infertility. Importantly, this has led to the discovery that infertile men with both normal and abnormal sperm parameters may have significantly higher sperm DNA damage. The major factors affecting sperm DNA integrity include DNA fragmentation and formation of DNA adducts (primary and secondary structure) and chromatin decondensation (tertiary structure). Oxidative stress (OS) is one of the major causes of DNA and chromatin damage (at least for primary and secondary structure) and sperm quality (Kao et al. 2008). OS causes DNA fragmentation, formation of a-basic sites, and formation of DNA adducts, which can be partially the result of chemical covalent interactions between by-products of lipid (polyunsaturated fatty acids, PUFAs) peroxidation, i.e. malonedialdehyde and 4 hydroxynonenal, with nuclear bases (Badouard et al. 2008). In theory, these chemical insults can be repaired by the zygote and the very early embryo using reserves accumulated in the growing oocyte (Menezo et al. 2007a; Jaroudi et al. 2009). The oocyte has an important and redundant, yet limited, DNA repair capacity that decreases with age. However, the oocyte must also repair female genome DNA damage (Lopes et al. 1998; Zenzes et al. 1998), thereby contributing to an overall increase in the total amount of DNA needing repair. Approximately two million DNA repair operations are needed during the first 24 h following fertilization (Menezo et al. 2010). If the DNA repair capacity is overwhelmed, the embryo will initiate apoptosis and developmental arrest. However, a point of greater concern is that some sperm DNA damage, if not repaired, may lead to mutations. Therefore, paternal transmission of damaged DNA may compromise embryonic development and subsequently alter post-natal development (Ji et al. 1997; Zenzes 2000; Zini and Sigman 2009; Robinson et al. 2012).

In animal models, ICSI using sperm with fragmented DNA leads to a high risk of genetic disease transmission and severe pathologies (Fernandez-Gonzalez et al. 2008).

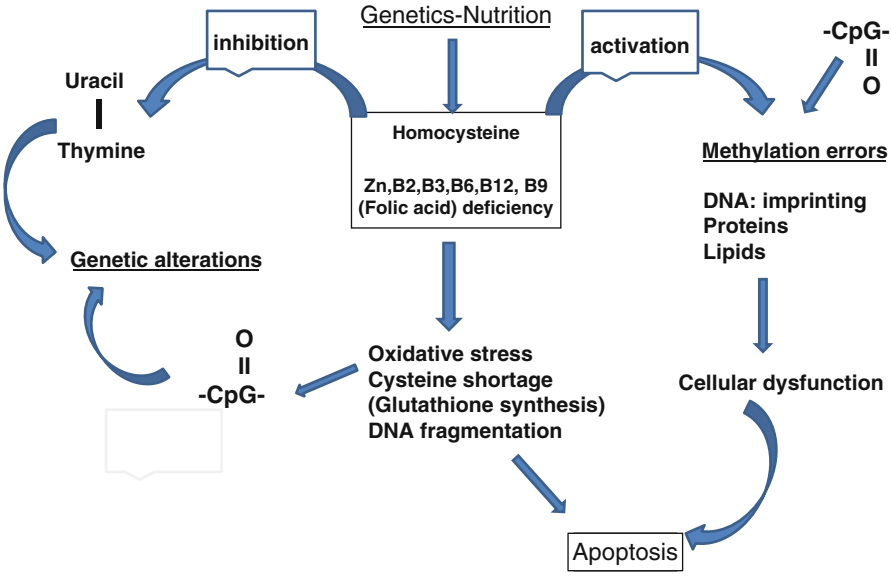

Fig. 11.1 Interrelations between Methylation, homocysteine and oxidative stress. CpG=O, CpG islands with oxidized guanine. CpG: phosphodiester bond between Cytosine (C) and Guanine (G)

There are at least 12 base oxidation products (Menezo et al. 2012); the most important is 8 oxo guanine (G). 8 oxo G causes G->T transversions, a source of mutations. If left unrepaired, 8 oxo G affects methylation of the adjacent cytosine (CpG, phosphodiester bond between the cytosine and guanine sites): these CpG sites in DNA represent mutational hotspots (Wachsman 1997; Franco et al. 2008). This observation strongly suggests a link between reactive oxygen species (ROS) decays, genetic alterations and carcinogenesis (Fig. 11.1) induced by aberrant DNA methylation (Cerda and Weitzman 1997; Franco et al. 2008). G is also an important component of telomeres (TTAGGG repeats). Critically short telomeres are associated with sperm DNA fragmentation (Rodriguez et al. 2005). Thus, OS, in a kind of vicious circle, also directly damages telomeres and accelerates their shortening. Telomere shortening induces defects in meiosis, fertilization and embryo development and thus leads to infertility (Keefe and Liu 2009). Telomere dysfunction is a masterpiece in reproductive aging. In addition, 8 oxo G may affect codons of the sulfur-containing amino acid cysteine (UGU, UGC), involved in the synthesis of glutathione, the universal "physiological" free radical scavenger. 8 oxo G may also affect the methionine (AUG) codon. Methionine is the major effector in methylation processes (through S adenosyl methionine: SAM): it yields the methyl groups, and the by-product formed is homocysteine. This aspect can be a supplementary link between defective methylation and OS. ROSs are at the crossroads between genetic and epigenetic changes, potentially affecting cell quality. Seminal plasma has a finite potential for scavenging ROSs either by enzymatic action [superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX)] or by small molecules:

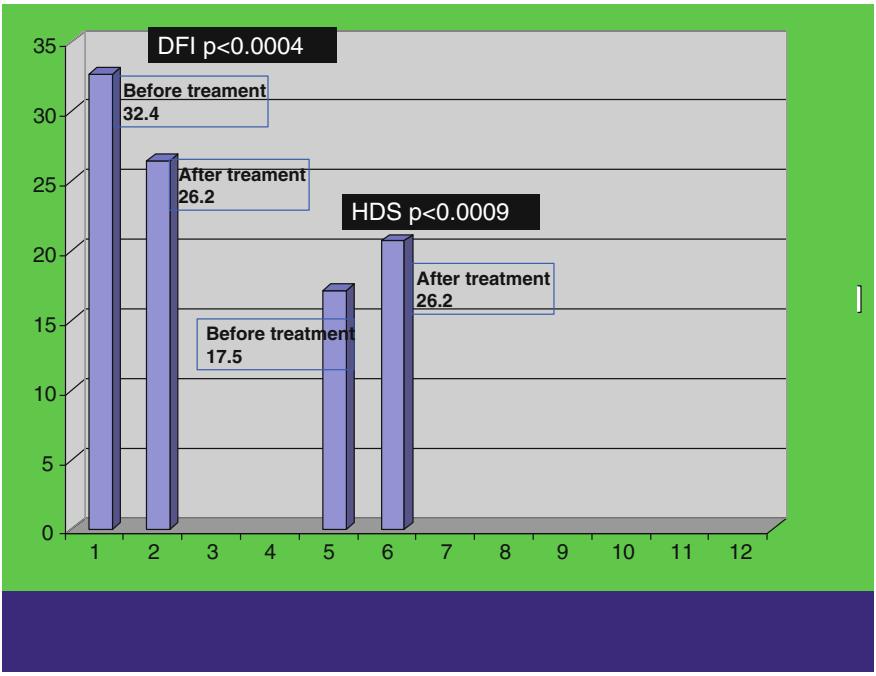

Fig. 11.2 variations in DFI and HDS, post 3 months treatment with vitamins C, E and A and Selenium (From Menezo et al. 2007b). The treatment decreases fragmentation (DFI) but increases the nucleus decondensation (HDS) inthe same order of magnitude

glutathione, hypotaurine, ascorbic acid and gamma tocopherol (and not beta tocopherol, the commercial form). For infertile men with high levels of sperm DNA fragmentation, it is tempting to increase the antioxidant capacity by providing nutraceutical antioxidants. The positive effect of such complementation is far from being demonstrated (Agarwal et al. 2004). Moreover, we recently demonstrated that complements may have a deleterious effect on sperm nucleus condensation (Menezo et al. 2007b) (Fig. 11.2). In this chapter, we will point out two types of complement treatment: (a) the modern theories on OS and (b) the effective action of these reducing substances on cell physiology, with particular emphasis on the overexposure that is often observed.

Approaches to Determining Oxidative Stress in Sperm

First of all, two important and unavoidable prerequisites must be mentioned: high-quality reproducible evaluations of the main DNA decays must be made. Determination needs to be made as to whether the decays observed are stable in time without any treatments or if there are fluctuations of known/unknown origin. DNA fragmentation, tertiary structure (compaction/decondensation), presence of

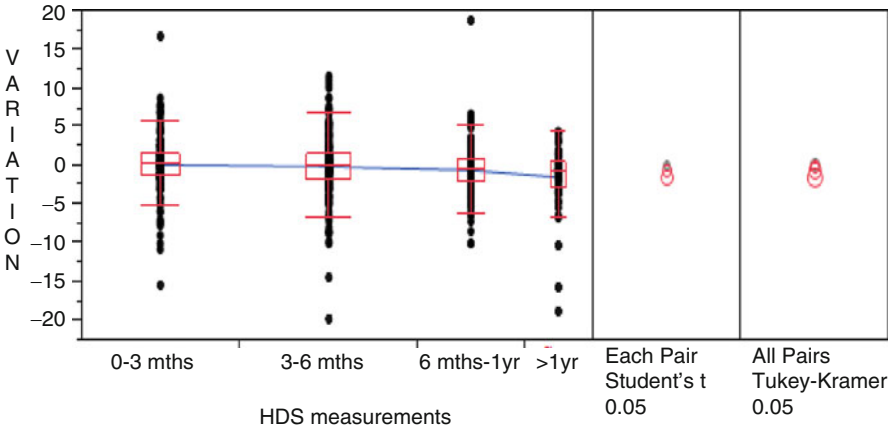

Fig. 11.3 Variations in HDS measurements (SCSA) over time. Each patient is its own control ($N=560$ patients). The curve shows that under normal conditions, without treatment, sperm nucleus condensation does not show any modification during 6 months and then it slightly decreases

adducts and oxidized bases are the key parameters linked to successful embryogenesis. Fragmentation can be estimated by two robust techniques: the SCSA test (Evenson et al. 1980, 2002), which measures by flow cytometry the percentage and extent of sperm DNA fragmentation, and the TUNEL (d-UTP nick-end labelling), which may have some artefacts (Muratori et al. 2010). Decondensation can be quantified using the SCSA test (HDS: high DNA stainability) and staining with toluidine/aniline blue (Hammadeh et al. 1996). Chromomycin A3 (CMA3), a glycosidic antibiotic from *Streptomyces griseus* which is used as a fluorescent stain of DNA, has been used to quantify sperm maturity, i.e. defect in protamination. But in reality CMA3 has no specificity towards histones or protamines: it is a DNA dye. Indeed, CMA3 seems to have the same feature as TUNEL in determining rather DNA decays (Belloc et al. 2009). Sperm DNA fragmentation and nuclear lack of condensation in the nucleus are largely, but not totally, independent parameters (Wyrobek et al. 2006). Failed condensation does not increase the risk of DNA fragmentation. In fact, the balance between the two parameters is clearly demonstrated by two observations: (1) age increases DNA fragmentation and decreases decondensation (Wyrobek et al. 2006); (2) strong antioxidants decrease fragmentation to a certain extent but increase decondensation (Menezo et al. 2007b). This likely means that an increase in ROS production and vulnerability (with age) allows for a better padlocking of the protamine-cysteine bridges. A balance must be respected between pro- and antioxidants, and the glutathione/glutathione peroxidase (GPX) system is the epicenter of the regulations involving padlocking of sperm nuclei and protection against ROS insults (Menezo et al. 2012).

Concerning the stability of the chromatin structure over time, our study in a group of 503 patients, which controlled for HDS after various time periods (0–3, 3–6, 6–12 months, more than 1 year), demonstrated that HDS values did not vary between 0 and 6 months. After 6 months, it slightly but significantly decreased, confirming the general feature observed for age ($r=-0.22$, $p<0.001$) (Fig. 11.3). The DFI is also

stable over time without treatment; the DFI measured in 45 patients (1 sample every month for 8 months) showed no significant variation. DFI is a much more stable parameter than the ones given by conventionnal semen analysis; moreover it is not subject to seasonal variatons contrariliy to sperm concentration and quantity.

8 OHdG is an important marker for OS. Quantification can be done using several techniques including both liquid chromatography and fluorescence, flow cytometry/immunofluorescence or mass spectometrty after DNA hydrolysis. These tests are not easy to carry out (Badouard et al. 2008). A strong correlation exists between DNA fragmentation and oxidative damage as determined by SCSA fragmentation and 8 OHdG (Oger et al. 2003) and TUNEL fragmentation and 8 OHdG (De Iuliis et al. 2009; Aitken et al. 2010). Malonedialdehyde (MDA) formation results from a peroxidation of lipids; it most likely induces the formation of DNA adducts (Badouard et al. 2008): MDA is a marker of sperm immaturity (Montjean et al. 2010) and the related excessive presence of polyunsaturated fatty acids (PUFAs). No correlation has been found between TUNEL and MDA sperm content. DNA adduct determination is complex and requires both liquid chromatography and mass spectrometry measurements.

To demonstrate the effect of antioxidant treatments, there are only two possibilities: (1) double blind versus placebo and (2) control before and after treatments (paired samples) once the stability of the parameter without treatment has been demonstrated. The patient is his own control.

Current Treatments and Their Pitfalls

Current treatments are generally based on bits of information from the literature. Therefore, supplements may vary widely in terms of composition and concentration. Oral intake of some molecules may increase their concentration in serum but not necessarily in the testes. It must be clear that the active compounds must pass into the blood and then various organs. Pregnancy should be the ultimate parameter in judging treatment efficacy.

Selenium

Selenium (Se) is a very popular compound in supplements, especially for anti-ageing. It could be a logical choice since it is a cofactor of phospholipid hydroxyperoxide glutathione peroxidase GPX4 or PhGPX (Ursini et al. 1997), involved in the compaction of the sperm nucleus through a padlocking of the protamine-cysteine bonds. However, there are negative effects that counteract this positive observation. First, there is no Se deficiency described in the scientific literature for the Caucasian population. Second, According to Bleau et al. (1984), seminal plasma Se must be in a strict range of values, between 50 and 70 nG/mL. Increased values are associated with decreased motility and a higher incidence of asthenospermia, followed by higher abortion rates.

This observation was confirmed by Li et al. (2012) in 100 patients: the concentration found for Cu, Mn and Se in the seminal plasma of pathological sperm was higher than in the normal sperm group (Cu, $p=0.024$; Se, $p=0.002$; Mn, $p=0.002$). Third, animal studies have demonstrated that if supplementation increases serum values, then it does not influence testicular values. Fourth, increasing Se serum values is not totally harmless. According to Hawkes et al. (2009), Se at high doses induces thyroid pathologies which reduce sperm motility: we have observed two cases in our hypofertile population. Finally, according to WHO regulations (Guidelines for drinking-water quality, fourth edition, World Health Organization 2011), a high selenium concentration in human drinking water, together with aluminium, bacterial contamination, radioactivity, pesticides and nitrates, is one of the six parameters used to regulate safety. According to Brack et al. (2013), serum Se value is never a systemic biomarker of oxidative stress in chronic human pathologies. From a basic point of view, selenites increase apoptosis through a generation of free radicals (superoxides) by mitochondria (Zhao et al. 2009). Mitochondria are already the primary source of DNA damage in sperm cells (through oxidative phosphorylation). Finally, Se at high concentrations could displace Zn and alter DNA methylation and, thus, impair genetic stability. Iron and copper are to be avoided, unless a strong deficiency is established; their role in increasing free radical formation is widely known (Haber-Weiss and Fenton reactions).

Zinc (Zn) Is an Interesting Divalent Cation

First of all, according to the CDC, the National Center for Health Statistics (NCHS), 15 % of the Caucasian population suffers from Zn deficiency. Zn is a cofactor in around 200 enzymes, and especially Zn superoxide dismutase and metallothionein (MT) capture of superoxide and hydroxyl radicals. The sperm of fertile men have mRNA coding for most of the MTs (Garcia Herrero et al. 2011). Zn counteracts the negative impact of cadmium. Zn restriction and repletion affect DNA integrity in healthy men (Song et al. 2009; Ho 2004), decreasing the DNA repair capacity. There is no reason that spermatogenesis should not be regulated by this important process. Zn is an important cofactor in homocysteine recycling, at two levels, dihydrofolate reductase and methionine synthase (Fig. 11.4). Homocysteine is a molecule considered to strongly disturb the reproductive system, in both males and females, causing as a consequence OS and inhibition of DNA methylation (Fig. 11.1). Thus, Zn supplementation seems important, if not mandatory. Omu et al. (2008), working on Zinc supplementation associated or not with vitamins C and E, observed no difference in the outcome measures between zinc only and zinc with vitamin E and a combination of vitamins E and C. However, it has been shown that most of the compounds added to supplements, i.e. Zn pipecolate, Zn oxide, Zn gluconate, Zn sulfate and others, are only marginally efficient due to their poor bioavailability. To be active, Zn must be added in a chelated form www.efsa.europa.eu/ (*European Food Safety authority: EFSA-Q-2005-035, EFSA-Q-2005-133, EFSA-Q-2005-034, EFSA-Q-2005- 038, EFSA-Q-2005-166, EFSA-Q-2005-033, EFSA-Q-2005-132, EFSA-Q-2005-036, EFSAQ-2005-130).*

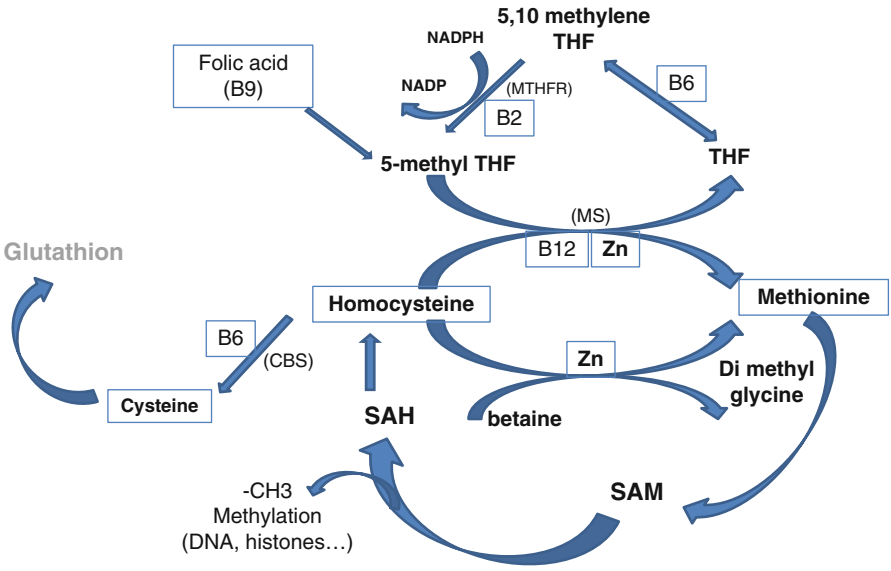

Fig. 11.4 The one carbon cycle. Importance of group B vitamins and Zinc in Homocysteine recycling SAH: S-Adenosyl Homocysteine; SAM: S-Adenosyl Methionine; THF tetrahydrofolate CBS: cystathionine beta synthase; MS Methionine synthase; MTHFR: Methyltetrahydrofolate reductase Zn: Zinc

Vitamin C (Ascorbic Acid)

Vitamin C is one of the natural antioxidants present in the testes, and its concentration in seminal plasma is ten times the concentration observed in serum (Lewis et al. 1997). It was the first supplement used by Fraga et al. (1991) to prevent smoking-related damage. But according to Kandar et al. (2011), vitamin C and uric acid concentrations are similar in the seminal plasma of smokers and non-smokers. It is probable that vitamin C intake can help to reduce DNA fragmentation to a certain extent. However, it is a handicap for sperm nucleus condensation (Menezo et al. 2007b). First, it prevents the primary oxidation of the sperm membrane, and second, it has the capacity to open the disulfide bonds padlocking the DNA. This capacity to open disulfide bonds is of general concern (Giustarini et al. 2008; Donnelly et al. 1999).

Vitamins A and E

These to vitamins may improve the quality of spermatogenesis (Almbro et al. 2011). Vitamin E is given in the form of alpha tocopherol. It may protect membrane lipids from peroxidation and thus increase sperm motility. It probably has no effect on DNA protection. However, the concentrations of the various forms of tocopherol in seminal plasma are very low when compared to serum (Benedetti et al. 2012),

so it is not obvious that an increase in the blood has any impact on testicular concentration. After its neutralization with a free radical, vitamin E may be regenerated by vitamin C and is therefore a better complement (Greco et al. 2005). A decrease in carotene is very rarely a marker of pathologies. This is not the case for vitamin E: its concentration is significantly lower in several pathologies (Brack et al. 2013).

CoQ10 (Ubiquinone)

CoQ10 deficiency is very rare; it occurs in cases of recessive autosomal mutations, some cancers, diabetes, and muscular and cardiovascular pathologies (Villalba et al. 2010), and not in the relatively young population consulting for infertility. Commercially available CoQ10 (ubiquinone) is poorly absorbed by the intestine (Liu and Hartmann Liu and Artmann 2009; Villalba et al. 2010). Marginal increases in CoQ10 can be observed in seminal plasma after long-term treatments but with no improvement in fertility (Mancini and Balercia 2011). In fact, the reduced form of ubiquinone, ubiquinol, which has only recently become available, affects DNA quality with a simultaneous increase in decondensation and, for some patients, a severe decrease in spermatogenesis (Amar et al., personnal communication).

Superoxide Dismutase

Our experience using superoxide dismutase (SOD) of vegetal origin, 'orally effective', (as modified to resist in the stomach environment), was that it yielded the same results as the mixture Se plus vitamins A, C, E: a decrease in DFI but a symmetric increase in nucleus decondensation (Menezo et al. 2007b) (Fig. 11.2).

Reduced Glutathione

Is the universal 'physiological' free radical scavenger. However, its 'effective' capacity after oral absorption is highly questionable for two reasons: (a) it is destroyed in the stomach and (b) it is not transported to the cells.

Carnitine

Carnitine is not, strictly speaking, an antioxidant molecule: it is a regulator of lipid metabolism and limits peroxidation of membrane phospholipids. A marginal positive effect has been described.

New Approaches to Treatment

Information given on the structure of the nucleus by spermiogrammes is scant: in extreme cases, abnormal forms can give an idea on fragmentation, but not on decondensation. In our experience, decondensation and fragmentation affect only marginally the fertilization process. There is no universal treatment and the type of sperm problem must be clearly established. There are so far four main DNA detrimental figures: (1) single defective condensation HDS > 25 %, (2) single moderate fragmentation 25 % < DFI < 35 %, (3) very high single fragmentation with DFI > 35 %, (4)simultaneously elevated fragmentation and decondensation. There exists a common consensus that DFI > 30 % is an upper tolerable level; beyond this limit fertility problems appear, especially for natural conception or even IUI, but less for IVF and ICSI (Bungun et al. 2007, 2011). Spermatozoa may be subject to a burst of ROS during their transit in the female genital tract and at the time of capacitation (De Lamirande and Gagnon 1995). It must be clear that the critical level depends upon the quality (i.e. DNA repair capacity) of the fertilized oocyte. Beyond a 50 % DFI, the chance of conceiving is virtually non-existent (we had one pregnancy with ICSI in a young woman 27 years of age). Concerning the lack of condensation, the problem is rather more complicated as the oocyte is not able to repair anomalies in the tertiary structure of the sperm nucleus. Our upper limit is 25 % for condensation (HDS) defects; this may vary with the technique: aniline/toluidine blue is more variable as it is operator dependent. Beyond these limits, embryo developmental arrests are observed as early as the one-cell stage.

One of the critical point in the understanding of ROS related DNA damages is the correlation between ROS insults and methylation (Tunc and Tremellen 2009). Homocysteine is the key parameter link between these two aspects (Fig. 11.1): "Homocysteine: cause and consequence of oxidative stress" in various pathologies (Tunc and Tremellen 2009; Hoffman 2011; Menezo et al. 2011). DNA damage and low folic acid are correlated (Boxmeer et al. 2009), but in general, folic acid and vitamin B12 are potent modulators of fertility (Ebisch et al. 2007; Boxmeer et al. 2009). As mentioned earlier, Zn (involved in homocysteine recycling) deficiency affects 15 % of the Caucasian population, and its importance has been underlined by Omu et al. (2008). Thus, it seems that all the group B vitamins must be present and not only folic acid (B9), associated with chelated Zinc, as blocks may occur at various steps of the recycling process (Fig. 11.4). Glutathione has a dual role in spermiogenesis – as a free radical scavenger but also an unavoidable partner in nucleus condensation. Quercetine, or quercetol, is a flavonoid (Fig. 11.5). It passes the blood barrier (Moskaug et al. 2005) and several organs such as muscle, and its metabolites formed in the blood, glucuronide, sulfate and methyl quercetin, have an extended life span. The protecting and supporting effect of quercetin has been widely known since the late 1980s. It has been demonstrated that it prevents sperm lipid peroxidation (Moretti et al. 2012) and protects sperm against various environmental toxicants (hydrocarbons), including estrogens or tar and ammonia originating from cigarette smoking (Bohn et al. 2010). Foods rich in quercetin increase the synthesis of 15 mRNAs involved in DNA repair and 11 mRNAs linked to apoptosis.

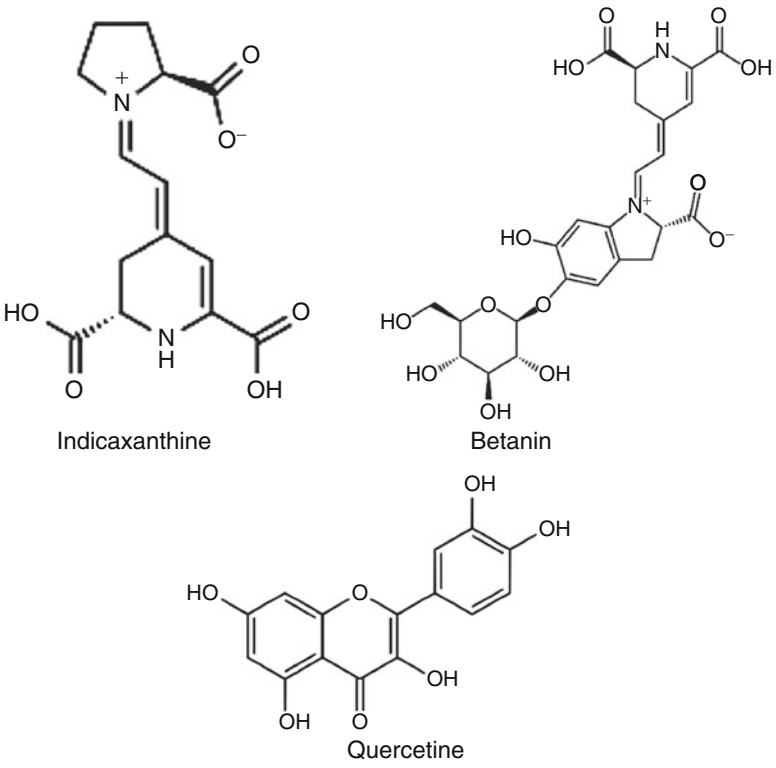

Fig. 11.5 Structure of Indicaxanthine, Betanin and quercetin

It up-regulates gamma-glutamylcysteine synthetase (CS) and glutathione synthetase expression and acts directly on promoters (Moskaug et al. 2005; Bohn et al. 2010). In vitro it may have a marginal protecting effect during sperm processing. Betalains are aromatic indole derivatives (yellow or red); they are antioxidant, lipoperoxyl radical scavengers (Tesoriere et al. 2004). They might have positive health effects in humans, slowing anarchistic cell division, and promote apoptosis of certain cancerous cells in vitro (Sreekanth et al. 2007). Betanine and indicaxanthine (Fig. 11.5) are the best known betalains. The most interesting fruit containing quercetine and betalains is *Opuntia* (prickly pear). We have used these observations in treating our patients.

Defective condensation (HDS > 25 %)

This study was performed in two private IVF units: 39 male patients with a severe history of infertility and numerous failed ART attempts were involved in our program. All of them had a sperm decondensation index (SDI), measured by aniline blue

Table 11.1 Effect of treatments with vitamins of group B, quercetine and betalains (Condensyl) on HDS treatment of 4 months (Wilcoxon test for paired samples)

	Decondensation (m, SD)		
	Before		After
All over patients (34)	36.4 (10.4)	$p<0.002$	23.6 (14.6)
Patients reacting positively (29)	34.4 (10.3)	$p<0.002$	23 (10.5)

staining, over the critical threshold of 25 %. The male patients took, orally, one pill containing all the B group vitamins (B2:1.4mG, B3: 16mG, B6: 2mG, B9: 200μG, B12:1 μG) and Zn: 15 mG (*under a chelated form of Zn bisglycinate*), which are involved in the recycling of homocysteine (Condensyl, Nurilia, France), for at least 4 months. Then a control of SDI was performed. Four patients did not make the post-treatment control. Two of them started a spontaneous pregnancy after the end of treatment, before the control. Of the 34 remaining patients, 5 showed no improvement. Overall (including the five stable patients), a highly significant effect of the treatment was observed (Table 11.1). To be precise, for three patients (not included in the study), a period of 6 months or more was necessary for a complete recovery of normal condensation under the critical threshold of 20 %.

Isolated Suboptimal DNA Fragmentation (25 %<DFI<35 %)

Our program has enrolled more than 100 patients in 4 different IVF units, patients with an infertility of 3 years or more with at least two failed ART procedures. The complement (Procrelia) contains all the ingredients of Condensyl plus citrus flavonids, green tea polyphenols and dunalliela carotenoids (Procrelia). The results, in terms of pregnancy, are not yet available (Menezo et al. in press); however, we have found a 27 % decrease in DNA fragmentation, but with no effect on decondensation. Tunc and Tremellen (2009), using a rather similar treatment (folic acid+lycopene+zinc+selenium+garlic oil, Menevit) observed a 50 % decrease in DNA fragmentation, but with no improvement in sperm numeration or motility. At this level of fragmentation the question could be whether we should treat these patients. The corresponding female age, and the oocyte capacity for DNA repair, is at this point one of the keys to answering that question: the answer is yes if the 'maternal' age is over 35.

Very High isolated DNA fragmentation (DFI>35 %)

Eighty patients are enrolled in this study. All of them had an infertility lasting at least 5 years, with 2 or more IVF/ICSI/IMSI failures. In this case, Fertibiol (Nurilia France) is used; it is a strong antioxidant cocktail containing all the group B vitamins,

chelated Zn, ubiquinol (the soluble form of CoQ10), astaxanthin, and green tea extract (containing more than 30 % polyphenols) and Pycnogenol. In a first approach with ten voluntary patients, we have observed that this treatment cannot be pursued over 5 weeks. Otherwise a strong decrease in spermatogenesis is observed in 20 % of the patients (Amar et al., personnal communication). After a 5-week treatment, a decrease in fragmentation is observed (up to 200 %), but a classic decondensation of the sperm nucleus is observed (Menezo et al. 2007b). Thus, the treatment for defective condensation is mandatory, and for these patients the 5-week Fertibiol treatment was followed by 4 months of Condensyl in order to fix nucleus compaction. Seventeen patients have completed the entire treatment regime. The DFI changed from 27.4 % (SD: 13.6) to 10.8 % (SD: 4.1) and the HDS from 22.4 % (SD: 10.8) to 11.9 % (SD: 6.1). Both variations before and after treatment are highly significant: $p < 0.001$, Wilcoxon test. Four ongoing spontaneous pregnancies obtained, three other have been obtained post IVF attempts (total 7/17: 41%). One pregnancy ended in a miscarriage.

High DFI and High Sperm Nucleus Decondensation (DFI and HDS > 25 %)

The same protocol as for isolated high DFI must be followed. DNA fragmentation must be corrected first (5 weeks of strong antioxidants, Fertibiol) followed by a correction of decondensation for 4 months. Sixty patients are enrolled in this protocol; the results on pregnancy rates are not yet available.

Conclusions

The negative impact of sperm DNA damage on human fertility is no longer a matter for debate. It is obvious that ROSs are strong effectors in compromising DNA quality. Aerobic conditions are normal in life, i.e. the generation of ROSs. ROSs are formed during the intermediate steps of oxygen reduction. However, modern life has obviously increased fertility problems through pesticide-induced ROS generation: xenoestrogens, endocrine-disrupting chemicals involved in plastic technology such as polychlorinated biphenyls, bisphenol A, phthalates and alkyl phenols, di-(2-ethylhexyl) phthalate (DEHP) (Ambruosi et al. 2011). There are little data as to whether it is possible to increase DNA repair capacity in the oocyte; that is why in ART, the yield in terms of take-home baby rate per oocyte retrieved is no higher than 5 % (Patrizio and Sakkas 2009). In addition, in vitro techniques to select the best spermatozoa are still weakly controlled. High-magnification sperm selection (IMSI) has not demonstrated any advantage in terms of selecting sperm cells with the best DNA secondary and tertiary structure (Montjean et al. 2012; Tanaka et al. 2012). Selection with hyaluronic acid seems more relevant and consistent

(Parmegiani et al. 2010; Wilding et al. 2012). A combined technique of IMSI and PICSI, termed PIMSI, has also been proposed (Wilding et al. 2012).

In any case, in vivo improvement of spermatozoa before starting a pregnancy or before ART is of paramount importance. Ingestion of vitamin and oligo-element cocktails is no longer reasonable. If we consider the Hippocratic "primum non nocere" precept, they are highly questionable. The real decays in sperm nuclei must be correctly defined, i.e. either DNA fragmentation (oxidation) or tertiary structure anomalies (nuclear condensation). It is obvious, however, that this type of treatment may be marginally useful for a part of the infertile population. Analyses of serum and, to a lesser extent, of seminal plasma are usually poor and give little indication of the real testicular situation. In infertility, the relationship between homocysteine recycling and OS seems particularly relevant: homocysteine seems to be the epicenter of both male and female problems, even if they are not completely understood. This is the case for the positive effect of homocysteine recycling, *via* methyl donor supply, on sperm nuclear condensation/maturity, which is probably more multifactorial than currently defined (Junca et al. 2012); methylation and condensation seem to be very tightly linked and interacting. Considering the B group vitamins, it is surprising that folic acid alone is sometimes given as nutraceutical complement. Looking at the methionine cycle (Fig. 11.4) it is obvious that at least B2, B6 and B12 are also strong partners in this process. A strict equilibrium must be maintained when reducing OS to avoid nuclear decondensation. In conclusion, a complete evaluation of sperm nucleus quality should be mandatory following IVF/ICSI failure(s).

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Erratum to

Structure of Chromatin in Spermatozoa

Lars Björndahl and Ulrik Kvist

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The publisher regrets the following information was mistakenly interpreted on legend for Figure 1.1 during the production process. The correct legend is as follows:

Fig. 1.1 Schematic view of DNA double helix in sperm chromatin and its relation to protamine compounds, indicating possible role of zinc to connect protamines: one zinc per protamine molecule for every ten base pairs (turn of the helix) of the DNA. If negative charges of phosphate groups in DNA are neutralized by protamines, tight packing of sperm DNA chromatin fibers is possible. With permission from Björndahl and Kvist (2010)

The online version of the original chapter can be found at
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