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Elisabetta Baldi
Monica Muratori *Editors*

Genetic Damage in Human Spermatozoa

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This book is dedicated to Prof. Gianni Forti, who guided our activities with great sapience.

Preface

The goal of the male gamete 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. Genetic abnormalities in spermatozoa can be generated in any phase of the sperm production and life and may be due to endogenous and exogenous conditions, the latter including in vitro manipulation for assisted reproduction and gonadotoxic therapies. In addition, emerging studies point out the importance of the damage to the sperm epigenome and address the mechanisms involved in generating it. All these abnormalities may have profound consequences for male fertility status and even for the health of the progeny. This book presents an updated overview of the various types of damage that may affect sperm chromatin. Besides the main mechanisms involved in the generation of de novo mutations and DNA strand breaks and oxidation, two chapters of the book are dedicated to sperm epigenome and epigenetic damage and their consequences for the progeny. In addition, as one of the most important issues regards the possible medical interventions to reduce or prevent sperm DNA fragmentation, one chapter faces the important aspect of pharmacological and surgical treatments, lifestyle modifications, and prevention against exposure to environmental and occupational toxicants.

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 the researchers in the topics of reproduction and serve as a reference for medical and technical staff working in assisted reproduction laboratories.

Florence, Italy

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Genetic Factors Affecting Sperm Chromatin Structure

1

Mélina Blanco and Julie Cocquet

Abstract

Spermatozoa genome has unique features that make it a fascinating field of investigation: first, because, with oocyte genome, it can be transmitted generation after generation; second, because of genetic shuffling during meiosis, each spermatozoon is virtually unique in terms of genetic content, with consequences for species evolution; and finally, because its chromatin organization is very different from that of somatic cells or oocytes, as it is not based on nucleosomes but on nucleoprotamines which confer a higher order of packaging. Histone-to-protamine transition involves many actors, such as regulators of spermatid gene expression, components of the nuclear envelop, histone-modifying enzymes and readers, chaperones, histone variants, transition proteins, protamines, and certainly many more to be discovered.

In this book chapter, we will present what is currently known about sperm chromatin structure and how it is established during spermiogenesis, with the aim to list the genetic factors that regulate its organization.

Keywords

Spermatozoa · Chromatin · Protamine · Nucleosome · Histone · Gene expression · Nucleus · Spermatids · Spermiogenesis

Introduction

Spermatozoa are produced through a multi-step process called spermatogenesis, during which spermatogonial stem cells at the base of the seminiferous tubules enter the differentiation pathway to ultimately give rise to spermatozoa, released in the lumen of the testicular seminiferous tubules. Spermatogenesis can be divided into three phases: mitotic phase, meiosis, and post-meiotic phase or spermiogenesis. During mitotic phase, spermatogonial stem cells undergo mitotic divisions to maintain the spermatogonial stem cell pool; some of them differentiate into primary spermatocytes. Each primary spermatocyte undergoes DNA replication and meiotic division to produce four haploid round spermatids. Round spermatids then differentiate into elongated spermatids in a process that involves dramatic morphological changes including cytoplasm removal, acrosome biogenesis, development of flagellum for motility, accumulation of mitochondria in the midpiece, and extensive chromatin remodeling that results in nuclear condensation and transcriptional silencing (Russell et al. 1990). The

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post-meiotic differentiation of round spermatids into spermatozoa is called spermiogenesis. During this step, spermatid chromatin is extensively modified and remodeled to give rise to a chromatin organization only found in spermatozoa. Indeed, in all other cells (somatic cells, female germ cells, and male germ cells until spermatid stage), the nucleosome is the core particle of chromatin structure (Luger et al. 1997). Histone proteins H2A, H2B, H3, and H4 assemble into an octamer around which 146 base pairs of DNA are wrapped, and this nucleosome structure occurs every 200 base pairs in the eukaryotic genome (Mcghee and Felsenfeld 1980; Luger et al. 1997). In sperm chromatin, the basal unit is not the nucleosome but the nucleoprotamine, formed of smaller, more basic proteins (richer in arginine) than histones: the protamines. Sperm chromatin is organized as toroids containing ~50–100kb of DNA, leading to a chromatin structure 5–10 times more condensed than nucleosome-based chromatin (Ward and Coffey 1991; Balhorn 2007). This tight compaction is essential to allow DNA to fit into a nucleus that is seven times smaller than an interphasic somatic cell nucleus (Ward and Coffey 1991) and to protect the paternal genome from physical and chemical damages. It is also possible that a small nucleus is a hydrodynamic advantage that confers a higher speed to spermatozoa during their transit (Braun 2001).

Briefly, the process of replacement of histones by protamines requires (i) opening of the histone-based chromatin structure facilitated by histone posttranslational modifications (PTM) – in particular histone hyperacetylation – and incorporation of histone variants, (ii) binding of bromodomain proteins to acetyl residues and recruitment of chromatin-remodeling proteins and of transition proteins, (iii) formation and repair of DNA breaks, and (iv) incorporation of protamines leading to a protamine-based compact chromatin structure. At the end of this process, most histones have been replaced by protamines. A small portion of histones (~1% in mice, ~10% in humans) is retained in the spermatozoa genome and contributes to the epigenetic program of the embryo (Balhorn et al. 1977; Gatewood et al. 1990; Hammoud et al. 2009; Brykczynska et al. 2010; Erkek et al. 2013; Ihara et al. 2014; Carone et al. 2014; Samans et al. 2014; Royo et al. 2016; Yoshida et al. 2018; Yamaguchi et al. 2018). [For review, see Champroux et al. (2018).]

Studying animal models (mostly knockout mice) and patient cases, researchers and clinicians have found many genes involved in histone-to-protamine transition, and many more will certainly be discovered. Each of them is a genetic factor which could alter chromatin structure when mutated. In this review, we will present their known or predicted roles while describing the key steps leading to the transition from a histone-based chromatin to protamine-based chromatin (see also Table 1.1).

Regulation of Spermatid Gene Expression

The differentiation of round spermatids into spermatozoa involves profound morphological and functional changes and requires a very specific genetic program with thousands of genes only expressed at that time and regulated at the transcriptional and post-transcriptional levels (Steger 1999; White-Cooper and Davidson 2011; Kleene 2013). Studies of gene expression dynamic throughout spermatogenesis have shown that this program starts as early as the pachytene phase of meiosis [see, for instance, da Cruz et al. (2016) and Chen et al. (2018)].

Among the genes of which expression is activated/upregulated during spermiogenesis are those required for histone-to-protamine transition such as histone variants, chaperones, histone-modifying enzymes, transition proteins, and, of course, protamines themselves. Hence, transcription regulators which control the spermatid gene expression program can indirectly impact on sperm chromatin structure via deregulating key genes of this process.

This is particularly true for regulators of *Protamine 1* (*Prm1*) and *Protamine 2* (*Prm2*) gene expression: in the mouse, *Prm1* and *Prm2* are transcribed into mRNAs that can be detected

Table 1.1 List of genes of which mutations have been shown to result in abnormal sperm chromatin structure

Gene name	Protein	Molecular role in spermiogenesis	Phenotype when mutated	Evidence of role in sperm chromatin structure	References
Genes encoding chromatin proteins					
Prm1/2	*Protamine 1/ 2*	DNA compaction in male germ cells	*Prm1*$^{+/-}$ and *Prm2*$^{+/-}$ chimeric male mice are infertile with abnormal chromatin compaction and sperm DNA damage Another study found that *Prm2*$^{+/-}$ males are fertile and that *Prm2*$^{-/-}$ males are infertile with chromatin compaction defect	Acridin orange assay on *Prm1*$^{+/-}$ and *Prm2*$^{+/-}$ chimeric mice and Comet assay on *Prm2*$^{+/-}$ chimeric mice sperm Electron microscopy on sperm from *Prm2*$^{+/-}$ chimeric mice and *Prm2*$^{-/-}$ mice	Cho et al. (2001, 2003); Schneider et al. (2016)
Tnp1/2	*Transition protein 1/2*	Intermediates in histone-to-protamine transition	*Tnp1*$^{-/-}$ and *Tnp2*$^{-/-}$ *mice* are hypofertile but present chromatin compaction defect and high level of unprocessed PRM2-precursor in sperm *Tnp1*$^{-/-}$ *Tnp2*$^{-/-}$ double knockout mice are infertile with chromatin compaction defect and unprocessed PRM2 precursor protein	Electron microscopy and western blot on spermatids at different stages	Yu et al. (2000); Zhao et al. (2001); Zhao et al. (2004)
H1fnt (H1t2)	*Testis-specific histone H1*	Testis specific Histone H1	Knockout male mice are infertile with sperm chromatin compaction defect, nuclear abnormalities in spermatids, and low protamine level in sperm	Quantification of propidium iodide in sperm DNA by FACS, western blot on sperm and electron microscopy on elongated spermatids	Tanaka et al. (2005); Martianov et al. (2005)
Th2a (Hist1h2aa) and Th2b (Hist1h2ba)	*TH2A (histone cluster 1 H2A family member a, Hist1h2aa) and TH2B (histone H2B type 1-A, Hist1h2ba)*	Testis specific Histone 2 variants	In *Th2b*$^{-/-}$ mouse, fertility is not altered. The absence of TH2B is compensated by the overexpression of H2B in testes However, transgenic mice, in which TH2B is fused to a C-terminal tag, are infertile, and elongating spermatids fail to differentiate and to compact their chromatin. TH2B is incorporated into chromatin but is not replaced by transition proteins or protamines in elongating spermatids In *Th2a*$^{-/-}$ *Th2b*$^{-/-}$ double mutant mice, TNPs and PRMs also fail to incorporate into chromatin, and H2B is overexpressed	Electron microscopy on sperm, MNase digestion in condensed spermatids, and immunostaining of spermatids at different stages Histone liquid chromatography and mass spectrometry	Montellier et al. (2013); Shinagawa et al. (2015)

(continued)

Table 1.1 (continued)

Gene name	Protein	Molecular role in spermiogenesis	Phenotype when mutated	Evidence of role in sperm chromatin structure	References
H3f3a, H3f3b	*Histone H3.3*	Histone H3 variant	Knockout male mice are infertile with abnormal sperm head shape, increase in H3K9me3, and decrease in H3K4me3 at *Prm1/2/3*, and *Tnp1* promoters are associated with decreased expression	Phase-contrast microscopy on sperm, ChIP-seq, and RT-qPCR on testes	Yuen et al. (2014)
H2al2 (H2al2a)	*H2A.L.2 (histone H2A-Bbd type 1)*	Spermatid specific histone variant	Knockout male mice are infertile with chromatin compaction defect and unprocessed PRM2 protein. Transition proteins are not bound to chromatin	Electron microscopy on sperm, immunoprecipitation, and immunostaining on condensing spermatids	Barral et al. (2017)
Regulators of gene expression: Direct or indirect effect on the expression of genes encoding chromatin proteins					
Act (Fhl5)	*Activator of cAMP-responsive element modulator in testis*	Activator of Crem, a major regulator of spermatid gene expression	Knockout male mice are fertile but with low sperm count, abnormal head shape, and chromatin compaction defect in sperm	Electron microscopy on *Act*-null spermatozoa	Kotaja et al. (2004)
Brdt	*Bromodomain testis-specific protein*	Driver of testis-specific gene expression program, histone acetylation reader	Knockout male mice are infertile with altered sperm morphology, failure of spermatids to elongate, and chromatin compaction defect. At the beginning of meiosis, BRDT regulates the expression of hundreds of meiotic and post-meiotic genes. After meiosis, during spermatid elongation, BRDT's first bromodomain is involved in the recognition of histone hyperacetylation prior to histone removal	Electron microscopy on elongating spermatids and epididymal spermatozoa, microarray-based gene expression profiling, and RT-qPCR on (juvenile and adult) whole testis RNA, immunostaining on whole testis sections, and elongating spermatids	Pivot-Pajot et al. (2003); Shang et al. (2007); Gaucher et al. (2012)
Brwd1	*Bromodomain and WD repeat containing protein 1*	Regulator of gene expression	Knockout male mice are infertile, with chromatin compaction defect and decreased expression of *Prm1*, *Tnp1*, and *Tnp2* in testes	Electron microscopy on spermatids, microarray-based gene expression profiling, and RT-qPCR on whole testis RNA	Philipps et al. (2008); Pattabiraman et al. (2015)
Chd5	*Chromodomain-helicase-DNA-binding protein 5*	Helicase	*Chd5* deficient mice are subfertile or sterile with chromatin compaction defect and spermiogenesis impairment in elongating spermatids. In spermatids, *Prm1* is upregulated and CHD5 appears to be enriched at its promoter. In late spermatids, histones are retained, and the level of non-processed PRM2 precursor is higher	Electron microscopy on sperm and sperm chromatin structure assay, western blots on spermatids, immunostaining on testis section, ChIP-qPCR and RT-qPCR on round spermatids	Li et al. (2014); Zhuang et al. (2014)

Cnr1	*Cannabinoid receptor 1*	Guanine nucleotide-binding protein-coupled receptor	Knockout male mice are fertile but their spermatozoa present abnormal chromatin compaction and higher rate of DNA damage and of retained histones. *Tnp2* expression is downregulated in testis	Acridine orange assay, Comet assay, Aniline blue staining, western blot detection of TNPs and PRMs on whole testis extract. Densitometry analysis of *Tnp2* cDNA	Chioccarelli et al. (2010)
Ctcf	*Transcriptional repressor CTCF*	Architectural protein. Regulates the 3D structure of chromatin	Male germ cell-specific knockout of *Ctcf* in mice leads to infertility with low sperm count, seminiferous tubule atrophy, and defects in sperm head formation and chromatin compaction. *H1fnt* expression is downregulated. KO sperm present reduced PRM1 level and defective histone retention	Electron microscopy on elongated spermatids, microarray on whole testis RNA, and western blot detection of PRM1 and PRM2 in spermatozoa	Hernandez-Hernandez et al. (2016)
Epc1	*Enhancer of polycomb homolog 1*	Component of the NuA4 histone acetyltransferase (HAT) complex	Knockout male mice are infertile with spermiogenesis arrest. At the molecular level, histone acetylation is lower, ubiquitination of H2A and H2B is increased, and *Tnp1*, *Tnp2*, *Prm1*, and *Prm2* are upregulated	Immunofluorescence on testicular section, western blot, and RNA-seq on round spermatids	Dong et al. (2017)
Kdm3a (Jhdm2a)	*Lysine demethylase 3A*	H3K9 demethylase. Binds *Tnp1* and *Prm1* promoter	Knockout male mice are infertile with low sperm count and chromatin compaction defects. Decreased KDM3A at the promoter of *Prm1* and *Tnp1* in round spermatids is linked with a decreased expression of these genes	Electron microscopy on spermatids, ChIP qPCR and RT-qPCR on round spermatids	Okada et al. (2007)
Pygo2	*Pygopus 2*	Unclear (belongs to a family of a co-activators of β-Catenin/Wnt signaling pathway)	Mice with mutations in *Pygo2* are sterile with defective elongation process and clear reduction of *Prm1*, *Prm2*, *Tnp2*, and *H1fnt* expression. In elongated spermatids, histone H3 acetylation is reduced, and a higher proportion of histones is retained indicative of defective histone-to-protamine transition. In testis, Pygo2 protein co-immunoprecipitates with a histone acetyl transferase (HAT) activity. The phenotype induced by *Pygo2* mutations appears to be independent of the β-Catenin/Wnt signaling pathway	Immunohistochemistry on testis section, RT-qPCR, and western blot on round spermatids	Nair et al. (2008)

(continued)

Table 1.1 (continued)

Gene name	Protein	Molecular role in spermiogenesis	Phenotype when mutated	Evidence of role in sperm chromatin structure	References
Setd2	*Histone-lysine N-methyltransferase SETD2*	H3K36 methyl transferase	*Setd2* conditional knockout in murine male germ cells leads to infertility with a spermiogenic arrest at step 8 round spermatids and decreased expression of *Tnp1/2* and *Prm1/2/3*	RNA-seq and RT-qPCR on round spermatids	Zuo et al. (2018)
Sly	*Sycp3 like Y-linked*	Regulator of gene expression	Knock-down male mice are hypofertile with reduced histone H3K79 dimethylation and H4 acetylation in elongating spermatids, histone retention in spermatozoa, and abnormal chromatin compaction. *Sly* KD leads to the deregulation of many genes including downregulation of the H3K79 histone methyl transferase *Dot1l* and upregulation of X- and Y-encoded *H2al* genes	Immunostaining on testis section, RT-qPCR, ChIP-qPCR, and western blot on spermatids, western blot on spermatozoa, comet assay, CMA3 staining, and electron microscopy on sperm	Riel et al. (2013); Moretti et al. (2017); El Kennani et al. (2018)
Sox30	*Transcription factor SOX-30*	Transcription factor binding *Tnp1* promoter	Knockout male mice are infertile with a spermiogenic arrest at the early round spermatid stage and reduced expression of *H1fnt*, *Hils1*, *H2afb1*, *H2al1n*, *H2al3*, *Tnp2*, and *Prm1/2/3* in spermatids	ChIP-seq and RNA-seq on spermatogonia, spermatocytes, and round spermatids	Bai et al. (2019); Zhang et al. (2018)
Taf7l	*TATA-binding protein associated factor 7l*	Activation complex of RNApol II. Binds *Prm1* promoter	Oligozoospermia and post-meiotic spermatogenesis arrest in knocked-out mice. Mutations of *Taf7l* have been found in oligozoospermic patients	Taf7l and Pol II ChIP-qPCR on adult testes	Cheng et al. (2007); Akinloye et al. (2007); Sediva et al. (2007); Zhou et al. (2013)
Direct or indirect regulators of chromatin proteins at the translational/posttranslational level					
Camk4	*Calcium/calmodulin-dependent protein kinase type IV*	Serine threonine kinase, phosphorylates PRM2	Knockout male mice are infertile with spermiogenesis defect in elongating spermatids, retention of TNP2, and absence of PRM2 in elongating spermatids	Immunostaining on testis section and western blot on testes protein extract	Wu et al. (2000)

Cdyl	*Chromodomain Y-like protein*	Crotonyl-CoA hydratase (i.e., negatively regulates histone crotonylation)	Overexpression of *Cdyl* in transgenic mice decreases male fertility, with lower sperm count and motility. In elongating spermatids, Kcr level (in particular H2BK12cr) is lower, and the levels of chromatin-bound TNP1 and PRM2 are decreased	Immunostaining on testis section, western blot on testes, and ChIP-qPCR on spermatocytes and round spermatids	Liu et al. (2017)
Dcr1	*Dicer*	Endoribonuclease acting in short dsRNA-mediated post-transcriptional gene silencing	Male germ cell-specific knockout of *Dcr1* in mice leads to infertility with low sperm count and disruption of spermatid elongation. KO spermatids have reduced H3 acetylation and PRM1 levels	Optic microscopy and electron microscopy of elongating spermatids, immunostaining of H3 acetylated and PRM1 on testis section	Korhonen et al. (2011)
Kat5 (Tip60)	*Histone acetyltransferase KAT5*	Catalytic subunit of the NuA4 histone acetyltransferase complex	*Kat5* conditional mouse knockout (induced at postnatal day 15) produces degenerative tubules characterized by loss of spermatocytes and spermatids. Acetylated histone H4 and TNP2 levels appear decreased	Immunofluorescence on testicular section, western blot on germ cells	Dong et al. (2017)
Nut	*Nuclear protein in testis*	Regulator of histone acetylation via recruitment of P300 and CREBBP	Knockout male mice are infertile with spermiogenesis arrest at condensing spermatid stage, histone-to-protamine transition failure, and decrease in H4 and H2A acetylation	Immunostaining in spermatids, high-performance liquid chromatography-tandem mass spectrometry on round and elongating spermatids	Shiota et al. (2018)
Parg	*Poly(ADP-ribose) glycohydrolase*	Degradation of poly(ADP-ribose)	*Parg* deficient mouse sperm presents chromatin compaction defect and H1T and TH2B retention	CMA3 immunostaining on sperm, western blot detection of histone marks, variants, and transition proteins in spermatozoa	Meyer-Ficca et al. (2011a)
***Piwil1 (Hiwi* in humans and *Miwi* in mice)**	*Piwi-like protein 1*	Endoribonuclease repressing the mobilization of transposable element	In humans, R218A/L221A mutation in *Hiwi* is associated with azoospermia Male mice with the same mutation are infertile but produce spermatozoa with abnormal motility, chromatin compaction defect, and increased nucleosome retention	Electron microscopy on sperm, immunostaining of histone on sperm, western blot of PRM1/2, and TPNP1 on spermatozoa	Gou et al. (2017)
Psme4 (Pa200)	*Proteasome activator complex subunit 4*	Proteasome activator acetylation dependent	Knockout male mice are hypofertile with lower sperm count. Core histones are not degraded in KO elongating spermatids, and their disappearance is delayed	Immunostaining on testis section and western blot on testis extracts	Qian et al. (2013); Khor et al. (2006)

(continued)

Table 1.1 (continued)

Gene name	Protein	Molecular role in spermiogenesis	Phenotype when mutated	Evidence of role in sperm chromatin structure	References
Rfx2	*DNA-binding protein RFX2*	Key transcription factor of spermatogenesis	Knockout male mice are infertile with spermatid elongation failure and TNP2 accumulation in round spermatid nucleus. Spermiogenesis arrests at step 7 in round spermatids	Immunohistochemistry on testis section	Kistler et al. (2015)
Rnf8	*E3 ubiquitin protein ligase RNF8*	H2A and H2B ubiquitination, recruitment of KAT8 acetyltransferase to chromatin	Knockout male mice are sterile with defective histone removal and protamine insertion, resulting in less condensed sperm chromatin Another group generated a similar KO model but did not observe chromatin compaction defects	Immunostaining and electron microscopy on sperm, western blot (TNPs and PRMs) on testicular protein extracts	Lu et al. (2010); Sin et al. (2012)
Sirt1	*NAD-dependent protein deacetylase sirtuin-1*	NAD-dependent protein deacetylase	Male germ cell-specific knockout of *Sirt1* in mice leads to male hypofertility with spermatogenesis failure at round spermatid stage and sperm chromatin condensation defect. Globally (in whole testes) levels of histone posttranslational modifications are altered (H4ac, H3K4me, H2BK16ac, and H2BK120ac levels are decreased and H2BK120ub is increased). A higher proportion of histones is retained in KO spermatozoa By immunostaining, TNP2 and PRM1 appear to be perinuclear rather than nuclear in *Sirt1*-KO elongated spermatids	Electron microscopy on elongating spermatids, western blot on testis extract for H4 acetylation, H3 acetylation and methylation, and H2B acetylation and ubiquitination. Western blot detection of TH2B on spermatozoa. Immunofluorescence on squash slides of stages X to XII tubules	Bell et al. (2014)
Tarbp2	*RISC-loading complex subunit TARBP2*	Required for translational activation of protamine mRNAs	Knockout male mice are infertile with severe oligozoospermia, and elongated spermatids present a mosaic pattern for *Prm1* expression	Gene reporter and immunostaining of testicular section	Lee et al. (1996); Zhong et al. (1999)
Tssk6	*Testis-specific serine/ threonine-protein kinase 6*	Serine threonine kinase	Knockout male mice are infertile with high level of PRM2 precursor and retained histones in sperm. H2AX phosphorylation in elongating spermatids is impaired	Immunostaining on testis section and western blot detection of γH2AX and of histones in spermatids and spermatozoa, respectively	Jha et al. (2017)

Regulators of the nuclear structure					
Dpy19l2	*Probable C-mannosyltransferase DPY19L2*	Nuclear envelope component	Mutations in humans lead to globozoospermia In *Dpy19l2* knockout mice, spermatid elongation is defective with chromatin compaction defect due to defective protamination and histone retention	Immunostaining on testis section and spermatozoa, acidic aniline blue, and CMA3 staining on spermatozoa	Yassine et al. (2015)
Gmcl1 (Mgcl-1)	*Germ cell-less protein-like 1*	Nuclear matrix component	Knockout male mice are infertile with multiple heads and flagella spermatozoa, spermatid chromatin compaction defect, disruption of nuclear structure of elongating spermatids, and accumulation of immature PRM2 in sperm	Electron microscopy on testis section. Western blot detection of sperm proteins	Kimura et al. (2003)
Lis1 (Pafah1b1)	*Platelet-activating factor acetylhydrolase IB subunit alpha*	Non-catalytic component of platelet-activating factor acetylhydrolase 1b (PAF-AH 1B). Associates with microtubules	Disruption of the testis-specific *Lis1* transcript in mice leads to male infertility caused by a spermiogenesis block. Spermatids present multiple defects: acrosome and flagellum malformations, defective nuclear condensation, and abnormal TNP2 location	Immunostaining and electron microscopy on testis section	Nayernia et al. (2003)
Others					
Aurkc	*Aurora kinase C*	Serine threonine kinase	Mutations in humans induce polyploid spermatozoa. Knockout male mice are fertile but present sperm chromatin compaction defect	Electron microscopy on sperm	Kimmins et al. (2007); Dieterich et al. (2007)

NB. Genes have been grouped according to their expected molecular role, but it is sometimes difficult to discriminate between an effect on regulation of gene expression and on regulation of chromatin structure

in round spermatids and stored as nonpolysomal, ribonucleoproteins in the cytoplasm until translation 3–7 days later (Hecht 1990; Kleene 1989). Among the key regulators of *Protamine* transcription and translation are TATA box proteins (TBPs), CREM transcription factor, Y box proteins (such as MSY2), and TARBP2 (aka PRBP in mice and TRBP in humans) [see Carrell et al. (2007) for review and also below]. For instance, mice lacking *Tarbp2* gene fail to translate *Protamine* mRNA which results in delayed replacement of transition proteins, oligozoospermia, and male infertility (Lee et al. 1996; Zhong et al. 1999) (see Table 1.1).

Taf7l is a component of a protein complex required for transcription of genes by RNA polymerase II in spermatids, such as *Prm1/2* (Zhou et al. 2013). Its knockout leads to decreased sperm count, reduced sperm motility, and hypofertility/sterility (Cheng et al. 2007; Zhou et al. 2013). Its impact on chromatin sperm structure has not been described, but chromatin immunoprecipitation experiments (ChIP-Seq) showed that TAF7L directly binds to the promoter of *Prm1* gene (Zhou et al. 2013) and as such could influence its expression and thus sperm chromatin structure. Mutations in *Taf7l* gene have been found in patients with oligozoospermia (Akinloye et al. 2007; Sediva et al. 2007) (see Table 1.1).

It is worth noting that, in cases of regulators of spermatid gene expression, mutations can induce a plethora of spermatid differentiation defects, with sometimes a block leading to no sperm at all (azoospermia). This is the case, for instance, of TATA box-binding protein-like 1 *Tbpl1* knockout (aka *Trf2*) which leads to a spermiogenic block at stage 7 (Zhang et al. 2001). *Papolb* (aka *Tpap*) encodes a testis-specific enzyme responsible for poly(A) tail extension of specific mRNAs in round spermatids (i.e., a poly(A) polymerase) and thus involved in post-transcriptional regulation of mRNAs. Its knockout also induces a stage 7 block during spermiogenesis (Kashiwabara et al. 2002). *Crem* encodes a master regulator of spermiogenesis as it controls the expression of many spermatid genes including *Tnp1 (Transition protein 1)*, *Tnp2 (Transition protein 2)*, *Prm1*, and *Prm2*. In its absence, spermatids fail to fully differentiate, and no spermatozoa are produced (Blendy et al. 1996; Nantel et al. 1996). Male mice lacking *Crem* activator, *Act* (aka *Fhl5*), produce spermatozoa in reduced number, with abnormal flagellum and heads yet are fertile (Kotaja et al. 2004). KIF17B is a regulator of CREM-ACT activity (Macho et al. 2002) and as such can influence expression of genes coding for protamines and other proteins essential for sperm structure (see Table 1.1). It is worth noting that KIF17B has been shown to interact with MIWI, an important regulator of sperm chromatin structure (Wang et al. 2015).

The arginine methyltransferase CARM1 has recently been shown to also control gene expression in spermatids: *Carm1*-KO male germ cells present post-meiotic gene deregulation associated with multiple spermiogenesis defects leading to male hypofertility. Investigation of the underlying mechanism has revealed that CARM1 negatively controls the transcriptional activity of CREM-ACT via methylating their co-activator, the histone acetyl transferase P300 protein (Bao et al. 2018). Yet, *Carm1*-KO does not seem to affect mRNA levels of genes known to be essential for histone-to-protamine transition such as *Prm1*, *Prm2*, *Tnp1*, and *Tnp2* but could impact on sperm chromatin condensation via another pathway. The consequence of *Carm1*-KO on sperm chromatin structure has not been described.

The histone H3K36 methyltransferase SETD2 is another protein that appears to control the expression of genes essential to histone-to-protamine transition. SETD2 is highly expressed in spermatocytes and spermatids in the mouse testis and localizes to the nucleus of spermatocytes and round spermatids. *Setd2* conditional knockout male mice are sterile and have arrested spermatogenesis at step 8 round spermatids. It leads to the downregulation of *Tnp1*, *Tnp2*, *Prm1*, *Prm2*, *Prm3*, *H1fnt*, and *H2afb1* genes in round spermatids (Zuo et al. 2018) (see Table 1.1).

Kdm3a (also known as *Jhdm2a*) encodes the lysine-specific demethylase 3A, a histone demethylase that is highly expressed in post-meiotic germ cells. *Jhdm2a*-null male mice are

infertile due to failure of round spermatids to differentiate into elongated spermatids. *Jhdm2a* is required for *Tnp1* and *Prm1* transcription, and ChIP experiments showed that JHDM2A is recruited to the promoter of *Tnp1* and *Prm1* in round spermatids (Okada et al. 2007). Thus loss of *Jhdm2a* in the testis leads to decreased expression of *Tnp1* and *Prm1*, resulting in chromatin condensation defects such as indistinct chromocenter, loss of heterochromatin polarity in steps 7–9 spermatids and defective chromatin condensation in step 13 spermatids (Okada et al. 2007) (see Table 1.1).

CTCF is an architectural protein that regulates gene expression via the 3D organization of the genome. The specific knockout of *Ctcf* gene in male germ cells leads to spermiogenesis defects and male infertility due to abnormal histone-to-protamine transition, in particular defective protamine incorporation (Hernandez-Hernandez et al. 2016) (see Table 1.1).

There are many other examples of regulators of spermatid gene expression, at the transcriptional or post-transcriptional level, of which knockout leads to an arrest during spermiogenesis, such as *Sox30* (Bai et al. 2019; Zhang et al. 2018; Feng et al. 2017) and *Rfx2* (Kistler et al. 2015). Their absence in mice precludes the formation of spermatozoa; however, mutations in these genes that do not induce a complete loss of function (heterozygous mutations, for instance) could lead to the production of sperm with an abnormal chromatin structure.

Small RNAs and associated proteins also contribute significantly to gene regulation during spermiogenesis. The chromatoid body is a perinuclear cloud-like/granule structure that starts to be formed in late pachytene spermatocytes and is predominant in round spermatids. It is involved in post-transcriptional gene regulation and mostly composed of RNA-binding proteins and small RNA (i.e., mostly piRNA and miRNA) (Meikar et al. 2014).

Dicer1 is a critical regulator of microRNA and siRNA biogenesis (Ha and Kim 2014). Recent studies in mouse models revealed that the ubiquitously expressed *Dicer1* gene has an essential role in spermatogenesis. *Dicer1* mRNA was found to be highest in spermatogonia and spermatocytes and decreases in spermatids. Germ cell-specific *Dicer1* knockout male mice are sterile, with reduced testis size, and spermatid elongation is severely affected indicating abnormal spermiogenesis. Elongating spermatids show abnormal head shapes and disrupted chromatin condensation and organization: elongating spermatids retain hyperacetylated H3, and protamine deposition in elongating spermatids is severely reduced; expression and localization of the histone variant H1T2 (see also below) are disrupted in knockout spermatids (Korhonen et al. 2011) (see Table 1.1).

Mutations in regulators of the piRNA pathway or in genes involved in the piRNA pathway could also affect sperm chromatin structure via their effect on gene expression. Absence of MIWI (encoded by *Piwil1/Miwi* in mice) leads to an arrest of germ cell differentiation at the beginning of the round spermatid stage, and therefore no sperm are produced (Deng and Lin 2002). In infertile patients, R218A/L221A mutation in *Hiwi* (human homolog of *Piwil1*) has been found associated with azoospermia (Gou et al. 2017). Yet, knock-in male mice generated to mimic this mutation are infertile with a different phenotype: spermatozoa are produced (albeit in reduced number) and present abnormal motility and chromatin compaction (Gou et al. 2017). The underlying mechanism producing this phenotype appears to be independent of the piRNA pathway: the mutation in the D-Box element prevents MIWI protein ubiquitination and degradation and leads to sequestration of the histone ubiquitination ligase RNF8. As a consequence, in mutant sperm, H2A and H2B posttranslational modifications are impaired which leads to nucleosome stabilization and retention, defective protamine deposition, and defective chromatin compaction (Gou et al. 2017) (see Table 1.1). This study exemplifies the complexity to predict the consequence of mutations found in human patients based on the study of animal models and, therefore, to provide an exhaustive list of the genes affecting sperm chromatin structure.

Importance of the Nuclear Envelope

Nuclear and chromatin remodeling during spermiogenesis also depends on the nuclear envelope and its components, via their effect on gene expression and/or on nucleus architecture. *Gmcl1* (also known as *Mgcl-1*) encodes a protein expected to regulate gene expression in male germ cells via its association with the nuclear envelope (Nili et al. 2001). It is highly expressed from pachytene spermatocytes and localizes to nuclear lamina; its deletion results in male infertility, abnormal nuclear architecture, and abnormal chromatin condensation (Kimura et al. 2003). Reduced levels of PRM1 and PRM2 proteins were observed in *Mgcl*-1-null sperm, and immature precursor PRM2 accumulate indicating abnormal posttranslational processing of protamines. It is yet unclear whether abnormal protamine processing and chromatin condensation are direct or indirect consequences of nuclear envelope abnormality (Kimura et al. 2003) (see Table 1.1).

LIS1 (encoded by *Lis1* also known as *Pafah1b1*) associates with dynein and microtubules and has been shown to affect nuclear structure; disruption of the testis-specific *Lis1* transcript leads to abnormal spermiogenesis, with abnormal formation of acrosome and flagellum and defective nuclear condensation in spermatids. As a result, spermiogenesis is almost fully blocked with only a few sperm found in the epididymides (Nayernia et al. 2003) (see Table 1.1). LIS1 and two other subunits compose PAF acetylhydrolase 1b. Disruption of LIS1-associated proteins has been shown to reduce LIS1 protein level; PAFAH α1 and α2 could therefore have an impact on spermatid nuclear condensation though knockout studies have found earlier spermatogenesis defects, at the meiotic stage (Yan et al. 2003a; Koizumi et al. 2003).

DPY19L2 is also presumed to be involved in nuclear envelop structure. It is highly expressed in human and mouse germ cells and co-localizes with the region of the inner nuclear membrane facing the acrosome (Pierre et al. 2012). Deletion of *Dpy19l2* gene is a major cause of globozoospermia, a rare male infertility condition characterized by deformed round sperm heads without an acrosome [see Ray et al. 2017 for review]. *Dpy19l2*-null male mice are infertile and have abnormal sperm head and chromatin condensation defects due to abnormal protamine deposition (Yassine et al. 2015) (see Table 1.1).

During spermiogenesis, acrosome development is tightly linked to nuclear shaping and nucleus compaction via its anchoring to a structure formed of F-actin and keratin called the acroplaxome (Kierszenbaum et al. 2003). Moreover, acrosome development and chromatin remodeling appear to be interconnected (De Vries et al. 2012), and there have been several reports of reduced ICSI (*intracytoplasmic sperm injection*) success rates (i.e., lower fertilization, pregnancy, and live birth rates) in patients with globozoospermia which could be due to sperm DNA damage resulting from poor chromatin condensation (Davila Garza and Patrizio 2013). It is therefore worth taking a closer look at genes other than *Dpy19l2* which have been linked to globozoospermia phenotype.

Mutations in two other genes have been suggested to be involved in human globozoospermia, though with a much lower prevalence than *Dpy19l2* mutations: *Pick1* (Liu et al. 2010) and *Spata16* (Dam et al. 2007). *Pick1* knockout leads to male infertility with a phenotype similar to human globozoospermia with defective acrosome formation, reduced sperm count, and deformed sperm nuclei (Xiao et al. 2009). A mouse model mimicking *Spata16* human mutation does not have spermatogenesis defect, but the deletion of its exon 4 produces male infertility with severe spermiogenesis defects and only few spermatozoa (Fujihara et al. 2017). The studies of *Pick1* knockout and of *Spata16* knockout mice did not present a detailed characterization of the nuclear morphology and composition of the produced sperm cells. So, the involvement of those factors in sperm chromatin structure is unclear (Xiao et al. 2009; Fujihara et al. 2017).

In the mouse, several other genes have been shown to cause globozoospermia-like phenotypes when knocked out and could therefore also be involved in chromatin compaction. This is the case for *Mfsd14a* (aka *Hiat1*) which encodes the

hippocampus abundant transcript 1 protein of which function is unknown: a mouse model with a *LacZ* gene insertion that disrupts the expression of the *Mfsd14a* gene displays spermiogenesis defects characterized by failure of acrosome formation abnormal sperm head condensation (as observed by electron microscopy) and mitochondrial mislocalization (Doran et al. 2016). Other genes of which KO in mice leads to globozoospermia, such as *Csnk2a2* (Xu et al. 1999), *Atg7* (Wang et al. 2014), *Gba2* (Yildiz et al. 2006), *Golga2* (Han et al. 2017), *Gopc* (Yao et al. 2002), *Hrb* (Kang-Decker et al. 2001), *Hsp90b1* (Audouard and Christians 2011), *Zpbp1* (Lin et al. 2007), *Vps54* (Paiardi et al. 2011), or *Smap2* (Funaki et al. 2013), could also be directly or indirectly involved in sperm chromatin condensation, but data on this particular phenotype are lacking or not always conclusive.

Remodeling of Chromatin Structure During Spermatid Differentiation

As mentioned, during spermatid differentiation, the chromatin is extensively remodeled with multiple posttranslational modifications (PTM) of histone residues and incorporation of many histone variants. These changes in the spermatid chromatin structure aid in destabilizing nucleosomes and loosening chromatin in preparation for histone-to-protamine transition (Braun 2001; Sassone-Corsi 2002). Deregulation of this process such as abnormal expression of histone-modifying enzymes, histone variants, associated factors, and chaperones can lead to abnormal histone eviction and is therefore expected to affect sperm chromatin structure.

Histone Posttranslational Modifications and Enzymes

One hallmark of histone-to-protamine transition is hyperacetylation of histones, predominantly of histone H4, but also, to a lesser extent, of other nucleosomal histones in stages 9–11 mouse spermatids (Hazzouri et al. 2000). Acetylation has been shown to be essential for histone degradation and eviction though it is not the sole mechanism for histone eviction during spermatid differentiation (Marushige et al. 1976; Oliva et al. 1987; Oliva and Mezquita 1986; Sassone-Corsi 2002; Awe and Renkawitz-Pohl 2010). Several histone acetyl transferases (HAT) have been suggested to be involved in this process, such as CREB-binding protein (encoded by *Crebbp*), P300 (encoded by *Ep300*), KAT8 (encoded by *Kat8* also known as *Mof*), enhancer of polycomb homolog 1 (encoded by *Epc1*), and KAT5 (encoded by *Kat5* also known as *Tip60*) (Boussouar et al. 2014, Dong et al. 2017, Lu et al. 2010). Yet the production of conditional KO mouse models of the genes encoding these HAT did not allow to fully demonstrate their implication, either (i) because gene KO was only partial and did not show the expected phenotype (Boussouar et al. 2014) or (ii) induced a phenotype or blockade of spermatogenesis before the stage at which histones are hyperacetylated (Dong et al. 2017; Jiang et al. 2018). It is also important to add that histone-modifying enzymes and histone PTMs are involved in the regulation of gene expression and contribute to sperm chromatin structure via their impact on spermatid gene expression program (as described in the previous paragraph).

Acetylation of H3 coincides with H4 acetylation and could also participate in histone removal as suggested by the study of PYGOPUS 2. PYGOPUS 2 (encoded by *Pygo2*), a co-activator of the beta-catenin/Wnt signaling pathway, has been studied during spermatid elongation: it is expressed in steps 8–12 elongated spermatids and co-immunoprecipitates with a histone acetyl transferase activity in the testis. Reduced levels of *Pygo2* in mice lead to male infertility associated with a decrease of H1 histone variant H1FNT level and of histone H3K9/K14 acetylation in elongating spermatids but not of H4K8/K12 acetylation (see Table 1.1). Though its precise role remains unclear, PYGOPUS 2 appears to be an essential co-regulator of histone PTM in spermatids (Nair et al. 2008) and as such could impact spermatid chromatin structure.

KAT6B (encoded by *Kat6b* also known as *Myst4*) is another HAT. In bovine testes, it has only been detected in the nuclei of elongating spermatids and therefore has been suggested to contribute to histone hyperacetylation in spermatids (Mcgraw et al. 2007). Functional studies remain to be performed.

Histone lysine crotonylation (Kcr) is also observed during spermatid differentiation in steps 9–11 spermatids, coincident with histone hyperacetylation (Tan et al. 2011; Liu et al. 2017). Kcr was also found enriched at transcription start sites of genes that are post-meiotically activated (Tan et al. 2011). It has recently been found that the histone acetyltransferase P300 can both acetylate and crotonylate histone lysine H3K18 depending on the intracellular concentration of acetyl-CoA or crotonyl-CoA (Sabari et al. 2015). Similarly, P300 and CREBBP are able to catalyze the addition of propionyl and butyryl (Chen et al. 2007) (which are other types of acyl groups together with acetyl, crotonyl, 2-hydroxyisobutyryl, β-hydroxybutyryl, succinyl, malonyl, glutaryl) onto histone lysines [see Sabari et al. (2015) for review]. Goudarzi et al. have found that P300 can also butyrylate H4K5 and K8 (Goudarzi et al. 2016). Tight regulation of acylation of histone lysines during spermiogenesis is therefore expected to have a critical role in histone-to-protamine transition; this is exemplified by the fact that BRDT, which is essential to histone removal (see below), can bind to acetylated H4K5 but not to butyrylated H4K5 and that butyrylated histones at H4K5/K8 persist longer than acetylated ones (Goudarzi et al. 2016).

The dynamic of acyl-CoA availability throughout spermatogenesis and the influence it has on histone modifications remain to be studied, but enzymes involved in acyl-CoA synthesis (acyl-CoA synthetases) could have an impact on sperm chromatin structure.

Collectively, the abovementioned studies point to a role of the histone acetyltransferase P300 in modulating multiple histone modifications depending on acyl-CoA availability. It remains to be demonstrated if other lysine residues can also be acylated by the same enzyme or by others. HATs are therefore essential in the setup of spermatozoa chromatin structure. Similarly, enzymes that regulate the removal of acyl are important in the regulation of this process: it has been shown that blocking histone deacetylases (with TSA) could mimic the role of HAT in vitro (Hazzouri et al. 2000); deregulation of histone deacetylases could therefore also affect histone-to-protamine transition. Chromodomain Y-like protein (CDYL) was initially described to have HAT activity with a predominance for H4 (Lahn et al. 2002). Shortly after, another study did not recapitulate this finding (Caron et al. 2003), and it was recently confirmed that in fact CDYL is not a HAT but a crotonyl-CoA hydratase that converts crotonyl-CoA into b-hydroxybutyryl-CoA, thereby inhibiting histone crotonylation (Liu et al. 2017). To better understand the role of CDYL during spermatogenesis, and since CDYL KO is embryonically/perinatally lethal (Wan et al. 2013), Liu et al. produced a transgenic mouse model in which CDYL is overexpressed. In these mice, histone lysine crotonylation (Kcr) levels and, in particular, H2BK12cr level were found reduced. Males were hypofertile with decreased sperm count and decreased sperm motility. TNP1 and PRM2 were significantly enriched in soluble testis fraction and significantly reduced in chromatin-bound fraction indicating defects in histone-to-protamine transition (Liu et al. 2017) (see Table 1.1).

Besides acylation, other posttranslational modifications of histone residues such as ubiquitination, phosphorylation, and methylation have been observed in elongating spermatids, for instance, H3K4me2/3 (Rathke et al. 2007; Godmann et al. 2007; Song et al. 2011) and H3K79me2/3 (Dottermusch-Heidel et al. 2014a, b).

Those PTM are also expected to facilitate histone eviction. For instance, ubiquitinated histones H2A and H2B are highly expressed in elongated spermatids just prior to histone removal (Baarends et al. 1999). Mouse knockout of the ubiquitin-conjugating enzyme E2 B (encoded by *Ube2b* aka *Hr6b*) leads to male infertility, with reduced testis weight and low number of spermatozoa which mostly appeared abnormally

shaped such as immobility and abnormal head morphology (Roest et al. 1996). It was initially thought that *Ube2b* knockout leads to abnormal histone-to-protamine transition (Roest et al. 1996) via its effect on H2A ubiquitination, but in *Ube2b*-KO elongating spermatids, H2A ubiquitination is normal (Baarends et al. 1999) suggesting that other enzymes are involved in this process. Since then, UBE2B has been involved in meiotic recombination (Baarends et al. 2003) and the regulation of genes encoded by the sex chromosomes during male meiosis and beyond (Mulugeta Achame et al. 2010).

In 2010, Lu et al. have shown that, in male germ cells, the E3 ubiquitin protein ligase RNF8 is required for H2A and H2B ubiquitination which is itself required for recruitment of KAT8 acetyltransferase (also known as MOF) to chromatin and subsequent H4K16 acetylation that facilitates histone eviction. The *Rnf8* KO mice they produced were infertile showing abnormal chromatin condensation, with a failure of histone eviction/protamine deposition (Lu et al. 2010) (see Table 1.1). However, in 2012, Sin et al. produced *Rnf8*-KO mice which did not have the same phenotype, as they display a deregulation of post-meiotic XY gene expression but no defects in chromatin compaction during spermatid elongation (Sin et al. 2012).

The NAD-dependent protein deacetylase sirtuin-1 (encoded by *Sirt1*) is highly expressed in meiotic germ cells and to a lesser extent in spermatids (Bell et al. 2014). *Sirt1*-null male mice are hypofertile with abnormally shaped sperm heads presenting a higher incidence of chromatin condensation and compaction defects. SIRT1 appears to be required for histone acetylation and subsequent histone eviction and protamine deposition (Bell et al. 2014) (see Table 1.1). Interestingly, SIRT1 has both histone and non-histone deacylase activities and has been shown to regulate the activity of the acetyl-CoA synthetase (encoded by *Acss2*), a key enzyme in the production of the substrate required for histone acylation, via its ability to de-acetylate this enzyme [for review, see Sabari et al. (2017)].

Calcium/calmodulin-dependent protein kinase type IV (CaMK IV encoded by *Camk4*) is a serine/threonine kinase involved in transcriptional regulation. In testes, it is highly expressed in elongating spermatids and associated to the chromatin and nuclear matrix (Wu and Means 2000). *Camk4*-KO mice are infertile with severe spermatogenesis defects (dramatic reduction of testis weight and sperm count) suggesting problems occurring before spermatid differentiation. CaMK IV has been suggested to be important for histone-to-protamine transition because *Camk4*-KO spermatids retain TNP2 longer and have lower level of PRM2 (see Table 1.1). Interestingly CaMK IV has been shown to phosphorylate PRM2 in vitro (Wu et al. 2000).

In mice, downregulation of the H3K79 methyl transferase DOT1L and of H3K79me2 level in spermatids is associated with hypofertility, chromatin condensation defects, and a higher proportion of retained histones than in wild-type mouse spermatozoa (Moretti et al. 2017). This has been observed in mice knocked down for *Sly*, a multicopy gene located on the mouse Y chromosome long arm (MSYq), which controls the expression of many spermatid-expressed genes (Moretti et al. 2017) (see Table 1.1). The sole impact of *Dot1l* knockout remains to be determined, as *Sly* knock down leads to the deregulation of many genes in addition to *Dot1l*, including X- and Y-encoded *H2al* genes (El Kennani et al. 2018) which could contribute to the chromatin remodeling defects of this mouse model (see below). It is worth adding that *Sly* is not conserved in humans, but its partners and target genes are and could therefore influence expression of genes involved in sperm chromatin organization in humans.

Histone Variants and Transition Proteins

In addition to multiple changes of histone PTM, the process of histone-to-protamine transition involves expression and incorporation of many histone variants, many of which are testis-specific or testis-enriched. Their incorporation into the chromatin is expected to result in weaker interaction with DNA, destabilization of the

nucleosomal structure, and finally histone displacement (Govin et al. 2006). Each nucleosome contains one molecule of the linker histone H1 that binds DNA in the nucleosome and linker DNA between nucleosomes (Wolffe 1997); several testis-specific H1 variants exist. The spermatid-specific linker histone H1-like protein (HILS) is specifically expressed in elongating spermatids and has been hypothesized to be involved in chromatin condensation at this stage (Yan et al. 2003b), yet a recent study demonstrated it is a poor condenser of chromatin (Mishra et al. 2018). ChIP-Seq experiments on elongating/condensing spermatids revealed HILS1 is preferentially located in the regions encoding LINE elements and specific histone PTM such as H3K9me3, H4K20me3, and H4K5ac (Mishra et al. 2018). Its molecular effect during spermiogenesis remains to be identified.

H1FNT (H1 histone family member N, testis-specific, encoded by *H1fnt* aka *H1t2*) is another histone variant highly expressed in spermatids. Immunohistochemical examination of histone H1FNT localization showed that it is expressed in steps 5–13 mouse spermatids (Tanaka et al. 2005) and appears as cap-like structure at the inner periphery of the nuclear membrane (Martianov et al. 2005). *H1fnt*-null male mice are infertile, and *H1fnt*-null sperm have abnormal sperm heads and abnormally condensed chromatin due to defective deposition of protamines (Martianov et al. 2005; Tanaka et al. 2005) (see Table 1.1).

Several spermatid-specific histone variants appear to be dispensable for this process as their knockout does not lead to any defects in spermatid differentiation nor sperm abnormalities. For instance, H1T (encoded by *Hist1h1t*) is a testis-specific variant of histone linker H1 only found in spermatocytes and round spermatids, but the knockout of its gene does not have any phenotype (Lin et al. 2000).

TH2B is a histone variant that replaces most of canonical H2B in male germ cells from the spermatocyte stage (Montellier et al. 2013). Surprisingly, sperm production and male fertility are normal in its absence (in *Th2b*-null mice), but spermatid chromatin structure is changed with a higher expression of H2B and changes in the level of multiple histone PTMs (including of H2B and of other nucleosomal histones such as H4) compared to WT (Montellier et al. 2013). Transgenic male mice expressing TH2B fused to a His, Flag, and HA C-terminal tag are however infertile due to a block at elongating/condensing spermatid stage. In this model, tagged TH2B appears to be assembled into nucleosomes of spermatocytes and round spermatids, normally. But later, in elongating spermatids, histone eviction and replacement by transition proteins are abnormal leading to defective chromatin compaction (Montellier et al. 2013). In another study that investigated the function of both TH2A and TH2B, double knockout male mice were found to be sterile, and their spermatids displayed abnormal nuclear morphology and chromatin structure despite overexpression of canonical H2B (Shinagawa et al. 2015) (see Table 1.1). Collectively, these data show that TH2A and TH2B are required for proper histone-to-protamine transition and that compensation mechanisms are at work and can induce a chromatin re-organization without impacting on protamine deposition and male fertility. One cannot exclude that those compensations could differ among species and that the deletion of one gene could have an impact in one species but no effect in another.

H3.3 histone variant appears to play multiple roles during spermatogenesis. H3.3 protein is encoded by two genes, *H3f3a* and *H3f3b*, (Yuen et al. 2014) and is incorporated into the chromatin of mouse sex chromosomes during meiosis when sex chromosomes are transcriptionally inactive (Van Der Heijden et al. 2007). It has been shown that a small proportion of H3.3 is retained in sperm chromatin and correlates with genes important for development of the early embryo (Erkek et al. 2013). In mice *H3f3a* KO leads to male infertility with reduced number of germ cells and abnormal sperm heads indicating defects in chromatin condensation (Yuen et al. 2014) (see Table 1.1). Another study reported that *H3f3b* KO mice died shortly after birth but that heterozygous mice were viable with male infertility characterized by spermatogenesis

arrest at the spermatid stage; in the same study, *H3f3a* KO was found to produce abnormally shaped spermatozoa and reduced male fertility (Tang et al. 2013). The exact molecular role of H3.3 during spermiogenesis remains unclear.

Transition proteins (TNPs) are the protein intermediates which are transiently incorporated in the spermatid chromatin (in steps 12–13 mouse spermatids) after histones have been removed. The biochemical properties of TNPs and protamines make them suitable for condensing DNA in the sperm nucleus. TNPs are rich in arginine, lysine, and serine (Dadoune 2003; Brewer et al. 2002). TNP1 and TNP2 are capable of condensing DNA at a rate similar to those of protamines, while dissociation rates from DNA for TNP1 and TNP2 are faster than those of protamines (Dadoune 2003, Brewer et al. 2002). Thus, TNPs can destabilize nucleosomes and facilitate histone eviction. They are then rapidly replaced by protamines. TNP1 and TNP2 are essential for male fertility, and the deletion of one TNP is partially compensated by overexpression of the other remaining TNPs. *Tnp1*- or *Tnp2*-null male mice are fertile though with reduced litter sizes, and spermatozoa present with abnormal chromatin condensation (Yu et al. 2000; Zhao et al. 2001). Double knockout leads to male infertility with abnormal sperm head morphology, abnormal nuclear shape and uncondensed nucleus, and abnormal chromatin condensation (Zhao et al. 2004) (see Table 1.1), yet, in the absence of TNPs, histones are removed and protamines deposited. Protamine 2 remains however as an uncleaved precursor, and overall protamine-mediated genome compaction is not normal since spermatozoa are unable to fertilize oocytes by ICSI (Zhao et al. 2004).

Like histones, TNP1 and TNP2 have been shown to carry posttranslational modifications, such as lysine methylation, arginine methylation, lysine acetylation, and serine phosphorylation. The histone-arginine methyltransferase CARM1 (encoded by *Carm1* also known as *Prmt4*) and the histone lysine N-methyltransferase SETD7 appear to be responsible for methylation of transition proteins on arginine or lysine residues, respectively (Gupta et al. 2015). Phosphorylation has been shown to be mediated by PKA and PKC kinases and to affect TNP properties (Levesque et al. 1998; Meetei et al. 2002; Ullas and Rao 2003). Acetylation of TNP2 by the P300 has been shown to reduce its DNA condensation ability (Pradeepa et al. 2009). Abnormal posttranslational modification(s) of TNPs could impair histone-to-protamine transition and thus sperm chromatin structure.

H2A.L.2 is a H2A variant specifically expressed in spermatids that is critical for sperm chromatin structure, as it is required for TNP incorporation into the chromatin during histone-to-protamine transition. And indeed, H2A.L.2-null male mice have a phenotype very similar to that of TNPs double knockout males: they are sterile and H2A.L.2-null sperm are unable to fertilize oocytes in vitro. Besides, H2A.L.2-null sperm contain unprocessed PRM2 protein, and, despite incorporation of protamines, genome compaction is defective in these mice (Barral et al. 2017) (see Table 1.1). H2A.L.2 variant was shown to be incorporated, together with TH2B, onto nucleosomes by Nap1L4 (nucleosome assembly protein 1-like 4) in elongating spermatids, which leads to a more open chromatin. H2A.L.2 is critical for TNP incorporation and then efficient protamine-mediated genome compaction (Barral et al. 2017).

Collectively, these data show that TNP's molecular role is not to displace histones, as previously thought, but rather to recruit and process PRM, which in turn displaces histones and compacts the paternal genome.

Chaperones and Readers

A recent study has identified the testis-specific NUT protein as an essential regulator of histone acetylation as it recruits P300 and/or CREB-binding protein to enhance histone acetylation. In its absence, histone H4 (in particular at K5 and K8) and H2A acetylation levels are dramatically decreased, and spermatid elongation is arrested (Shiota et al. 2018) (see Table 1.1).

Acetylated/acylated histones are recognized by histone readers before being displaced; in par-

ticular, acetylated histones are recognized by Bromodomain proteins. BRDT, a testis-specific protein with 2 bromodomains, has been shown to be essential for histone-to-protamine transition and thus sperm chromatin structure (see Table 1.1). Male mice in which the first bromodomain of BRDT has been removed have a block in spermatid elongation (Shang et al. 2007) (similar to that of *Nut* KO) due to a failure to remove hyperacetylated histones. TNP and PRM proteins are normally synthesized but are not incorporated in the chromatin and accumulate in the perinuclear region (Gaucher et al. 2012). In vitro studies have shown that BRDT recognition of acetylated histones can induce chromatin compaction (Pivot-Pajot et al. 2003; Govin et al. 2006). In humans, single nucleotide polymorphisms in Bromodomain testis-specific protein are associated with severe oligozoospermia (Aston et al. 2010). In addition to its role during spermatid chromatin remodeling, BRDT has an earlier role in meiotic cells, as it controls the expression of many genes at this stage. The complete deletion of BRDT results in spermatogenesis arrest at the end of the spermatocyte stage (Gaucher et al. 2012). It is worth adding that, like other proteins cited before, BRDT is also involved in the epigenetic regulation of sex chromosome gene expression during meiosis (Manterola et al. 2018).

BRWD1 (Bromodomain and WD repeat-containing protein 1), another bromodomain protein expressed (though not specifically) in the testis, also appears to be important for gene regulation during spermatogenesis. Its knockout leads to downregulation of hundreds of spermatid-specific genes (Pattabiraman et al. 2015), resulting in male infertility, with reduced sperm count and motility, and abnormally shaped sperm (Philipps et al. 2008). Defective chromatin condensation was also observed (Philipps et al. 2008) but could be an indirect effect of spermatid gene deregulation (see Table 1.1).

Histone acetylation is known to be required for histone degradation: when calf thymus chromatin was acetylated in vitro with acetic anhydride, it was found that histones were degraded and removed from DNA and that their binding to DNA became weaker. However, this could not be achieved at physiologic ionic strength indicating that other mechanisms that aid in histone degradation must exist (Marushige et al. 1976). It has been shown that the degradation of acetylated histones is mediated by a polyubiquitin-independent proteasome machinery which involves the proteasome activator PA200 (Qian et al. 2013). Indeed, PA200 mutant male mice have significantly impaired fertility characterized by reduced sperm production, increased apoptotic germ cell death, and defects in spermatid differentiation (Khor et al. 2006). Retention of core histones, in particular of acetylated histones in the soluble fraction of PA200-deficient testicular extracts, suggests that apoptosis of spermatids during elongation is caused by deficient degradation of acetylated core histone (Qian et al. 2013) (see Table 1.1). In mouse embryonic fibroblasts (MEF), in response to DNA double-strand breaks (caused by irradiations in this model), PA200 is involved in the degradation of acetylated core histones, so that proteins of the DNA damage repair pathway can access the sites of breaks. A similar mechanism could be at work during spermatogenesis (Qian et al. 2013).

In *Drosophila*, CAF1-p75 subunit has been found to bind to protamines and to be important for their deposition, but since, in this model, protamines are not essential for male fertility, loss of CAF1-p75 does not affect male fertility (Doyen et al. 2013).

Chd5 encodes the chromodomain-helicase-DNA-binding protein 5 which is involved in regulating sperm chromatin structure (see Table 1.1). During spermiogenesis, CHD5 appears to have multiple roles, as it is involved in transcriptional and post-transcriptional regulation of gene expression (in particular of genes encoding histone variants, TNP and PRM). It also appears to have a direct impact on histone-to-protamine transition via its effects on histone H4 hyperacetylation, nucleosome eviction, and DNA damage repair (Li et al. 2014). Consequently, *Chd5*-null male mice are infertile, with reduced sperm count, impaired sperm motility, and abnormal sperm head morphology. In particular, *Chd5*-null sperm chromatin integrity and compaction are

impaired (Li et al. 2014; Zhuang et al. 2014). These data indicate that CHD5 is an important regulator of chromatin structure in spermatid and sperm.

Regulation of DNA Breaks

The testis-specific serine/threonine kinase 6 (TSSK6) is another enzyme-regulating sperm chromatin structure (see Table 1.1). It is located in steps 11–12 elongating spermatids where it co-localizes with the phosphorylated form (at ser139) of H2A.X variant (γH2AX). γH2AX is a marker of DNA breaks and is detected as foci in spermatocytes during meiotic recombination (Mahadevaiah et al. 2001). Slightly later, in pachytene spermatocytes, γH2AX marks the inactive sex chromosomes independent of the occurrence of DNA breaks (Fernandez-Capetillo et al. 2003). Then, in elongating spermatids at the time where histones are displaced, γH2AX foci are also observed and are presumed to mark the transient double-strand breaks required for histone removal [see Leduc et al. (2008)].

Tssk6-null male mice are sterile, and their spermatozoa have abnormal shape and motility (Sosnik et al. 2009), with a high proportion of retained histones and abnormal processing of PRM2 (Jha et al. 2017). Interestingly, γH2AX signal in elongating spermatids is absent in TSSK6-KO elongating spermatids; DNA breaks are however formed normally. These data suggest that TSSK6 is required for phosphorylation of H2AX at that time but that its role is independent of DNA breaks (Jha et al. 2017).

Production of poly ADP-ribose is associated with activation of DNA damage signaling pathways resulting from the endogenous formation of DNA breaks during histone-to-protamine transition. Enzymes involved in the regulation of poly (ADP-ribose) metabolism such as poly ADP-ribose polymerases 1 and 2 (PARP1, PARP2) and poly ADP-ribose glycohydrolase (PARG) are therefore regulators of sperm chromatin structure. Mice with a deficient PAR [poly (ADP-ribose)] metabolism, such as *Parg*-null mice, have fertility defects with abnormal sperm chromatin condensation defects (see Table 1.1). *Parg*-null sperm present with a higher proportion of retained histones and protamine insufficiency are incorporated as a result of reduced poly ADP-ribose degradation (Meyer-Ficca et al. 2011a).

Topoisomerase II beta (TOP2B) expression strongly correlates with the appearance of DNA strand breaks in elongating spermatids of many species (Roca and Mezquita 1989; Mcpherson and Longo 1993; Leduc et al. 2008) and is presumed to be involved in this process. Interestingly, its activity is regulated by PARP metabolism and is expected to produce physiological DNA strand breaks (marked by γH2AX) in elongating spermatids (Meyer-Ficca et al. 2011b).

Other Genes Involved in Sperm Chromatin Structure

Aurora kinase C (AURKC) is a serine/threonine kinase involved in the regulation of chromosome segregation and cytokinesis during meiosis. It is highly expressed in human and mouse testes (Kimmins et al. 2007). Human patients with *AURKC* mutations are infertile due to macrozoospermia, a rare sperm defect characterized by the presence of large-headed multiflagellar spermatozoa (Dieterich et al. 2007; Ben Khelifa et al. 2011). The underlying molecular mechanism is a chromosomal segregation defect at the stage of meiosis and often leads to polyploid and aneuploid sperm. *AurkC*-null mice are subfertile, and *AurkC*-null spermatozoa are abnormally shaped with increased chromatin condensation defects (Kimmins et al. 2007) (see Table 1.1).

The cannabinoid receptor 1 (*Cnr1*) gene is a member of the guanine nucleotide-binding protein (G-protein)-coupled receptor family. It is expressed chiefly in the brain, but the effect of its KO on spermatogenesis has been investigated. *Cnr1*-null sperm show higher incidence of chromatin condensation defects and higher incidence of DNA damage in sperm (see Table 1.1). However, *Cnr1*-null mice do not have fertility defects (Chioccarelli et al. 2010).

Seipin (encoded by *Bscl2*) is an integral endoplasmic reticulum protein, highly expressed in

the brain and the testis and required for differentiation of preadipocytes to adipocytes and required for male fertility in mice and humans (Jiang et al. 2014; El Zowalaty et al. 2015). Protein folding enzymes and molecular chaperones that are required for maturation of proteins are in the endoplasmic reticulum (ER) lumen. Mutations that result in a misfolded seipin protein cause ER stress and activation of the unfolded protein response (UPR). *Bscl2*-null male mice are infertile, with reduced sperm count, reduced sperm motility, and arrest of spermatogenesis during spermatid differentiation. Abnormal chromatin structure in round spermatids and fragmented chromocenter in round spermatids and high incidence of chromatin vacuoles in elongating spermatids of *Bscl2*-null male mice were observed indicating a requirement for Seipin in regulating chromatin structure in spermatids (El Zowalaty et al. 2015).

In another context than spermiogenesis, the transcription factor YY1 has been shown to enhance ER stress and to recruit histone-modifying enzymes such as histone methyltransferase PRMT1 and acetyltransferase p300 induction (Baumeister et al. 2005). There could be a relationship between development of ER stress and histone acetylation leading to abnormal chromatin structure, but this requires further studies.

Protamines

Protamines are essential structural proteins of sperm chromatin (see Table 1.1). Many mammals only produce one type of protamine, but mice and humans express PRM1 and PRM2, though PRM1/PRM2 ratio is lower in mice than in humans. Aberrant PRM1/2 ratios are associated with male infertility in humans (Balhorn et al. 1988; Aoki et al. 2006). Protamines are small, very basic proteins rich in cysteines and lysines. PRM2 is translated as a precursor protein of which N-terminal region is cleaved to obtain a mature PRM2 protein. Both PRM1 and 2 are required for tight DNA condensation of sperm chromatin: intermolecular disulfide bonds (Cys38-Cys38 and Cys5-Cys22) that form between cysteine residues in protamine proteins are essential to stabilize the chromatin structure in the sperm nucleus (Vilfan et al. 2004; Balhorn 2007). Protamine molecules bind to DNA in its major groove and wrap around the DNA helix. This binding neutralizes the negative charge of phosphodiester backbone, condenses DNA into toroidal chromatin subunits that are ~50–70 nm in diameter and contain ~50 kb of DNA (doughnut-loop model) [see Balhorn (2007) for review]. This leads to extreme DNA condensation, and DNA molecules are packed in ~1/20 the volume of somatic nucleus (Balhorn 2007). Changes in the amount of protamines affect nuclear integrity and chromatin assembly. In mice, haploinsufficiency of *Prm1* or of *Prm2* has been initially reported to result in male infertility with abnormal sperm chromatin condensation, sperm DNA damage, and embryo lethality (Cho et al. 2001). A recent study has observed that *Prm2* complete KO rather than deletion of one allele leads to these defects. They also found that *Prm2* knockout affects sperm membrane integrity and leads to immobility (Schneider et al. 2016). In 2016, another study reported that progeny could be obtained using spermatozoa lacking *Prm1* encoding DNA (Takeda et al. 2016). Yet, those spermatozoa came from $Prm1^{+/-}$ males (with one functional *Prm1* allele), and, because spermatids share most of their cytoplasmic content via a pseudo-syncytium (Braun et al. 1989), those $Prm1^{-}$ sperm cells are expected to have incorporated some PRM1 proteins (coming from $Prm1^{+}$ "brother" sperm cells). All these data show that both PRM1 and 2 are required and that little/no compensation mechanisms exist, contrary to what is observed for histone variants or transition proteins.

Like for histones and TNPs, posttranslational modifications of protamines could affect their properties, in particular DNA-binding capacities (Willmitzer et al. 1977). Serine 55 residue of PRM2 has been shown to be phosphorylated (in vitro) by CaMK IV (Wu and Means 2000). Recent proteomic analyses have identified other sites of posttranslational modifications in protamines (11 in total) (Brunner et al. 2014).

Conclusion

The reorganization of chromatin structure during spermiogenesis is a tightly and timely regulated process involving many actors. Mutation in one of these actors produces sperm with a suboptimal chromatin compaction often resulting in male infertility. Abnormal chromatin compaction is associated with increased sperm DNA damage and lower in vitro fertilization rates. After fecundation, those damages need to be repaired to maintain paternal genome integrity. Recent studies indicate that sperm chromatin structure ("epigenome") can impact on the development and health of the future embryo/progeny (Siklenka et al. 2015; Teperek et al. 2016). It will therefore be important to clarify the consequences that the use of abnormally compacted spermatozoa by ICSI can have on the zygote genome and epigenome integrity.

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References

Akinloye O, Gromoll J, Callies C, Nieschlag E, Simoni M (2007) Mutation analysis of the X-chromosome linked, testis-specific TAF7L gene in spermatogenic failure. Andrologia 39:190–195

Aoki VW, Liu L, Jones KP, Hatasaka HH, Gibson M, Peterson CM, Carrell DT (2006) Sperm protamine 1/protamine 2 ratios are related to in vitro fertilization pregnancy rates and predictive of fertilization ability. Fertil Steril 86:1408–1415

Aston KI, Krausz C, Laface I, Ruiz-Castane E, Carrell DT (2010) Evaluation of 172 candidate polymorphisms for association with oligozoospermia or azoospermia in a large cohort of men of European descent. Hum Reprod 25:1383–1397

Audouard C, Christians E (2011) Hsp90beta1 knockout targeted to male germline: a mouse model for globozoospermia. Fertil Steril 95:1475–7 e1–1475–7 e4

Awe S, Renkawitz-Pohl R (2010) Histone H4 acetylation is essential to proceed from a histone- to a protamine-based chromatin structure in spermatid nuclei of Drosophila melanogaster. Syst Biol Reprod Med 56:44–61

Baarends WM, Hoogerbrugge JW, Roest HP, Ooms M, Vreeburg J, Hoeijmakers JHJ, Grootegoed JA (1999) Histone ubiquitination and chromatin remodeling in mouse spermatogenesis. Dev Biol 207:322–333

Baarends WM, Wassenaar E, Hoogerbrugge JW, Van Cappellen G, Roest HP, Vreeburg J, Ooms M, Hoeijmakers JH, Grootegoed JA (2003) Loss of HR6B ubiquitin-conjugating activity results in damaged synaptonemal complex structure and increased crossing-over frequency during the male meiotic prophase. Mol Cell Biol 23:1151–1162

Bai S, Fu K, Yin H, Cui Y, Yue Q, Li W, Cheng L, Tan H, Liu X, Guo Y, Zhang Y, Xie J, He W, Wang Y, Feng H, Xin C, Zhang J, Lin M, Shen B, Sun Z, Guo X, Zheng K, Ye L (2019, 10) Sox30 initiates transcription of haploid genes during late meiosis and spermiogenesis in mouse testes. Development 146

Balhorn R (2007) The protamine family of sperm nuclear proteins. Genome Biol 8:227

Balhorn R, Gledhill BL, Wyrobek AJ (1977) Mouse sperm chromatin proteins: quantitative isolation and partial characterization. Biochemistry 16:4074–4080

Balhorn R, Reed S, Tanphaichitr N (1988) Aberrant protamine 1/protamine 2 ratios in sperm of infertile human males. Experientia 44:52–55

Bao J, Rousseaux S, Shen J, Lin K, Lu Y, Bedford MT (2018) The arginine methyltransferase CARM1 represses p300∗ACT∗CREMtau activity and is required for spermiogenesis. Nucleic Acids Res 46:4327–4343

Barral S, Morozumi Y, Tanaka H, Montellier E, Govin J, De Dieuleveult M, Charbonnier G, Coute Y, Puthier D, Buchou T, Boussouar F, Urahama T, Fenaille F, Curtet S, Hery P, Fernandez-Nunez N, Shiota H, Gerard M, Rousseaux S, Kurumizaka H, Khochbin S (2017) Histone variant H2A.L.2 guides transition protein-dependent protamine assembly in male germ cells. Mol Cell 66:89–101 e8

Baumeister P, Luo S, Skarnes WC, Sui G, Seto E, Shi Y, Lee AS (2005) Endoplasmic reticulum stress induction of the Grp78/BiP promoter: activating mechanisms mediated by YY1 and its interactive chromatin modifiers. Mol Cell Biol 25:4529–4540

Bell EL, Nagamori I, Williams EO, Del Rosario AM, Bryson BD, Watson N, White FM, Sassone-Corsi P, Guarente L (2014) SirT1 is required in the male germ cell for differentiation and fecundity in mice. Development 141:3495–3504

Ben Khelifa M, Zouari R, Harbuz R, Halouani L, Arnoult C, Lunardi J, Ray PF (2011) A new AURKC mutation causing macrozoospermia: implications for human spermatogenesis and clinical diagnosis. Mol Hum Reprod 17:762–768

Blendy JA, Kaestner KH, Weinbauer GF, Nieschlag E, Schütz G (1996) Severe impairment of spermatogenesis in mice lacking the CREM gene. Nature 380:162–165

Boussouar F, Goudarzi A, Buchou T, Shiota H, Barral S, Debernardi A, Guardiola P, Brindle P, Martinez G, Arnoult C, Khochbin S, Rousseaux S (2014) A specific CBP/p300-dependent gene expression pro-

gramme drives the metabolic remodelling in late stages of spermatogenesis. Andrology 2:351–359
Braun RE (2001) Packaging paternal chromosomes with protamine. Nature Genetics 28:10–12
Braun RE, Behringer RR, Peschon JJ, Brinster RL, Palmiter RD (1989) Genetically haploid spermatids are phenotypically diploid. Nature 337:373–376
Brewer L, Corzett M, Balhorn R (2002) Condensation of DNA by spermatid basic nuclear proteins. J Biol Chem 277:38895–38900
Brunner AM, Nanni P, Mansuy IM (2014) Epigenetic marking of sperm by post-translational modification of histones and protamines. Epigenetics Chromatin 7:2
Brykczynska U, Hisano M, Erkek S, Ramos L, Oakeley EJ, Roloff TC, Beisel C, Schubeler D, Stadler MB, Peters AH (2010) Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. Nat Struct Mol Biol 17:679–687
Caron C, Pivot-Pajot C, Van Grunsven LA, Col E, Lestrat C, Rousseaux S, Khochbin S (2003) Cdyl: a new transcriptional co-repressor. EMBO Rep 4:877–882
Carone BR, Hung JH, Hainer SJ, Chou MT, Carone DM, Weng Z, Fazzio TG, Rando OJ (2014) High-resolution mapping of chromatin packaging in mouse embryonic stem cells and sperm. Dev Cell 30:11–22
Carrell DT, Emery BR, Hammoud S (2007) Altered protamine expression and diminished spermatogenesis: what is the link? Hum Reprod Update 13:313–327
Champroux A, Cocquet J, Henry-Berger J, Drevet JR, Kocer A (2018) A decade of exploring the mammalian sperm epigenome: paternal epigenetic and transgenerational inheritance. Front Cell Dev Biol 6:50
Chen Y, Sprung R, Tang Y, Ball H, Sangras B, Kim SC, Falck JR, Peng J, Gu W, Zhao Y (2007) Lysine propionylation and butyrylation are novel post-translational modifications in histones. Mol Cell Proteomics 6:812–819
Chen Y, Zheng Y, Gao Y, Lin Z, Yang S, Wang T, Wang Q, Xie N, Hua R, Liu M, Sha J, Griswold MD, Li J, Tang F, Tong MH (2018) Single-cell RNA-seq uncovers dynamic processes and critical regulators in mouse spermatogenesis. Cell Res 28:879–896
Cheng Y, Buffone MG, Kouadio M, Goodheart M, Page DC, Gerton GL, Davidson I, Wang PJ (2007) Abnormal sperm in mice lacking the taf7l gene. Mol Cell Biol 27:2582–2589
Chioccarelli T, Cacciola G, Altucci L, Lewis SE, Simon L, Ricci G, Ledent C, Meccariello R, Fasano S, Pierantoni R, Cobellis G (2010) Cannabinoid receptor 1 influences chromatin remodeling in mouse spermatids by affecting content of transition protein 2 mRNA and histone displacement. Endocrinology 151:5017–5029
Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB, Eddy EM (2001) Haploinsufficiency of protamine-1 or -2 causes infertility in mice. Nat Genet 28:82–86
Cho C, Jung-Ha H, Willis WD, Goulding EH, Stein P, Xu Z, Schultz RM, Hecht NB, Eddy EM (2003) Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. Biol Reprod 69:211–7
da Cruz I, Rodriguez-Casuriaga R, Santinaque FF, Farias J, Curti G, Capoano CA, Folle GA, Benavente R, Sotelo-Silveira JR, Geisinger A (2016) Transcriptome analysis of highly purified mouse spermatogenic cell populations: gene expression signatures switch from meiotic-to postmeiotic-related processes at pachytene stage. BMC Genomics 17:294
Dadoune JP (2003) Expression of mammalian spermatozoal nucleoproteins. Microsc Res Tech 61:56–75
Dam AH, Koscinski I, Kremer JA, Moutou C, Jaeger AS, Oudakker AR, Tournaye H, Charlet N, Lagier-Tourenne C, Van Bokhoven H, Viville S (2007) Homozygous mutation in SPATA16 is associated with male infertility in human globozoospermia. Am J Hum Genet 81:813–820
Davila Garza SA, Patrizio P (2013) Reproductive outcomes in patients with male infertility because of Klinefelter's syndrome, Kartagener's syndrome, round-head sperm, dysplasia fibrous sheath, and 'stump' tail sperm: an updated literature review. Curr Opin Obstet Gynecol 25:229–246
De Vries M, Ramos L, Housein Z, De Boer P (2012) Chromatin remodelling initiation during human spermiogenesis. Biol Open 1:446–457
Deng W, Lin H (2002) miwi, a murine homolog of piwi, encodes a cytoplasmic protein essential for spermatogenesis. Dev Cell 2:819–830
Dieterich K, Soto Rifo R, Faure AK, Hennebicq S, Ben Amar B, Zahi M, Perrin J, Martinez D, Sele B, Jouk PS, Ohlmann T, Rousseaux S, Lunardi J, Ray PF (2007) Homozygous mutation of AURKC yields large-headed polyploid spermatozoa and causes male infertility. Nat Genet 39:661–665
Dong Y, Isono KI, Ohbo K, Endo TA, Ohara O, Maekawa M, Toyama Y, Ito C, Toshimori K, Helin K, Ogonuki N, Inoue K, Ogura A, Yamagata K, Kitabayashi I, Koseki H (2017) EPC1/TIP60-mediated histone acetylation facilitates spermiogenesis in mice. Mol Cell Biol 37(19)
Doran J, Walters C, Kyle V, Wooding P, Hammett-Burke R, Colledge WH (2016) Mfsd14a (Hiat1) gene disruption causes globozoospermia and infertility in male mice. Reproduction 152:91–99
Dottermusch-Heidel C, Gartner SM, Tegeder I, Rathke C, Barckmann B, Bartkuhn M, Bhushan S, Steger K, Meinhardt A, Renkawitz-Pohl R (2014a) H3K79 methylation: a new conserved mark that accompanies H4 hyperacetylation prior to histone-to-protamine transition in Drosophila and rat. Biol Open 3:444–452
Dottermusch-Heidel C, Klaus ES, Gonzalez NH, Bhushan S, Meinhardt A, Bergmann M, Renkawitz-Pohl R, Rathke C, Steger K (2014b) H3K79 methylation directly precedes the histone-to-protamine transition in mammalian spermatids and is sensitive to bacterial infections. Andrology 2:655–665
Doyen CM, Moshkin YM, Chalkley GE, Bezstarosti K, Demmers JA, Rathke C, Renkawitz-Pohl R, Verrijzer CP (2013) Subunits of the histone chaperone CAF1 also mediate assembly of protamine-based chromatin. Cell Rep 4:59–65

El Kennani S, Adrait A, Permiakova O, Hesse AM, Ialy-Radio C, Ferro M, Brun V, Cocquet J, Govin J, Pflieger D (2018) Systematic quantitative analysis of H2A and H2B variants by targeted proteomics. Epigenetics Chromatin 11:2

El Zowalaty AE, Baumann C, Li R, Chen W, De La Fuente R, Ye X (2015) Seipin deficiency increases chromocenter fragmentation and disrupts acrosome formation leading to male infertility. Cell Death Dis 6:e1817

Erkek S, Hisano M, Liang CY, Gill M, Murr R, Dieker J, Schubeler D, Van Der Vlag J, Stadler MB, Peters AH (2013) Molecular determinants of nucleosome retention at CpG-rich sequences in mouse spermatozoa. Nat Struct Mol Biol 20:868–875

Feng CA, Spiller C, Merriner DJ, O'Bryan MK, Bowles J, Koopman P (2017) SOX30 is required for male fertility in mice. Sci Rep 7:17619

Fernandez-Capetillo O, Mahadevaiah SK, Celeste A, Romanienko PJ, Camerini-Otero RD, Bonner WM, Manova K, Burgoyne P, Nussenzweig A (2003) H2AX is required for chromatin remodeling and inactivation of sex chromosomes in male mouse meiosis. Dev Cell 4:497–508

Fujihara Y, Oji A, Larasati T, Kojima-Kita K, Ikawa M (2017) Human globozoospermia-related gene spata16 is required for sperm formation revealed by CRISPR/Cas9-mediated mouse models. Int J Mol Sci 18(10)

Funaki T, Kon S, Tanabe K, Natsume W, Sato S, Shimizu T, Yoshida N, Wong WF, Ogura A, Ogawa T, Inoue K, Ogonuki N, Miki H, Mochida K, Endoh K, Yomogida K, Fukumoto M, Horai R, Iwakura Y, Ito C, Toshimori K, Watanabe T, Satake M (2013) The Arf GAP SMAP2 is necessary for organized vesicle budding from the trans-Golgi network and subsequent acrosome formation in spermiogenesis. Mol Biol Cell 24:2633–2644

Gatewood JM, Cook GR, Balhorn R, Schmid CW, Bradbury EM (1990) Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones. J Biol Chem 265:20662–20666

Gaucher J, Boussouar F, Montellier E, Curtet S, Buchou T, Bertrand S, Hery P, Jounier S, Depaux A, Vitte AL, Guardiola P, Pernet K, Debernardi A, Lopez F, Holota H, Imbert J, Wolgemuth DJ, Gerard M, Rousseaux S, Khochbin S (2012) Bromodomain-dependent stage-specific male genome programming by Brdt. EMBO J 31:3809–3820

Godmann M, Auger V, Ferraroni-Aguiar V, Di Sauro A, Sette C, Behr R, Kimmins S (2007) Dynamic regulation of histone H3 methylation at lysine 4 in mammalian spermatogenesis. Biol Reprod 77:754–764

Gou LT, Kang JY, Dai P, Wang X, Li F, Zhao S, Zhang M, Hua MM, Lu Y, Zhu Y, Li Z, Chen H, Wu LG, Li D, Fu XD, Li J, Shi HJ, Liu MF (2017) Ubiquitination-deficient mutations in human piwi cause male infertility by impairing histone-to-protamine xchange during spermiogenesis. Cell 169:1090–1104 e13

Goudarzi A, Zhang D, Huang H, Barral S, Kwon OK, Qi S, Tang Z, Buchou T, Vitte AL, He T, Cheng Z, Montellier E, Gaucher J, Curtet S, Debernardi A, Charbonnier G, Puthier D, Petosa C, Panne D, Rousseaux S, Roeder RG, Zhao Y, Khochbin S (2016) Dynamic competing histone H4 K5K8 acetylation and butyrylation are hallmarks of highly active gene promoters. Mol Cell 62:169–180

Govin J, Lestrat C, Caron C, Pivot-Pajot C, Rousseaux S, Khochbin S (2006) Histone acetylation-mediated chromatin compaction during mouse spermatogenesis. Ernst Schering Res Found Workshop (57):155–172

Gupta N, Madapura MP, Bhat UA, Rao MR (2015) Mapping of post-translational modifications of transition proteins, TP1 and TP2, and identification of protein arginine methyltransferase 4 and lysine methyltransferase 7 as methyltransferase for TP2. J Biol Chem 290:12101–12122

Ha M, Kim VN (2014) Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol 15:509–524

Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR (2009) Distinctive chromatin in human sperm packages genes for embryo development. Nature 460:473–478

Han F, Liu C, Zhang L, Chen M, Zhou Y, Qin Y, Wang Y, Chen M, Duo S, Cui X, Bao S, Gao F (2017) Globozoospermia and lack of acrosome formation in GM130-deficient mice. Cell Death Dis 8:e2532

Hazzouri M, Pivot-Pajot C, Faure AK, Usson Y, Pelletier R, Sele B, Khochbin S, Rousseaux S (2000) Regulated hyperacetylation of core histones during mouse spermatogenesis: involvement of histone deacetylases. Eur J Cell Biol 79:950–960

Hecht NB (1990) Regulation of 'haploid expressed genes' in male germ cells. J Reprod Fert 88:679–693

Hernandez-Hernandez A, Lilienthal I, Fukuda N, Galjart N, Hoog C (2016) CTCF contributes in a critical way to spermatogenesis and male fertility. Sci Rep 6:28355

Ihara M, Meyer-Ficca ML, Leu NA, Rao S, Li F, Gregory BD, Zalenskaya IA, Schultz RM, Meyer RG (2014) Paternal poly (ADP-ribose) metabolism modulates retention of inheritable sperm histones and early embryonic gene expression. PLoS Genet 10:e1004317

Jha KN, Tripurani SK, Johnson GR (2017) TSSK6 is required for gammaH2AX formation and the histone-to-protamine transition during spermiogenesis. J Cell Sci 130:1835–1844

Jiang H, Gao Q, Zheng W, Yin S, Wang L, Zhong L, Ali A, Khan T, Hao Q, Fang H, Sun X, Xu P, Pandita TK, Jiang X, Shi Q (2018) MOF influences meiotic expansion of H2AX phosphorylation and spermatogenesis in mice. PLoS Genet 14:e1007300

Jiang M, Gao M, Wu C, He H, Guo X, Zhou Z, Yang H, Xiao X, Liu G, Sha J (2014) Lack of testicular seipin causes teratozoospermia syndrome in men. Proc Natl Acad Sci U S A 111:7054–7059

Kang-Decker N, Mantchev GT, Juneja SC, Mcniven MA, Van Deursen JM (2001) Lack of acrosome formation in Hrb-deficient mice. Science 294:1531–1533

Kashiwabara S, Noguchi J, Zhuang T, Ohmura K, Honda A, Sugiura S, Miyamoto K, Takahashi S, Inoue K, Ogura A, Baba T (2002) Regulation of spermatogenesis by testis-specific, cytoplasmic poly(A) polymerase TPAP. Science 298:1999–2002

Khor B, Bredemeyer AL, Huang CY, Turnbull IR, Evans R, Maggi LB Jr, White JM, Walker LM, Carnes K, Hess RA, Sleckman BP (2006) Proteasome activator PA200 is required for normal spermatogenesis. Mol Cell Biol 26:2999–3007

Kierszenbaum AL, Rivkin E, Tres LL (2003) Acroplaxome, an F-actin-keratin-containing plate, anchors the acrosome to the nucleus during shaping of the spermatid head. Mol Biol Cell 14:4628–4640

Kimmins S, Crosio C, Kotaja N, Hirayama J, Monaco L, Hoog C, Van Duin M, Gossen JA, Sassone-Corsi P (2007) Differential functions of the Aurora-B and Aurora-C kinases in mammalian spermatogenesis. Mol Endocrinol 21:726–739

Kimura T, Ito C, Watanabe S, Takahashi T, Ikawa M, Yomogida K, Fujita Y, Ikeuchi M, Asada N, Matsumiya K, Okuyama A, Okabe M, Toshimori K, Nakano T (2003) Mouse germ cell-less as an essential component for nuclear integrity. Mol Cell Biol 23:1304–1315

Kistler WS, Baas D, Lemeille S, Paschaki M, Seguin-Estevez Q, Barras E, Ma W, Duteyrat JL, Morle L, Durand B, Reith W (2015) RFX2 Is a major transcriptional regulator of spermiogenesis. PLoS Genet 11:e1005368

Kleene KC (1989) Poly(A) shortening accompanies the activation of translation of five mRNAs during spermiogenesis in the mouse. Development 106:367–373

Kleene KC (2013) Connecting cis-elements and trans-factors with mechanisms of developmental regulation of mRNA translation in meiotic and haploid mammalian spermatogenic cells. Reproduction 146:R1–19

Koizumi H, Yamaguchi N, Hattori M, Ishikawa TO, Aoki J, Taketo MM, Inoue K, Arai H (2003) Targeted disruption of intracellular type I platelet activating factor-acetylhydrolase catalytic subunits causes severe impairment in spermatogenesis. J Biol Chem 278:12489–12494

Korhonen HM, Meikar O, Yadav RP, Papaioannou MD, Romero Y, Da Ros M, Herrera PL, Toppari J, Nef S, Kotaja N (2011) Dicer is required for haploid male germ cell differentiation in mice. PLoS One 6:e24821

Kotaja N, De Cesare D, Macho B, Monaco L, Brancorsini S, Goossens E, Tournaye H, Gansmuller A, Sassone-Corsi P (2004) Abnormal sperm in mice with targeted deletion of the act (activator of cAMP-responsive element modulator in testis) gene. Proc Natl Acad Sci U S A 101:10620–10625

Lahn BT, Tang ZL, Zhou J, Barndt RJ, Parvinen M, Allis CD, Page DC (2002) Previously uncharacterized histone acetyltransferases implicated in mammalian spermatogenesis. Proc Natl Acad Sci U S A 99:8707–8712

Leduc F, Maquennehan V, Nkoma GB, Boissonneault G (2008) DNA damage response during chromatin remodeling in elongating spermatids of mice. Biol Reprod 78:324–332

Lee K, Fajardo MA, Braun RE (1996) A testis cytoplasmic RNA-binding protein that has the properties of a translational repressor. Mol Cell Biol 16:3023–3034

Levesque D, Veilleux S, Caron N, Boissonneault G (1998) Architectural DNA-binding properties of the sperma-tidal transition proteins 1 and 2. Biochem Biophys Res Commun 252:602–609

Li W, Wu J, Kim SY, Zhao M, Hearn SA, Zhang MQ, Meistrich ML, Mills AA (2014) Chd5 orchestrates chromatin remodelling during sperm development. Nat Commun 5:3812

Lin Q, Sirotkin A, Skoultchi AI (2000) Normal spermatogenesis in mice lacking the testis-specific linker histone H1t. Mol Cell Biol 20:2122–2128

Lin YN, Roy A, Yan W, Burns KH, Matzuk MM (2007) Loss of zona pellucida binding proteins in the acrosomal matrix disrupts acrosome biogenesis and sperm morphogenesis. Mol Cell Biol 27:6794–6805

Liu G, Shi QW, Lu GX (2010) A newly discovered mutation in PICK1 in a human with globozoospermia. Asian J Androl 12:556–560

Liu S, Yu H, Liu Y, Liu X, Zhang Y, Bu C, Yuan S, Chen Z, Xie G, Li W, Xu B, Yang J, He L, Jin T, Xiong Y, Sun L, Liu X, Han C, Cheng Z, Liang J, Shang Y (2017) Chromodomain protein CDYL acts as a crotonyl-CoA hydratase to regulate histone crotonylation and spermatogenesis. Mol Cell 67:853–866 e5

Lu LY, Wu J, Ye L, Gavrilina GB, Saunders TL, Yu X (2010) RNF8-dependent histone modifications regulate nucleosome removal during spermatogenesis. Dev Cell 18:371–384

Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389:251–260

Macho B, Brancorsini S, Fimia GM, Setou M, Hirokawa N, Sassone-Corsi P (2002) CREM-dependent transcription in male germ cells controlled by a kinesin. Science 298:2388–2390

Mahadevaiah SK, Turner JMA, Baudat F, Rogakou EP, De Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM, Burgoyne PS (2001) Recombinational DNA double-strand breaks in mice precede synapsis. Nat Genet 27:271–276

Manterola M, Brown TM, Oh MY, Garyn C, Gonzalez BJ, Wolgemuth DJ (2018) BRDT is an essential epigenetic regulator for proper chromatin organization, silencing of sex chromosomes and crossover formation in male meiosis. PLoS Genet 14:e1007209

Martianov I, Brancorsini S, Catena R, Gansmuller A, Kotaja N, Parvinen M, Sassone-Corsi P, Davidson I (2005) Polar nuclear localization of H1T2, a histone H1 variant, required for spermatid elongation and DNA condensation during spermiogenesis. Proc Natl Acad Sci U S A 102:2808–2813

Marushige K, Marushige Y, Wong TK (1976) Complete displacement of somatic histones during transformation of spermatid chromatin: a model experiment. Biochemistry 15:2047–2053

Mcghee JD, Felsenfeld G (1980) Nucleosome structure. Annu Rev Biochem 49:1115–1156

Mcgraw S, Morin G, Vigneault C, Leclerc P, Sirard MA (2007) Investigation of MYST4 histone acetyltransferase and its involvement in mammalian gametogenesis. BMC Dev Biol 7:123

Mcpherson SM, Longo FJ (1993) Nicking of rat spermatid and spermatozoa DNA: possible involvement of DNA topoisomerase II. Dev Biol 158:122–130

Meetei AR, Ullas KS, Vasupradha V, Rao MR (2002) Involvement of protein kinase A in the phosphorylation of spermatidal protein TP2 and its effect on DNA condensation. Biochemistry 41:185–195

Meikar O, Vagin VV, Chalmel F, Sostar K, Lardenois A, Hammell M, Jin Y, Da Ros M, Wasik KA, Toppari J, Hannon GJ, Kotaja N (2014) An atlas of chromatoid body components. RNA 20:483–495

Meyer-Ficca ML, Ihara M, Lonchar JD, Meistrich ML, Austin CA, Min W, Wang ZQ, Meyer RG (2011a) Poly(ADP-ribose) metabolism is essential for proper nucleoprotein exchange during mouse spermiogenesis. Biol Reprod 84:218–228

Meyer-Ficca ML, Lonchar JD, Ihara M, Meistrich ML, Austin CA, Meyer RG (2011b) Poly(ADP-ribose) polymerases PARP1 and PARP2 modulate topoisomerase II beta (TOP2B) function during chromatin condensation in mouse spermiogenesis. Biol Reprod 84:900–909

Mishra LN, Shalini V, Gupta N, Ghosh K, Suthar N, Bhaduri U, Rao MRS (2018) Spermatid-specific linker histone HILS1 is a poor condenser of DNA and chromatin and preferentially associates with LINE-1 elements. Epigenetics Chromatin 11:43

Montellier E, Boussouar F, Rousseaux S, Zhang K, Buchou T, Fenaille F, Shiota H, Debernardi A, Hery P, Curtet S, Jamshidikia M, Barral S, Holota H, Bergon A, Lopez F, Guardiola P, Pernet K, Imbert J, Petosa C, Tan M, Zhao Y, Gerard M, Khochbin S (2013) Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B. Genes Dev 27:1680–1692

Moretti C, Serrentino ME, Ialy-Radio C, Delessard M, Soboleva TA, Tores F, Leduc M, Nitschke P, Drevet JR, Tremethick DJ, Vaiman D, Kocer A, Cocquet J (2017) SLY regulates genes involved in chromatin remodeling and interacts with TBL1XR1 during sperm differentiation. Cell Death Differ 24:1029–1044

Mulugeta Achame E, Wassenaar E, Hoogerbrugge JW, Sleddens-Linkels E, Ooms M, Sun ZW, Van IWF, Grootegoed JA, Baarends WM (2010) The ubiquitin-conjugating enzyme HR6B is required for maintenance of X chromosome silencing in mouse spermatocytes and spermatids. BMC Genomics 11:367

Nair M, Nagamori I, Sun P, Mishra DP, Rheaume C, Li B, Sassone-Corsi P, Dai X (2008) Nuclear regulator Pygo2 controls spermiogenesis and histone H3 acetylation. Dev Biol 320:446–455

Nantel F, Monaco L, Foulkes NS, Masquiller D, Lemeur M, Henriksén K, Dierich A, Parvinen M, Sassone-Corsi P (1996) Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. Nature 380:159–162

Nayernia K, Vauti F, Meinhardt A, Cadenas C, Schweyer S, Meyer BI, Schwandt I, Chowdhury K, Engel W, Arnold HH (2003) Inactivation of a testis-specific Lis1 transcript in mice prevents spermatid differentiation and causes male infertility. J Biol Chem 278:48377–48385

Nili E, Cojocaru GS, Kalma Y, Ginsberg D, Copeland NG, Gilbert DJ, Jenkins NA, Berger R, Shaklai S, Amariglio N, Brok-simoni F, Simon AJ, Rechavi G (2001) Nuclear membrane protein LAP2beta mediates transcriptional repression alone and together with its binding partner GCL (germ-cell-less). J Cell Sci 114:3297–3307

Okada Y, Scott G, Ray MK, Mishina Y, Zhang Y (2007) Histone demethylase JHDM2A is critical for Tnp1 and Prm1 transcription and spermatogenesis. Nature 450:119–123

Oliva R, Bazett-Jones D, Mezquita C, Dixon GH (1987) Factors affecting nucleosome disassembly by protamines in vitro. Histone hyperacetylation and chromatin structure, time dependence, and the size of the sperm nuclear proteins. J Biol Chem 262:17016–17025

Oliva R, Mezquita C (1986) Marked differences in the ability of distinct protamines to disassemble nucleosomal core particles in vitro. Biochemistry 25:6508–6511

Paiardi C, Pasini ME, Gioria M, Berruti G (2011) Failure of acrosome formation and globozoospermia in the wobbler mouse, a Vps54 spontaneous recessive mutant. Spermatogenesis 1:52–62

Pattabiraman S, Baumann C, Guisado D, Eppig JJ, Schimenti JC, De La Fuente R (2015) Mouse BRWD1 is critical for spermatid postmeiotic transcription and female meiotic chromosome stability. J Cell Biol 208:53–69

Philipps DL, Wigglesworth K, Hartford SA, Sun F, Pattabiraman S, Schimenti K, Handel M, Eppig JJ, Schimenti JC (2008) The dual bromodomain and WD repeat-containing mouse protein BRWD1 is required for normal spermiogenesis and the oocyte-embryo transition. Dev Biol 317:72–82

Pierre V, Martinez G, Coutton C, Delaroche J, Yassine S, Novella C, Pernet-Gallay K, Hennebicq S, Ray PF, Arnoult C (2012) Absence of Dpy19l2, a new inner nuclear membrane protein, causes globozoospermia in mice by preventing the anchoring of the acrosome to the nucleus. Development 139:2955–2965

Pivot-Pajot C, Caron C, Govin J, Vion A, Rousseaux S, Khochbin S (2003) Acetylation-dependent chromatin reorganization by BRDT, a testis-specific bromodomain-containing protein. Mol Cell Biol 23:5354–5365

Pradeepa MM, Nikhil G, Hari Kishore A, Bharath GN, Kundu TK, Rao MR (2009) Acetylation of transition protein 2 (TP2) by KAT3B (p300) alters its DNA condensation property and interaction with putative histone chaperone NPM3. J Biol Chem 284:29956–29967

Qian MX, Pang Y, Liu CH, Haratake K, Du BY, Ji DY, Wang GF, Zhu QQ, Song W, Yu Y, Zhang XX, Huang HT, Miao S, Chen LB, Zhang ZH, Liang YN, Liu S, Cha H, Yang D, Zhai Y, Komatsu T, Tsuruta F, Li H, Cao C, Li W, Li GH, Cheng Y, Chiba T, Wang L, Goldberg AL, Shen Y, Qiu XB (2013) Acetylation-mediated proteasomal degradation of core histones during DNA repair and spermatogenesis. Cell 153:1012–1024

Rathke C, Baarends WM, Jayaramaiah-Raja S, Bartkuhn M, Renkawitz R, Renkawitz-Pohl R (2007) Transition from a nucleosome-based to a protamine-based chromatin configuration during spermiogenesis in Drosophila. J Cell Sci 120:1689–1700

Ray PF, Toure A, Metzler-Guillemain C, Mitchell MJ, Arnoult C, Coutton C (2017) Genetic abnormalities leading to qualitative defects of sperm morphology or function. Clin Genet 91:217–232

Roca J, Mezquita C (1989) DNA topoisomerase II activity in nonreplicating, transcriptionally inactive, chicken late spermatids. EMBO J 8:1855–1860

Roest HP, Van Klaveren J, De Wit J, Van Gurp CG, Koken MH, Vermey M, Van Roijen JH, Hoogerbrugge JW, Vreeburg JT, Baarends WM, Bootsma D, Grootegoed JA, Hoeijmakers JH (1996) Inactivation of the HR6B ubiquitin-conjugating DNA repair enzyme in mice causes male sterility associated with chromatin modification. Cell 86:799–810

Royo H, Stadler MB, Peters A (2016) Alternative computational analysis shows no evidence for nucleosome enrichment at repetitive sequences in mammalian spermatozoa. Dev Cell 37:98–104

Russell LD, Hikim APS, Ettlin RA, Clegg ED (1990) Histological and histopathological evaluation of the testis. Cache River Press, Clearwater

Riel JM, Yamauchi Y, Sugawara A, Li HY, Ruthig V, Stoytcheva Z, Ellis PJ, Cocquet J, Ward MA (2013) Deficiency of the multi-copy mouse Y gene Sly causes sperm DNA damage and abnormal chromatin packaging. J Cell Sci 126:803–13

Sabari BR, Tang Z, Huang H, Yong-Gonzalez V, Molina H, Kong HE, Dai L, Shimada M, Cross JR, Zhao Y, Roeder RG, Allis CD (2015) Intracellular crotonyl-CoA stimulates transcription through p300-catalyzed histone crotonylation. Mol Cell 58:203–215

Sabari BR, Zhang D, Allis CD, Zhao Y (2017) Metabolic regulation of gene expression through histone acylations. Nat Rev Mol Cell Biol 18:90–101

Samans B, Yang Y, Krebs S, Sarode GV, Blum H, Reichenbach M, Wolf E, Steger K, Dansranjavin T, Schagdarsurengin U (2014) Uniformity of nucleosome preservation pattern in Mammalian sperm and its connection to repetitive DNA elements. Dev Cell 30:23–35

Sassone-Corsi P (2002) Unique chromatin remodeling and transcriptional regulation in spermatogenesis. Science 296:2176–2178

Schneider S, Balbach M, Jan FJ, Fietz D, Nettersheim D, Jostes S, Schmidt R, Kressin M, Bergmann M, Wachten D, Steger K, Schorle H (2016) Re-visiting the Protamine-2 locus: deletion, but not haploinsufficiency, renders male mice infertile. Sci Rep 6:36764

Sediva A, Smith CI, Asplund AC, Hadac J, Janda A, Zeman J, Hansikova H, Dvorakova L, Mrazova L, Velbri S, Koehler C, Roesch K, Sullivan KE, Futatani T, Ochs HD (2007) Contiguous X-chromosome deletion syndrome encompassing the BTK, TIMM8A, TAF7L, and DRP2 genes. J Clin Immunol 27:640–646

Shang E, Nickerson HD, Wen D, Wang X, Wolgemuth DJ (2007) The first bromodomain of Brdt, a testis-specific member of the BET sub-family of double-bromodomain-containing proteins, is essential for male germ cell differentiation. Development 134:3507–3515

Shinagawa T, Huynh LM, Takagi T, Tsukamoto D, Tomaru C, Kwak HG, Dohmae N, Noguchi J, Ishii S (2015) Disruption of Th2a and Th2b genes causes defects in spermatogenesis. Development 142:1287–1292

Shiota H, Barral S, Buchou T, Tan M, Coute Y, Charbonnier G, Reynoird N, Boussouar F, Gerard M, Zhu M, Bargier L, Puthier D, Chuffart F, Bourova-Flin E, Picaud S, Filippakopoulos P, Goudarzi A, Ibrahim Z, Panne D, Rousseaux S, Zhao Y, Khochbin S (2018) Nut directs p300-dependent, genome-wide H4 hyperacetylation in male germ cells. Cell Rep 24:3477–3487 e6

Siklenka K, Erkek S, Godmann M, Lambrot R, Mcgraw S, Lafleur C, Cohen T, Xia J, Suderman M, Hallett M, Trasler J, Peters AH, Kimmins S (2015) Disruption of histone methylation in developing sperm impairs offspring health transgenerationally. Science 350(6261): aab2006

Sin HS, Barski A, Zhang F, Kartashov AV, Nussenzweig A, Chen J, Andreassen PR, Namekawa SH (2012) RNF8 regulates active epigenetic modifications and escape gene activation from inactive sex chromosomes in post-meiotic spermatids. Genes Dev 26:2737–2748

Song N, Liu J, An S, Nishino T, Hishikawa Y, Koji T (2011) Immunohistochemical analysis of histone H3 modifications in germ cells during mouse spermatogenesis. Acta Histochem Cytochem 44:183–190

Sosnik J, Miranda PV, Spiridonov NA, Yoon SY, Fissore RA, Johnson GR, Visconti PE (2009) Tssk6 is required for Izumo relocalization and gamete fusion in the mouse. J Cell Sci 122:2741–2749

Steger K (1999) Transcriptional and translational regulation of gene expression in haploid spermatids. Anat Embryol (Berl) 199:471–487

Takeda N, Yoshinaga K, Furushima K, Takamune K, Li Z, Abe S, Aizawa S, Yamamura K (2016) Viable offspring obtained from Prm1-deficient sperm in mice. Sci Rep 6:27409

Tan M, Luo H, Lee S, Jin F, Yang JS, Montellier E, Buchou T, Cheng Z, Rousseaux S, Rajagopal N, Lu Z, Ye Z, Zhu Q, Wysocka J, Ye Y, Khochbin S, Ren B, Zhao Y (2011) Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell 146:1016–1028

Tanaka H, Iguchi N, Isotani A, Kitamura K, Toyama Y, Matsuoka Y, Onishi M, Masai K, Maekawa M, Toshimori K, Okabe M, Nishimune Y (2005) HANP1/H1T2, a novel histone H1-like protein involved in nuclear formation and sperm fertility. Mol Cell Biol 25:7107–7119

Tang MC, Jacobs SA, Wong LH, Mann JR (2013) Conditional allelic replacement applied to genes encoding the histone variant H3.3 in the mouse. Genesis 51:142–146

Teperek M, Simeone A, Gaggioli V, Miyamoto K, Allen GE, Erkek S, Kwon T, Marcotte EM, Zegerman P, Bradshaw CR, Peters AH, Gurdon JB, Jullien J (2016) Sperm is epigenetically programmed to regulate gene transcription in embryos. Genome Res 26:1034–1046

Ullas KS, Rao MR (2003) Phosphorylation of rat spermatidal protein TP2 by sperm-specific protein kinase A and modulation of its transport into the haploid nucleus. J Biol Chem 278:52673–52680

Van Der Heijden GW, Derijck AA, Posfai E, Giele M, Pelczar P, Ramos L, Wansink DG, Van Der Vlag J, Peters AH, De Boer P (2007) Chromosome-wide nucleosome replacement and H3.3 incorporation during mammalian meiotic sex chromosome inactivation. Nat Genet 39:251–258

Vilfan ID, Conwell CC, Hud NV (2004) Formation of native-like mammalian sperm cell chromatin with folded bull protamine. J Biol Chem 279:20088–20095

Wan L, Hu XJ, Yan SX, Chen F, Cai B, Zhang XM, Wang T, Yu XB, Xiang AP, Li WQ (2013) Generation and neuronal differentiation of induced pluripotent stem cells in Cdyl-/- mice. Neuroreport 24:114–119

Wang G, Zhang H, Wang L, Wang Y, Huang H, Sun F (2015) Ca(2+)/calmodulin-dependent protein kinase IV promotes interplay of proteins in chromatoid body of male germ cells. Sci Rep 5:12126

Wang H, Wan H, Li X, Liu W, Chen Q, Wang Y, Yang L, Tang H, Zhang X, Duan E, Zhao X, Gao F, Li W (2014) Atg7 is required for acrosome biogenesis during spermatogenesis in mice. Cell Res 24:852–869

Ward WS, Coffey DS (1991) DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. Biol Reprod 44:569–574

White-Cooper H, Davidson I (2011) Unique aspects of transcription regulation in male germ cells. Cold Spring Harb Perspect Biol 3(7)

Willmitzer L, Bode J, Wagner KG (1977) Phosphorylated protamines. I. Binding stoichiometry and thermal stability of complexes in DNA. Nucleic Acids Res 4:149–162

Wolffe AP (1997) Histone H1. Int J Biochem Cell Biol 29:1463–1466

Wu JY, Means AR (2000) Ca(2+)/calmodulin-dependent protein kinase IV is expressed in spermatids and targeted to chromatin and the nuclear matrix. J Biol Chem 275:7994–7999

Wu JY, Ribar TJ, Cummings DE, Burton KA, Mcknight GS, Means AR (2000) Spermiogenesis and exchange of basic nuclear proteins are impaired in male germ cells lacking Camk4. Nat Genet 25:448–452

Xiao N, Kam C, Shen C, Jin W, Wang J, Lee KM, Jiang L, Xia J (2009) PICK1 deficiency causes male infertility in mice by disrupting acrosome formation. J Clin Invest 119:802–812

Xu X, Toselli PA, Russell LD, Seldin DC (1999) Globozoospermia in mice lacking the casein kinase II alpha' catalytic subunit. Nat Genet 23:118–121

Yan W, Assadi AH, Wynshaw-Boris A, Eichele G, Matzuk MM, Clark GD (2003a) Previously uncharacterized roles of platelet-activating factor acetylhydrolase 1b complex in mouse spermatogenesis. Proc Natl Acad Sci U S A 100:7189–7194

Yan W, Ma L, Burns KH, Matzuk MM (2003b) HILS1 is a spermatid-specific linker histone H1-like protein implicated in chromatin remodeling during mammalian spermiogenesis. Proc Natl Acad Sci U S A 100:10546–10551

Yao R, Ito C, Natsume Y, Sugitani Y, Yamanaka H, Kuretake S, Yanagida K, Sato A, Toshimori K, Noda T (2002) Lack of acrosome formation in mice lacking a Golgi protein, GOPC. Proc Natl Acad Sci U S A 99:11211–11216

Yassine S, Escoffier J, Martinez G, Coutton C, Karaouzene T, Zouari R, Ravanat JL, Metzler-Guillemain C, Lee HC, Fissore R, Hennebicq S, Ray PF, Arnoult C (2015) Dpy19l2-deficient globozoospermic sperm display altered genome packaging and DNA damage that compromises the initiation of embryo development. Mol Hum Reprod 21:169–185

Yildiz Y, Matern H, Thompson B, Allegood JC, Warren RL, Ramirez DM, Hammer RE, Hamra FK, Matern S, Russell DW (2006) Mutation of beta-glucosidase 2 causes glycolipid storage disease and impaired male fertility. J Clin Invest 116:2985–2994

Yoshida K, Muratani M, Araki H, Miura F, Suzuki T, Dohmae N, Katou Y, Shirahige K, Ito T, Ishii S (2018) Mapping of histone-binding sites in histone replacement-completed spermatozoa. Nat Commun 9:3885

Yu YE, Zhang Y, Unni E, Shirley CR, Deng JM, Russell LD, Weil MM, Behringer R, Meistrich M (2000) Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice. Proc Natl Acad Sci U S A 97:4683–4688

Yuen BT, Bush KM, Barrilleaux BL, Cotterman R, Knoepfler PS (2014) Histone H3.3 regulates dynamic chromatin states during spermatogenesis. Development 141:3483–3494

Yamaguchi K, Hada M, Fukuda Y, Inoue E, Makino Y, Katou Y, Shirahige K, OKADA Y (2018) Re-evaluating the Localization of Sperm-Retained Histones Revealed the Modification-Dependent Accumulation in Specific Genome Regions. Cell Rep 23:3920–3932

Zhang D, Penttila TL, Morris PL, Teichmann M, Roeder RG (2001) Spermiogenesis deficiency in mice lacking the Trf2 gene. Science 292:1153–1155

Zhang D, Xie D, Lin X, Ma L, Chen J, Zhang D, Wang Y, Duo S, Feng Y, Zheng C, Jiang B, Ning Y, Han C (2018) The transcription factor SOX30 is a key regulator of mouse spermiogenesis. Development 145(11)

Zhao M, Shirley CR, Hayashi S, Marcon L, Mohapatra B, Suganuma R, Behringer RR, Boissonneault G, Yanagimachi R, Meistrich ML (2004) Transition nuclear proteins are required for normal chromatin condensation and functional sperm development. Genesis 38:200–213

Zhao M, Shirley CR, Yu YE, Mohapatra B, Zhang Y, Unni E, Deng JM, Arango NA, Terry NH, Weil MM, Russell LD, Behringer RR, Meistrich ML (2001) Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice. Mol Cell Biol 21:7243–7255

Zhong J, Peters AH, lee K, Braun RE (1999) A double-stranded RNA binding protein required for activation of repressed messages in mammalian germ cells. Nat Genet 22:171–174

Zhou H, Grubisic I, Zheng K, He Y, Wang PJ, Kaplan T, Tjian R (2013) Taf7l cooperates with Trf2 to regulate spermiogenesis. Proc Natl Acad Sci U S A 110:16886–16891

Zhuang T, Hess RA, Kolla V, Higashi M, Raabe TD, Brodeur GM (2014) CHD5 is required for spermiogenesis and chromatin condensation. Mech Dev 131:35–46

Zuo X, Rong B, Li L, Lv R, Lan F, Tong MH (2018) The histone methyltransferase SETD2 is required for expression of acrosin-binding protein 1 and protamines and essential for spermiogenesis in mice. J Biol Chem 293:9188–9197

2 Age-Dependent De Novo Mutations During Spermatogenesis and Their Consequences

Francesca Cioppi, Elena Casamonti, and Csilla Krausz

Abstract

Spermatogenesis is a highly complex biological process during which germ cells undergo recurrent rounds of DNA replication and cell division that may predispose to random mutational events. Hence, germ cells are vulnerable to the introduction of a range of de novo mutations, in particular chromosomal aberrations, point mutations and small indels. The main mechanisms through which mutations may occur during spermatogenesis are (i) errors in DNA replication, (ii) inefficient repair of non-replicative DNA damage between cell divisions and (iii) exposure to mutagens during lifetime. Any genetic alteration in the spermatozoa, if not repaired/eliminated, can be passed on to the offspring, potentially leading to malformations, chromosomal anomalies and monogenic diseases. Spontaneous de novo mutations tend to arise and accumulate with a higher frequency during testicular aging. In fact, there is an increased incidence of some chromosomal aberrations and a greater risk of congenital disorders, collectively termed paternal age effect (PAE), in children conceived by fathers with advanced age. PAE disorders are related to well-characterized de novo point mutations leading to a selective advantage on the mutant spermatogonial stem cells that cause a progressive enrichment over time of mutant spermatozoa in the testis.

The purpose of this chapter is to provide a summary on the spontaneous genetic alterations that occur during spermatogenesis, focusing on their underlying mechanisms and their consequences in the offspring.

Keywords

Genetics · Spermatogenesis · Y microdeletions · PAE mutations · Paternal aging · Spontaneous mutations · Infertility · Spermatozoa · PAE disorders · Genomic anomalies

Introduction

One marked difference between the male and female gametes is that there are many more germline cell divisions in the life history of spermatozoa relative to that of an egg. By the time of birth, germ cells in the developing ovary have already completed their proliferative phase, and

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all postnatal phases of germ cell development are meiotic. In contrast, spermatogenesis requires regular mitotic division of spermatogonial stem cells (SSCs) throughout male reproductive life. In fact, SSCs divide by mitosis approximately every 16 days, both maintaining the spermatogonial stem cell pool and generating differentiated spermatogonial cells which produce sperm cells, through an additional round of mitosis followed by meiosis (Crow 2000). Because this process involves recurrent rounds of DNA replication and cell division, random copy-error mutational events are predicted to arise mainly in the male germline (Penrose 1955), explaining the elevated male-to-female mutation ratio (ranging between 2 and 7) observed for the majority of spontaneous point mutations and small deletions (Makova and Li 2002; Taylor et al. 2006). It is interesting to note that de novo mutations do not occur completely random across the genome (Michaelson et al. 2012), but in some part of the genome, they can arise because of intrinsic characteristics of the region itself, related to its sequence composition and functional context (Shendure and Akey 2015). In fact, mutational hotspots for genomic rearrangements are largely determined by the underlying genomic architecture. For instance, the distribution and orientation of segmental duplications on the long arm of chromosome Y are known to create "hotspots for structural variation" mediating de novo recurrent Y-chromosome microdeletions by non-allelic homologous recombination (NAHR) (Repping et al. 2003).

As stated above, testis appears to be an ideal place where de novo mutations can occur, and the main mechanisms are (i) errors in DNA replication during gametogenesis, (ii) inefficient repair of DNA damages (Gao et al. 2016) and (iii) exposure to mutagens during lifetime.

Since spermatozoa are produced continuously throughout reproductive life, the number of chromosome replications and cell divisions increases with age (Crow 2000). In addition, it is worth noting that the efficiency of endogenous defence systems against radical oxygen species (ROS) and of DNA repair mechanisms seems to decline with age (Paul and Robaire 2013; Goriely 2016). In fact, recent whole genome/exome sequencing studies of parent–offspring trios show that most mutations (mainly nucleotide substitutions) originate from the paternal germline (Francioli et al. 2015; Rahbari et al. 2016; Goldmann et al. 2018) and their frequency increases with the father's age (Kong et al. 2012; Besenbacher et al. 2015). This issue is of particular significance given the demographic shift to delayed reproduction in many populations (Paul and Robaire 2013). De novo mutation(s) arising in the adult testis and leading to gonadal mosaicism might result in a clinical disorder in the offspring in whom the above-mentioned mutation(s) is transmitted as a germline event (Campbell et al. 2014). It is well known that damaging de novo point mutations and indels affecting important genes in development have been established as a prominent cause of both rare and common genetic disorders (Vissers et al. 2010; O'Roak et al. 2011; de Ligt et al. 2012; Rauch et al. 2012; Epi4K Consortium et al. 2013; Hoischen et al. 2014; Iossifov et al. 2014; Chong et al. 2015; Deciphering Developmental Disorders Study et al. 2015). Among them, several monogenic conditions show an extreme paternal bias in origin (Risch et al. 1987) and an epidemiological paternal age effect (Green et al. 2010), collectively termed as paternal age effect (PAE) disorders.

This chapter aims to describe the mechanisms of clinically relevant de novo mutational events during spermatogenesis, such as chromosomal anomalies and PAE mutations, with special focus on their health consequences on the offspring.

De Novo Chromosomal Anomalies During Spermatogenesis

Sperm chromosomal anomalies generally result from meiotic errors occurring during early spermatogenesis and are divided into aneuploidies (i.e. abnormalities of chromosome number) and structural aberrations (see below) (Martin and Rademaker 1987; Luetjens et al. 2002).

Aneuploidies

In humans, aneuploidies represent the most common heritable chromosomal anomaly, with approximately 0.33% of infants born with an altered number of chromosomes (Hassold and Hunt 2001). This genomic rearrangement derives mainly from non-disjunction events during meiotic divisions [both meiosis I (MI) and meiosis II (MII)].

Most constitutional aneuploidies are generated de novo during parental meiosis. Although chromosomal non-disjunctions originate mainly in the female germline (Hassold and Hunt 2009), over 50 publications have demonstrated that all men produce approximately 3–5% of aneuploid sperm (Ioannou, Fortun and Tempest 2018).

The introduction of fluorescent in situ hybridization (FISH) in spermatozoa in the early 1990s allowed a quicker labour- and cost-saving analysis of sperm aneuploidy.

Sperm FISH studies have mainly evaluated the effect of male aging on sperm autosomal aneuploidies, showing 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 (Chianese et al. 2014). The rest provide evidence that errors in MI and MII are more likely to occur with the advancement of age. As for non-disjunctions 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 (Griffin et al. 1995; Asada et al. 2000; Guttenbach et al. 2000; Bosch et al. 2001; Lowe et al. 2001) and a two- to threefold higher frequency of producing X or Y disomic spermatozoa as a consequence of MII errors (Martin et al. 1995; Kinakin et al. 1997; Robbins et al. 1997; Rubes et al. 1998).

Consequences on the offspring The vast majority of chromosome aneuploidies are not compatible with foetal development, leading to failing to implant or spontaneous abortion. In fact, analyses on foetal material retrieved from abortuses show that 60% of all aneuploidies consist in 45, XO monosomy and trisomies of chromosomes 16, 21 and 22 (Hassold and Hunt 2001). However, an aneuploid state for a number of chromosomes (13, 18, 21, X and Y) is compatible with viable foetus. For instance, trisomies 13, 18 and 21 cause Patau, Edwards and Down syndromes, respectively. The paternal contribution is considerably high for aneuploidies involving the sex chromosomes (Hassold et al. 1993) such as Klinefelter syndrome (47, XXY), Jacob's syndrome (47, XYY) and Turner syndrome (45, X0). The incidence of both Klinefelter syndrome and Jacob's syndrome is estimated to occur in 1 in 500 to 1 in 1000 male live births (Morris et al. 2008). Individuals with Klinefelter syndrome typically present with non-obstructive azoospermia and signs of androgen deficiency, ranging from hypogonadism with gynaecomastia and eunuchoid body proportions to variable levels of undervirilization (Aksglaede and Juul 2013). In addition, the 47, XXY karyotype may also be associated to a series of general health problems, including metabolic syndrome, autoimmune diseases, venous thromboembolism and cognitive/psychiatric disturbances (Calogero et al. 2017). Individuals with Jacob's syndrome show a great diversity in the degree of spermatogenic disturbance, ranging from severe oligozoospermia to

normozoospermia; the majority of cases may have no phenotypic abnormalities, whereas some individuals may have a greater risk for behavioural problems, mild learning disabilities and tall stature (Kim et al. 2013).

Turner syndrome has a prevalence of 1 in 2000–2500 live-born female children, and 45, X0 girls have significant variability in their clinical presentation, including mainly short stature, ovarian insufficiency, cardiac and renal abnormalities, sensorineural hearing loss, ophthalmologic/thyroid problems, metabolic syndrome, inflammatory bowel disease and neurocognitive issues (Shankar and Backeljauw 2018).

Structural Aberrations

The incidence of structural chromosomal abnormalities is lower compared to aneuploidies at birth (0.25% vs. 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). Thomas et al. observed a paternal derivation of de novo unbalanced structural chromosomal abnormalities, with 84% interstitial deletions and 58% duplications and rings (Thomas et al. 2006). 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 non-recurrent 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. 2010).

The de novo structural chromosomal aberrations may occur during pre-meiotic mitosis and meiotic recombination. The mechanisms involved in these rearrangements are different and can be divided into recurrent and non-recurrent rearrangements. The majority of them are not recurrent and are due to non-homologous end-joining (NHEJ) and microhomology-mediated break-induced replication (MMBIR) (for review see Weckselblatt, Hermetz and Rudd (2015)). Recurrent translocations are likely to be mediated by non-allelic homologous recombination (NAHR) between segmental duplications or paralogous interspersed repeats or palindromic AT-rich repeats (typical for chromosomes 3, 8, 11, 17, 22). Besides translocations and inversions, NAHR can cause also deletion/duplications, which will be described in more details in relationship with Y-chromosome microdeletions (see below).

The development of assays that allow the detection of structural chromosomal aberrations directly within spermatozoa represented an important incentive for the evaluation of age-related increase of the formation rate of chromosomal anomalies in a man's sperm population. The insemination of hamster eggs with human spermatozoa, a method known as the "hamster-egg penetration test", was described for the first time by Yanagimachi and colleagues (Yanagimachi et al. 1976) for the assessment of the fertilizing capacity of human spermatozoa. It provides information on the ability of sperm to undergo capacitation, fuse with the egg membrane and decondense the sperm head within the cytoplasm of the oocyte resulting in the formation of the male pronucleus. The results of this test have been correlated with the likelihood of success with in vitro fertilization. This test revealed itself a relevant tool also for the detection of spermatozoa bearing structural chromosomal abnormalities, such as unrejoined breaks and acentric fragments, of which 75% resulted in unstable aberrations (Martin and Rademaker 1987). The examination of 1582 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. (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 post-meiotic

DNA-repair-free spermatids. Another human-sperm/hamster-egg study (Sartorelli, Mazzucatto and de Pina-Neto 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). Another study based on a multicolour, 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%) (Templado et al. 2011).

Consequences on the offspring Conception with spermatozoa carrying balanced structural chromosomal rearrangements, i.e. translocations or inversions, is unlikely to affect embryogenesis or development. Children with this type of germline rearrangements are usually phenotypically normal, although problems during meiosis of their gametes may arise (Morin et al. 2017). For instance, individuals carrying balanced reciprocal translocation are subject to meiosis non-disjunction risk: the mispairing of translocated chromosomes during the first meiotic division can give rise to different forms of segregation, which can result in aneuploidy of the translocated chromosomes (Morin et al. 2017). This meiotic issue is the main cause of the increased risk of recurrent pregnancy loss in these couples.

On the other side, the vast majority of unbalanced sperm results in early embryonic arrest or spontaneous abortions due to the incompatibility of partial trisomies and monosomies with embryogenesis (Ioannou and Tempest 2015). However, some unbalanced segregation products may be clinically viable, potentially resulting in congenital malformations and/or cognitive impairment depending on the chromosome involved and the size of unbalanced segments (Wapner et al. 2012; Weckselblatt et al. 2015).

Y-Chromosome Rearrangements: The AZF Region Deletions/ Duplications

Y-chromosome microdeletions removing entirely or partially the AZF (azoospermia factor) regions occur in about 1 in 4000 men in the general population (Krausz et al. 2014). The relatively high frequency of AZF deletion indicates that this chromosome is particularly susceptible to the spontaneous loss of genetic material. The Y chromosome should be considered a genetically dynamic chromosome prone to significant variation owing to the high proportion of segmental duplications (paralogue sequences). The paralogues with same orientation provide the structural basis for the generation of copy number variations (CNVs), including AZF deletions/duplications. The most likely cellular origin of Y-chromosome CNVs are the germ cells but an embryonic origin cannot be excluded a priori (Aitken and Krausz 2001).

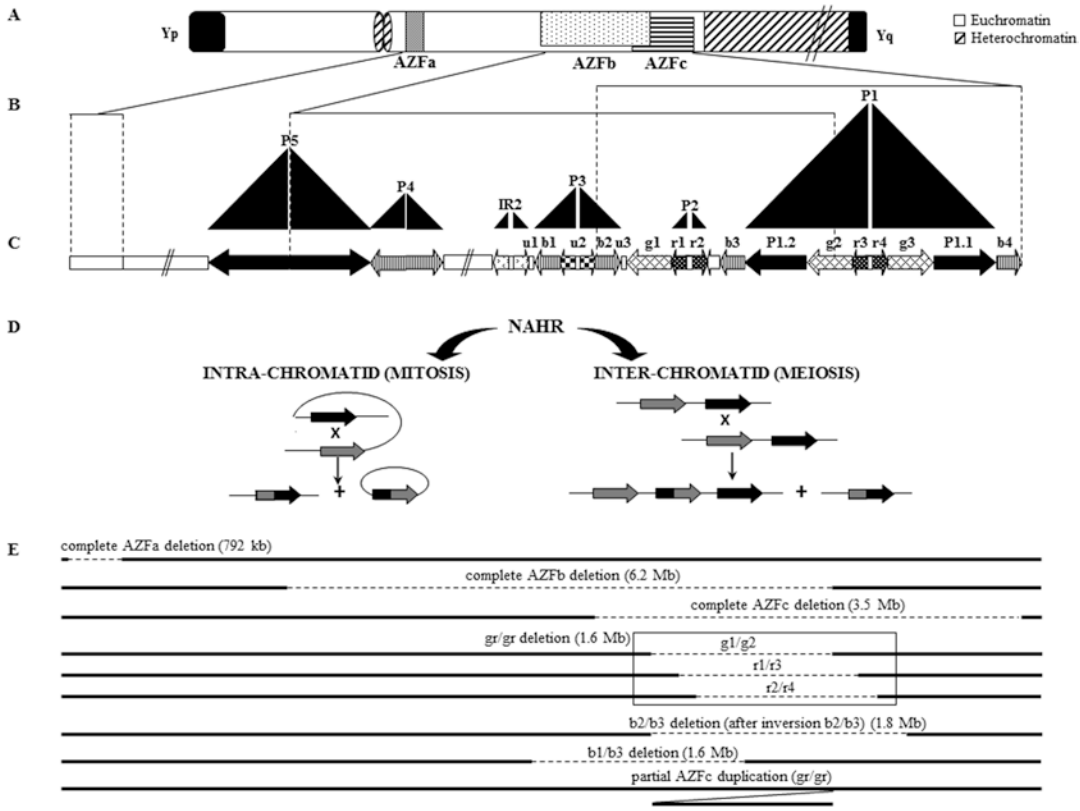

Fig. 2.1 Schematic representation of the Y chromosome, of the different amplicons involved in NAHR and of the Y-linked copy number variations. (**a**) Three azoospermia factor regions (AZFa, b and c) are located on the long arm of the Y chromosome (Yq) with an overlap between AZFb and c regions. (**b**) Triangles represent the relative sizes and the arm locations of palindromes in AZF regions (gaps between opposed triangles are the non-duplicated Y sequences). (**c**) The arrows with the same motifs represent Y-chromosome amplicons which may undergo NAHR. (**d**) Two classes of NAHR, intra-chromatid and inter-chromatid, between two duplicated sequences, shown as black and grey arrows. (**e**) The most frequent complete AZF deletions and partial AZFc deletions (gr/gr, b2/b3, b1/b3) are shown. The gr/gr deletion is depicted with three alternative breakpoints. An example of partial AZFc duplication (gr/gr) is shown (similar to the gr/gr deletion, different breakpoints may give origin to different types of gr/gr duplications)

During spermatogenesis, deletions may originate in mitotic and meiotic cells through a mechanism of non-allelic homologous recombination (Fig. 2.1). NAHR can take place in two ways: intra-chromatid during mitosis and inter-chromatid during meiosis. In the first one, paralogues on the same chromatid are involved in the recombination, and this type of NAHR generates a deletion and a circular DNA molecule, which lacks a centromere and cannot segregate at cell division. The second occurs between sister chromatids and results in reciprocal deletion and duplication. Turner and collaborators have calculated the average deletion rate, per generation, in the AZFa-HERV hotspot on the Y chromosome ($2.16 \times 10^{-5} \pm 6.69 \times 10^{-6}$) during spermatogenesis. Interestingly enough, the duplication rate was lower ($5.26 \times 10^{-6} \pm 1.58 \times 10^{-6}$) showing a ratio of deletion:duplication 4:11 (Turner et al. 2008).

The third theoretically possible cellular origin concerns the zygote. The DNA fragmentation, which appears to be commonplace in spermatozoa (Aitken 1999), has the potential to generate deletions as the chromatin unravels at fertilization. Oxidative stress appears to be particularly important since it may induce DNA fragmenta-

tion, which constitutes a promutagenic change. When the level of oxidative stress does not completely damage sperm plasma membrane, fertilization may occur, but the oocyte must repair the DNA strand breaks before the initiation of the first cleavage division. It is at this moment that deletions or sequence errors may be introduced. Any double-stranded DNA breaks would normally be repaired by homologous recombination in the few hours that elapse between fertilization and the initiation of the first cleavage division. However, this repair mechanism cannot apply to the non-recombining region of the Y chromosome, including the AZF regions, where recombination repair is impossible (Aitken and Krausz 2001).

Y-linked clinically relevant CNVs can be divided into three categories: (i) complete AZF deletions, (ii) partial AZFa and AZFb deletions and (iii) partial AZFc deletions/duplications.

The clinical consequences of the germline transmission of AZF deletions vary according to the type of deletions. In fact, the deletion intervals contain distinct genes with different roles in spermatogenesis leading to different deletion phenotypes ranging from Sertoli cell-only syndrome (SCOS) to oligozoospermia. A total of 26 genes are present in the AZF regions, and the majority of them are testis specific or overexpressed in the testis.

Complete AZF deletions The smallest deletion type, AZFa, occurs within the retroviral sequences in the same orientation HERVyq1 and HERVyq2 (Blanco et al. 2000; Kamp et al. 2000; Sun et al. 2000). The deletions of the entire AZFb and entire AZFc regions are caused by recombination of the palindrome P5 with proximal P1 and the palindrome P3 (amplicon b2) with P1 (amplicon b4), respectively (Kuroda-Kawaguchi et al. 2001; Repping et al. 2002). The deletion breakpoints indicate that the complete AZFb deletion removes also part of the AZFc region (Repping et al. 2002). The deletion, which includes simultaneously both the entire AZFb and the entire AZFc regions, is called AZFbc and has two potential breakpoints between palindromes: (i) P4/distal P1 and (ii) P5/distal P1. The AZFabc deletion involves the three AZF regions; therefore, it is most likely related to abnormal karyotype such as 46,XX male or iso(Y) (Lange et al. 2009). The most frequent Y-chromosome deletion type is the AZFc region deletion (~80%) followed by AZFa (0.5–4%), AZFb (1–5%) and AZFbc (1–3%) deletion (Krausz et al. 2014).

Consequences on the offspring The germline transmission of these deletions causes severe spermatogenic impairment in the deletion carrier. The deletions of the entire AZFa and AZFb regions are associated with azoospermia due to Sertoli cell-only syndrome (SCOS) and spermatogenic arrest (SGA). Therefore, in carriers of these two types of complete deletions, the probability of sperm recovery by testis biopsy is virtually zero. However, it is important to outline that the definition of "complete" AZFb deletion requests careful analysis of the breakpoints since "non-classical" apparently complete AZFb deletions can be compatible with intratesticular sperm production (Plotton et al. 2010; Stouffs et al. 2017; Zhang et al. 2017). The complete AZFc deletion is responsible for a variable semen phenotype ranging from severe oligozoospermia (mainly below two million spermatozoa/ml) to azoospermia (from SCOS to hypospermatogenesis) (Krausz and Casamonti 2017). Oligozoospermic AZFc deletion carriers are at risk for a progressive decrease of sperm concentration over time, therefore sperm cryoconservation could be advised as a preventive treatment (McElreavey and Krausz 1999; Krausz and Degl'Innocenti 2006) (references therein).

Partial AZFa and AZFb deletions Partial deletions of AZFa and AZFb regions are extremely rare. Concerning the AZFa region, none of the deletions occurred because of NAHR and thus are likely to be unique, supporting the extreme rarity of the occurrence of these events (Tyler-Smith and Krausz 2009). On the contrary, AZFb region is rich in palindromes which can be responsible for partial deletions based on NAHR (Soares et al. 2012).

Consequences on the offspring Concerning the AZFa region, all the five confirmed deletions (with the definition of the breakpoints) removed totally or partially the *USP9Y* gene belonging to the AZFa region leaving intact the second AZFa gene *DDX3Y* (Tyler-Smith and Krausz 2009). The semen/testis phenotype is largely variable among *USP9Y* deletion carriers (from azoospermia caused by hypospermatogenesis to normozoospermia) indicating that this gene rather acts as a fine tuner than an essential factor for spermatogenesis (Krausz et al. 2006; Luddi et al. 2009; Tyler-Smith and Krausz 2009). Since the phenotypic range of the above partial AZFa deletions contrasts with the effect of the complete AZFa deletion (associated with SCOS), a key role for *DDX3Y* in spermatogenesis is expected and further supported by its expression profile in premeiotic spermatogonia (Tyler-Smith and Krausz 2009).

Carriers of the partial AZFb deletion show various breakpoints inside the AZFb region. For instance, some of them are associated with the retention of proximal AZFb gene copies, such as *XKRY, CDY2* and *HSFY* (Rolf et al. 2002; Soares et al. 2012).

Partial AZFc deletions/duplications The high number of amplicons in AZFc predisposes this region to a series of rearrangements including partial deletions or duplications and complex rearrangements, i.e. deletions followed by single or multiple duplication of the remaining region. Three of them have been extensively studied in the literature: gr/gr, b2/b3 and b1/b3 deletions, which occur in the general population in about 2.4%, 1.1% and 0.10%, respectively (Rozen et al. 2012). The clinically most relevant is the gr/gr, which received its name after the fluorescent probes ("green" and "red") used for its discovery by Repping and colleagues (Repping et al. 2003). This rearrangement has three potential breakpoints between amplicons: (i) g1/g2, (ii) r1/r3 and (iii) r2/r4. After inversion b3/b4, amplicons involved in NAHR are (i) g1/g3, (ii) r1/r4 and (iii) r2/r3.

The b2/b3 deletion has two potential breakpoints, whereas b1/b3 has only one. The amplicons involved in NAHR responsible of b2/b3 and b1/b2 rearrangements are g1/g3 (after inversion b2/b3) or b2/b3 (after inversion gr/gr) and b1/b3, respectively.

In AZFc regions, partial duplications may occur based on NAHR and are reciprocal events to the above reported partial AZFc deletions (Repping et al. 2006).

Consequences on the offspring The gr/gr deletion represents a significant risk factor for spermatogenic impairment, indeed, according to five meta-analyses, it increases by 2–2.5-fold the risk for reduced sperm output (Tüttelmann et al. 2007; Visser et al. 2009; Navarro-Costa et al. 2010; Stouffs et al. 2011; Bansal et al. 2016). However, the entity of this risk varies between populations and shows the highest values in the Mediterranean area conferring an almost sixfold increased risk for impaired sperm production in the Italian population (Krausz and Casamonti 2017). Although this partial deletion is significantly higher in oligozoospermic men, the rearrangement is associated with a highly variable phenotype ranging from azoo- to normozoospermia. This variability in semen phenotype is largely dependent on the ethnic and geographic origin of the study population, on the basis of the Y-chromosome background; for example, in specific Y haplogroups, such as D2b, Q3 and Q1, common in Japan and certain areas of China, the deletion is fixed and apparently does not have negative effects on spermatogenesis (Sin et al. 2010; Yang et al. 2010).

The b2/b3 deletion seems to have a strong association with male infertility only in Chinese, Moroccan and South Indian populations (Wu et al. 2007; Lu et al. 2009, 2014; Eloualid et al. 2012; Vijesh et al. 2015). Concerning b1/b3, data are scarce due to its low frequency, but the analysis of 20,000 Y chromosomes found 2.5-fold increased risk of developing severe spermatogenic failure in men carrying this partial deletion (Rozen et al. 2012).

The clinical consequences of Y-chromosome duplications are still debated. In some populations, such as Han Chinese, Chinese Yi and Dutch, these are associated with impaired spermatogenesis (Lin et al. 2007; Noordam et al. 2011; Ye et al. 2013; Yang et al. 2015), whereas in the Mediterranean populations these do not seem to affect spermatogenesis (Giachini et al. 2008; Lo Giacco et al. 2014).

De Novo Point Mutation Rate During Spermatogenesis

Based on whole-genome sequencing studies of parent–offspring trios, the average generational mutation rate of single base substitutions in humans has been estimated to be ~1–1.5 × 10^{-8} (Roach et al. 2010; Conrad et al. 2011; Kong et al. 2012; Michaelson et al. 2012; Campbell and Eichler 2013). Notably, it has been calculated that one to three de novo mutations are added to the germline mutational load of the offspring for each additional year in the father's age at conception, although the magnitude of this effect differs by a factor > twofold between families (Francioli et al. 2015; Rahbari et al. 2016; Goldmann et al. 2018). The most plausible reason lies in the increasing number of cell divisions in the male germline: sperm cells have undergone approximately 100–150 mitoses in a 20-year-old man, but this number reaches 600 in a 40-year-old man, given that the male germline adds 23 mitoses per year (Crow 2000; Wilson Sayres and Makova 2011). Considering both the natural rate of de novo mutations during each replication as ~1–1.5 × 10^{-8} and the number of spermatogenesis cycles per year, it has been estimated that a 20-year-old man could acquire up to an average of 21 de novo mutations per year, accumulating an average of 420 over the subsequent 20 years (Rahbari et al. 2016). As stated in the introduction, this high number of spontaneous mutation in the male germline may explain the elevated male-to-female mutation ratio.

The mutational load during spermatogenesis further increases due to errors in mismatch repair mechanisms related to aging (Kong et al. 2012). In addition, certain non-random germline mutations provide a selective advantage on mutated spermatogonial cells, resulting in a favourable expansion of these mutated clones during spermatogenesis (see below) (Maher et al. 2014). Once this mutational event has occurred, the subsequent enrichment of mutated SSCs in the testes increases proportionally with the men's age. Thus, the major factor influencing the mutational burden inherited by offspring is the paternal age, entailing an increased risk of pathological conditions in children carrying harmful mutations.

Paternal Age Effect Mutations

The paternal age effect is an epidemiological concept describing the fact that some sporadic disorders tend to arise more frequently in the progeny of older men (Penrose 1955; Risch et al. 1987).

Beyond this epidemiological issue, de novo point mutations affecting a few well-characterized exonic sites in genes involved in RAS-MAPK pathway have been observed up to 1000-fold more frequently in offspring of older fathers (Goriely and Wilkie 2012).

Direct analysis of sperm DNA from healthy men sampled from the general population has confirmed that these mutations are present at levels substantially above the background mutation rate in most men (Maher et al. 2014), and the average mutation levels correlate with paternal age. This phenomenon cannot be explained only by the copy-error hypothesis (age-dependent accumulation of recurrent mutations taking place within localized hypermutable DNA hotspots), but a clonal expansion of SSCs expressing proteins with gain-of-function properties is also needed to the relative enrichment of mutant sperm over time. The combination of these two mechanisms would account for the observed paternal age effect and the relatively high birth rate associated with these specific mutations (Goriely and Wilkie 2012). Certainly, initial copy errors have to take place during spermatogenesis for this process to occur; therefore, the endogenous mutability and local sequence context of a nucleotide contribute to this process. In addition,

over time, SSCs carrying these mutations undergo positive selection owing to higher self-renewal than surrounding wild-type cells and expand in the testis (Maher et al. 2016a). This selective process taking place in the testis and likely occurring in all men has been termed selfish spermatogonial selection. A localized clonal expansion mechanism has been demonstrated for selfish mutations in five genes (*FGFR2*, *FGFR3*, *PTPN11*, *RET* and *KRAS*) by dissecting whole human testis into approximately 200 pieces and testing them for specific mutations, suggesting that these de novo mutations have a restricted spatial distribution with small regions containing high number of mutated cells, surrounded by larger mutation-free regions (Lim et al. 2012; Maher et al. 2014; Maher et al. 2016a, b).

Beyond these traditionally PAE genes, very recently, by combining systematic dissection of testicular biopsies with massively parallel simplex PCR and ultradeep sequencing of mutational hotspots dysregulating RAS-MAPK signalling, six new genes (*BRAF*, *CBL*, *MAP2K1*, *MAP2K2*, *RAF1* and *SOS1*) have been associated with this selfish selection in the male germline (Maher et al. 2018).

It is important to underline that these selfish mutations in spermatogonia have considerable implications not only for congenital disease in offspring (see below), but also for tumorigenesis in the host (Hansen et al. 2005). In fact, selfish behaviour is a characteristic of certain mutations driving cancer as they lead to positive cellular selection despite being harmful for the organism. Accordingly, several mutations behaving selfishly in SSCs have also been identified as somatic events driving clonal growth in tumorigenesis of several types of cancers, including endometrial and bladder cancers (Maher, Goriely and Wilkie 2014). Clonal expansion associated with the selective advantage conferred to the mutant SSCs by selfish mutation can also contribute to the pathogenesis of spermatocytic seminoma (Goriely et al. 2009), a rare germ cell tumour comprising less than 5% of seminomas. Unlike classical seminoma that mostly affects young adults and originates during embryonic gonadal development, spermatocytic seminoma derives from adult spermatogonia and is found specifically in older men (the mean age at diagnosis is around 54 years) (Eble 1994; Rajpert-De Meyts 2007; Lim et al. 2011). By the screening of 30 spermatocytic seminoma samples for mutations in PAE-associated and other candidate genes, it was found that mutation-positive samples (especially in *FGFR3* and *HRAS* genes) were from significantly older patients than the mutation-negative ones, suggesting that two genetically and epidemiologically distinct groups of spermatocytic seminomas may exist (Goriely et al. 2009; Giannoulatou et al. 2013). In the light of this evidence, PAE mutations should also be considered as "somatic" mutations, potentially leading to both a specific syndrome through germline transmission and an oncogenic process in the testis.

Consequences of PAE Mutations in the Offspring

The most common clinical outcome of the selfish spermatogonial selection is the birth of a child with a PAE disorder (Table 2.1). This condition, caused by dominant heterozygous mutation in a candidate gene, has been characterized by (i) an extreme bias in paternal origin of mutations (defined as male-to-female mutation ratio > 20) (Risch et al. 1987), (ii) a strong paternal age effect and (iii) a high apparent germline mutation rate ($>10^{-6}$ for particular individual mutations) (Green et al. 2010).

Among PAE disorders, achondroplasia, the most common cause of dwarfism, and Apert syndrome, characterized by craniosynostosis and severe syndactyly of both hands and feet, are the best representative examples. Both conditions are characterized by specific mutations in the fibroblast growth factor receptor (*FGFR*) genes. In particular, about 99% of patients with Apert syndrome carries one of the two mutations (c.755C > G; p.Ser252Trp or c.758C > G;p.Pro253Arg) of the *FGFR2* gene (Wilkie et al. 1995). On the other hand, about 98% of achondroplasia cases are caused by c.1138G > A; p.Gly380Arg mutation of *FGFR3* gene, and only in about 1% the transversion G to C at position 1138 was found (Rousseau et al. 1994; Bellus et al. 1995).

Table 2.1 Loci with evidence for selfish selection in human testis causing a germline disorder. Each PAE gene with chromosomal location, protein family, selfish mutations and associated germline disorder was reported together with corresponding references

Gene symbol	Chr. location	Protein family	Selfish mutation	Germline disorder	References
FGFR2	10q26.13	Receptor tyrosine kinase	c.755C > G; p.Ser252Trp	Apert syndrome	Goriely et al. (2003, 2005), Qin et al. (2007), Choi et al. (2008), Yoon et al. (2009), Maher et al. (2018)
			c.755C > T; p.Ser252Trp	Crouzon/Pfeiffer syndrome	Goriely et al. (2003, 2005)
			c. 758C > G; p.Pro253Arg	Apert syndrome	Choi et al. (2008), Yoon et al. (2009), Maher et al. (2016a, b, 2018)
			c.866A > C;p.Gln289Pro	Crouzon syndrome	Maher et al. (2018)
			c.870G > T; p.Trp290Cys	Pfeiffer syndrome	Maher et al. (2016a, 2018)
			c.1019A > G; p.Tyr340Cys	Pfeiffer syndrome	Maher et al. (2016a)
			c.1024T > A; p.Cys342Ser	Crouzon/Pfeiffer syndrome	Lim et al. (2012), Maher et al. (2016a, 2018)
			c.1052C > G; p.Ser351Cys	Pfeiffer syndrome	Maher et al. (2018)
			c.1115C > G; p.Ser372Cys	Beare–Stevenson syndrome	Maher et al. (2018)
FGFR3	4p16.3	Receptor tyrosine kinase	c.742C > T; p.Arg248Cys	Thanatophoric dysplasia I	Maher et al. (2016a)
			c.749C > G; p.Pro250Arg	Muenke syndrome	Rannan-Eliya et al. (2004)
			c.1118A > G; p.Tyr373Lys	Thanatophoric dysplasia I	Maher et al. (2016a)
			c.1138G > A; p.Gly380Arg	Achondroplasia	Tiemann-Boege et al. (2002), Dakouane Giudicelli et al. (2008), Shinde et al. (2013)
			c.1620G > T/C; p.Asn540Lys	Hypochondroplasia	Maher et al. (2018)
			c.1948A > G; p.Lys650Glu	Thanatophoric dysplasia, type II	Goriely et al. (2009), Maher et al. (2016a)
			c.1948A > C; p.Lys650Gln	Hypochondroplasia, acanthosis nigricans	Goriely et al. (2009)
			c.1949A > C; p.Gly650Thr	Acanthosis nigricans, short stature	Goriely et al. (2009)
			c.1949A > T; p.Gly650Met	Severe achondroplasia, developmental delay, acanthosis nigricans; thanatophoric dysplasia I	Goriely et al. (2009)
			c.1950G > T; p.Gly650Asn	Hypochondroplasia	Goriely et al. (2009)
HRAS	3q29	Small GTP-ase	c.34G > A; p.Gly12Ser	Costello syndrome	Giannoulatou et al. (2013)
			c.34G > T; p.Gly12Cys		
			c.35G > A; p.Gly12Asp		
			c.35G > T; p.Gly12Val		

(continued)

Table 2.1 (continued)

Gene symbol	Chr. location	Protein family	Selfish mutation	Germline disorder	References
PTPN11	12q24.13	Protein tyrosine phosphatase	c.182A > C; p.Asp61Ala	Noonan syndrome	Maher et al. (2018)
			c.182A > G; p.Asp61Gly		
			c.211T > C; p.Phe71Leu		
			c.214G > C; p.Ala72Pro		
			c.215C > G; p.Ala72Gly		
			c.218C > T; p.Thr73Ile		
			c.417G > T; p.Glu139Asp		
			c.854T > G; p.Phe285Cys		
			c.922A > G; p.Asn308Asp		Yoon et al. (2013), Eboreime et al. (2016), Maher et al. (2018)
			c.1510A > G; p.Met504Val		Maher et al. (2018)
RET	10q11.21	Receptor tyrosine kinase	c.2370G > C/T;p.Leu790Phe	Men2A	Maher et al. (2018)
			c.2692G > T; p.Asp898Tyr	Men2	Maher et al. (2018)
			c.2753T > C; p.Met918Tyr	Men2B	Choi et al. (2012)
RAF1	3p25.2	Serine/threonine kinase	c.770C > T; p.Ser257Leu	Noonan syndrome	Maher et al. (2018)
			c.781C > G; p.Pro261Ala		

Among the other *FGFR*-associated disorders belonging to the PAE diseases, Crouzon and Pfeiffer syndromes are clinically overlapping conditions typically caused by any of the more than 50 specific activating point mutations in *FGFR2* (Kan et al. 2002), whereas Muenke syndrome is characterized by coronal craniosynostosis and caused by a single transversion in *FGFR3* gene (c.749C > G; p.Pro250Arg) (Vajo, Francomano and Wilkin 2000).

Among the other PAE disorders, Costello and Noonan syndromes are part of a larger family of neuro-cardio-facial-cutaneous syndromes or RASopathies, referring to the dysregulation of the RAS signalling pathway (Aoki et al. 2008). Up to 90% of Costello syndrome patients carries a mutation in the *HRAS* gene (c.34G > A; p.Gly12Ser) at a well-known mutation hotspot in tumorigenesis, whereas about half of Noonan syndrome mutations is detected within the *PTPN11* gene (encoding SHP2-containing tyrosine phosphatase) (Aoki et al. 2008; Kratz et al. 2009; Tartaglia, Zampino and Gelb 2010).

The other two PAE disorders, multiple endocrine neoplasia types 2A (Men2A) and 2B (Men2B), are caused by mutations within the RET receptor tyrosine kinase and are complex syndromes of multiple endocrine neoplasms, characteristically including medullary thyroid carcinoma. Men2A is mostly caused by a handful of activating mutations at crucial cysteine residues located in the extracellular portion of RET, whereas most cases of Men2B, the most aggressive form, are caused by a single substitution (c.2753T > C; p.Met918Thr) at a residue located in the catalytic core of the tyrosine kinase domain (Raue and Frank-Raue 2010).

It is worth noting that other pathological conditions exhibiting a large phenotypic and molecular overlap with the disorders described above, including thanatophoric dysplasia (caused by *FGFR3* mutations) and cardio-facio-cutaneous syndrome (associated with *BRAF*, *MAP2K1* and *MAP2K2* mutations) are likely to belong to the PAE class, although some of the experimental evidence for meeting all three PAE criteria is still lacking (Goriely and Wilkie 2012).

It is important to underline that PAE mutations are unlikely to segregate for many generations given the severe phenotypes they cause; thus, PAE disorders fortunately have a low reproductive fitness. Conversely, selfish mutations associated with a weaker selective advantage in the testis and leading to lower levels of enrichment in sperm can be transmitted over many generations, representing a contribution to genetic variability. In this context, there is a growing body of epidemiological studies linking advanced paternal age with several common neurodevelopmental diseases, including schizophrenia (Malaspina et al. 2001, 2002; Brown et al. 2002; Byrne et al. 2003) and autism (Durkin et al. 2008; Tsuchiya et al. 2008; Grether et al. 2009; D'Onofrio et al. 2014). In fact, a 2.96-fold relative risk of schizophrenia among offspring of fathers >50 years of age compared to those of 20–24 years old has been observed (Malaspina et al. 2001). In addition, a strong association between offspring with autism spectrum disorders and advanced paternal age has been demonstrated by D'Onofrio and colleagues, indicating that father's age > 45 years old increases by 3.45-fold, 13.1-fold and 2.07-fold the risk for autism, attention deficit hyperactivity disorder and psychosis, respectively (D'Onofrio et al. 2014). Although some of these studies show confounding factors, such as maternal age at conception, selfish selection provides one plausible mechanism. In fact, RAS-MAPK signalling plays also an important role in brain development, learning memory, synaptic plasticity and cognition (Krab et al. 2008; Samuels et al. 2009), and alterations of this pathway may contribute to learning disability, neurocognitive disorders (Goriely et al. 2013), autism (Pinto et al. 2010; Gilman et al. 2011) and schizophrenia (Kalkman 2006; Klejbor et al. 2006; Kéri et al. 2009; Kim et al. 2009). Unfortunately, no information on parental origin and paternal age is available in most of these studies, and further evidences are needed to confirm the role of selfish selection in the pathogenesis of neurocognitive disorders.

Conclusions

Advances in sequencing technologies have allowed the analysis of de novo mutations at genome scale providing insight into the origins of these muta-

tions. The trio-based sequencing of children and their parents has shown that most of mutations originate from the paternal germline (Francioli et al. 2015; Rahbari et al. 2016; Goldmann et al. 2018). Hence, the male germline appears to be particularly susceptible to spontaneous mutations, which are essential to create the genetic diversity that promoted evolution. However, the occurrence of such alterations in male germ cells has also important health implications since any damage to reproductive cells might produce negative effects on the descendants, with potential consequences on the fitness of future generations. In this chapter, we gave an overview on paternally derived genetic diseases resulting from chromosomal aneuploidies, from structural rearrangements (including AZF deletions) and from selfish monogenic mutations. All these anomalies tend to occur and accumulate over time in germ cells leading to a higher risk of germline transmission of chromosomal anomalies and specific PAE mutations. Given the ever-spreading phenomenon of postponing fatherhood until older ages, it is important that both clinicians and couples are aware about potential age-related health risks due to spontaneous mutations during spermatogenesis.

References

Aitken RJ (1999) The Amoroso Lecture. The human spermatozoon--a cell in crisis? J Reprod Fertil 115(1):1–7

Aitken RJ, Krausz C (2001) Oxidative stress, DNA damage and the Y chromosome. Reproduction (Cambridge, England) 122(4):497–506

Aksglaede L, Juul A (2013) Therapy of endocrine disease: testicular function and fertility in men with Klinefelter syndrome: a review. Eur J Endocrinol 168(4):R67–R76

Aoki Y et al (2008) The RAS/MAPK syndromes: novel roles of the RAS pathway in human genetic disorders. Hum Mutat 29(8):992–1006

Asada H et al (2000) The effects of age and abnormal sperm count on the nondisjunction of spermatozoa. J Assist Reprod Genet 17(1):51–59

Bansal SK et al (2016) Gr/gr deletions on Y-chromosome correlate with male infertility: an original study, meta-analyses, and trial sequential analyses. Sci Rep 6(1):19798

Baptista J et al (2008) Breakpoint mapping and array CGH in translocations: comparison of a phenotypically normal and an abnormal cohort. Am J Hum Genet 82(4):927–936

Bellus GA et al (1995) Achondroplasia is defined by recurrent G380R mutations of FGFR3. Am J Hum Genet 56(2):368–373

Besenbacher S et al (2015) Novel variation and de novo mutation rates in population-wide de novo assembled Danish trios. Nat Commun 6(1):5969

Blanco P et al (2000) Divergent outcomes of intrachromosomal recombination on the human Y chromosome: male infertility and recurrent polymorphism. J Med Genet 37(10):752–758

Bosch M et al (2001) Linear increase of diploidy in human sperm with age: a four-colour FISH study. Eur J Hum Genet 9(7):533–538

Bosch M et al (2003) Linear increase of structural and numerical chromosome 9 abnormalities in human sperm regarding age. Eur J Hum Genet 11(10):754–759

Brown AS et al (2002) Paternal age and risk of schizophrenia in adult offspring. Am J Psychiatry 159(9):1528–1533

Byrne M et al (2003) Parental age and risk of schizophrenia: a case-control study. Arch Gen Psychiatry 60(7):673–678

Calogero AE et al (2017) Klinefelter syndrome: cardiovascular abnormalities and metabolic disorders. J Endocrinol Invest 40(7):705–712

Campbell CD, Eichler EE (2013) Properties and rates of germline mutations in humans. Trends Genet 29(10):575–584

Campbell IM et al (2014) Parent of origin, mosaicism, and recurrence risk: probabilistic modeling explains the broken symmetry of transmission genetics. Am J Hum Genet 95(4):345–359

Chianese C, Brilli S, Krausz C (2014) Genomic changes in spermatozoa of the aging male. Adv Exp Med Biol 791:13–26

Choi S-K et al (2008) A germ-line-selective advantage rather than an increased mutation rate can explain some unexpectedly common human disease mutations. Proc Natl Acad Sci U S A 105(29):10143–10148

Choi S-K et al (2012) Positive selection for new disease mutations in the human germline: evidence from the heritable cancer syndrome multiple endocrine neoplasia type 2B. PLoS Genet. Payseur BA (ed) 8(2):e1002420

Chong JX et al (2015) The genetic basis of mendelian phenotypes: discoveries, challenges, and opportunities. Am J Hum Genet 97(2):199–215

Conrad DF et al (2011) Variation in genome-wide mutation rates within and between human families. Nat Genet 43(7):712–714

Crow JF (2000) The origins, patterns and implications of human spontaneous mutation. Nat Rev Genet 1(1):40–47

D'Onofrio BM et al (2014) Paternal age at childbearing and offspring psychiatric and academic morbidity. JAMA Psychiat 71(4):432

Dakouane Giudicelli M et al (2008) Increased achondroplasia mutation frequency with advanced age and evidence for G1138A mosaicism in human testis biopsies. Fertil Steril 89(6):1651–1656

de Ligt J et al (2012) Diagnostic exome sequencing in persons with severe intellectual disability. N Engl J Med 367(20):1921–1929

Deciphering Developmental Disorders Study, Fitzgerald TW, et al (2015) Large-scale discovery of novel genetic causes of developmental disorders. Nature 519(7542):223–228

Durkin MS et al (2008) Advanced parental age and the risk of autism spectrum disorder. Am J Epidemiol 168(11):1268–1276

Eble JN (1994) Spermatocytic seminoma. Hum Pathol 25(10):1035–1042

Eboreime J et al (2016) Estimating exceptionally rare germline and somatic mutation frequencies via next generation sequencing. PLoS One. Lo AWI (ed) 11(6):e0158340

Eloualid A et al (2012) Association of spermatogenic failure with the b2/b3 partial AZFc deletion. PLoS One. Chadwick BP (ed) 7(4):e34902

Epi4K Consortium et al (2013) De novo mutations in epileptic encephalopathies. Nature 501(7466):217–221

Francioli LC et al (2015) Genome-wide patterns and properties of de novo mutations in humans. Nat Genet 47(7):822–826

Gao Z et al (2016) Interpreting the dependence of mutation rates on age and time. PLoS Biol. Barton NH (ed) 14(1):e1002355

Giachini C et al (2008) Partial AZFc deletions and duplications: clinical correlates in the Italian population. Hum Genet 124(4):399–410

Giannoulatou E et al (2013) Contributions of intrinsic mutation rate and selfish selection to levels of de novo HRAS mutations in the paternal germline. Proc Natl Acad Sci 110(50):20152–20157

Gilman SR et al (2011) Rare de novo variants associated with autism implicate a large functional network of genes involved in formation and function of synapses. Neuron 70(5):898–907

Goldmann JM et al (2018) Author Correction: parent-of-origin-specific signatures of de novo mutations. Nat Genet 50(11):1615

Goriely A (2016) Decoding germline de novo point mutations. Nat Genet 48(8):823–824

Goriely A, Wilkie AOM (2012) Paternal age effect mutations and selfish Spermatogonial selection: causes and consequences for human disease. Am J Hum Genet 90(2):175–200

Goriely A et al (2003) Evidence for selective advantage of pathogenic FGFR2 mutations in the male germ line. Science (New York, NY) 301(5633):643–646

Goriely A et al (2005) Gain-of-function amino acid substitutions drive positive selection of FGFR2 mutations in human spermatogonia. Proc Natl Acad Sci 102(17):6051–6056

Goriely A et al (2009) Activating mutations in FGFR3 and HRAS reveal a shared genetic origin for congenital disorders and testicular tumors. Nat Genet 41(11):1247–1252

Goriely A et al (2013) Selfish spermatogonial selection: a novel mechanism for the association between advanced paternal age and neurodevelopmental disorders. Am J Psychiatry. Europe PMC Funders 170(6):599–608

Green RF et al (2010) Association of paternal age and risk for major congenital anomalies from the National Birth Defects Prevention Study, 1997 to 2004. Ann Epidemiol 20(3):241–249

Grether JK et al (2009) Risk of autism and increasing maternal and paternal age in a large north American population. Am J Epidemiol 170(9):1118–1126

Griffin DK et al (1995) Non-disjunction in human sperm: evidence for an effect of increasing paternal age. Hum Mol Genet 4(12):2227–2232

Guttenbach M et al (2000) Meiotic nondisjunction of chromosomes 1, 17, 18, X, and Y in men more than 80 years of age. Biol Reprod 63(6):1727–1729

Hansen RMS et al (2005) Fibroblast growth factor receptor 2, gain-of-function mutations, and tumourigenesis: investigating a potential link. J Pathol 207(1):27–31

Hassold TJ (1998) Nondisjunction in the human male. Curr Top Dev Biol 37:383–406

Hassold T, Hunt P (2001) To err (meiotically) is human: the genesis of human aneuploidy. Nat Rev Genet 2(4):280–291

Hassold T, Hunt P (2009) Maternal age and chromosomally abnormal pregnancies: what we know and what we wish we knew. Curr Opin Pediatr 21(6):703–708

Hassold T, Hunt PA, Sherman S (1993) Trisomy in humans: incidence, origin and etiology. Curr Opin Genet Dev 3(3):398–403

Hoischen A, Krumm N, Eichler EE (2014) Prioritization of neurodevelopmental disease genes by discovery of new mutations. Nat Neurosci 17(6):764–772

Ioannou D, Tempest HG (2015) Meiotic nondisjunction: insights into the origin and significance of aneuploidy in human spermatozoa. Adv Exp Med Biol 868:1–21

Ioannou D, Fortun J, Tempest H (2018) Meiotic nondisjunction and sperm aneuploidy in humans. Reproduction. https://doi.org/10.1530/REP-18-0318. PMID: 30390610

Iossifov I et al (2014) The contribution of de novo coding mutations to autism spectrum disorder. Nature 515(7526):216–221

Kalkman HO (2006) The role of the phosphatidylinositide 3-kinase-protein kinase B pathway in schizophrenia. Pharmacol Ther 110(1):117–134

Kamp C et al (2000) Two long homologous retroviral sequence blocks in proximal Yq11 cause AZFa microdeletions as a result of intrachromosomal recombination events. Hum Mol Genet 9(17):2563–2572

Kan S et al (2002) Genomic screening of fibroblast growth-factor receptor 2 reveals a wide spectrum of mutations in patients with syndromic craniosynostosis. Am J Hum Genet 70(2):472–486

Kéri S et al (2009) Neuregulin 1-stimulated phosphorylation of AKT in psychotic disorders and its relationship with neurocognitive functions. Neurochem Int 55(7):606–609

Kim JY et al (2009) DISC1 regulates new neuron development in the adult brain via modulation of AKT-mTOR signaling through KIAA1212. Neuron 63(6):761–773

Kim IW et al (2013) 47,XYY syndrome and male infertility. Rev Urol 15(4):188–196
Kinakin B, Rademaker A, Martin R (1997) Paternal age effect of YY aneuploidy in human sperm, as assessed by fluorescence in situ hybridization. Cytogenet Cell Genet 78(2):116–119
Klejbor I et al (2006) Fibroblast growth factor receptor signaling affects development and function of dopamine neurons – inhibition results in a schizophrenia-like syndrome in transgenic mice. J Neurochem 97(5):1243–1258
Kong A et al (2012) Rate of de novo mutations and the importance of father's age to disease risk. Nature 488(7412):471–475
Krab LC, Goorden SMI, Elgersma Y (2008) Oncogenes on my mind: ERK and MTOR signaling in cognitive diseases. Trends Genet 24(10):498–510
Kratz CP et al (2009) Craniosynostosis in patients with Noonan syndrome caused by germline KRAS mutations. Am J Med Genet A 149A(5):1036–1040
Krausz C, Casamonti E (2017) Spermatogenic failure and the Y chromosome. Hum Genet 136(5):637–655
Krausz C, Degl'Innocenti S (2006) Y chromosome and male infertility: update, 2006. Front Biosci 11:3049–3061
Krausz C et al (2006) Natural transmission of USP9Y gene mutations: a new perspective on the role of AZFa genes in male fertility. Hum Mol Genet 15(18):2673–2681
Krausz C et al (2014) EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions: state-of-the-art 2013. Andrology 2(1):5–19
Kurahashi H et al (2009) Recent advance in our understanding of the molecular nature of chromosomal abnormalities. J Hum Genet 54(5):253–260
Kuroda-Kawaguchi T et al (2001) The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. Nat Genet 29(3):279–286
Lange J et al (2009) Isodicentric Y chromosomes and sex disorders as byproducts of homologous recombination that maintains palindromes. Cell 138(5):855–869
Lim J et al (2011) OCT2, SSX and SAGE1 reveal the phenotypic heterogeneity of spermatocytic seminoma reflecting distinct subpopulations of spermatogonia. J Pathol 224(4):473–483
Lim J et al (2012) Selfish spermatogonial selection: evidence from an immunohistochemical screen in testes of elderly men. PLoS One. Shipley J (ed) 7(8):e42382
Lin Y-W et al (2007) Partial duplication at AZFc on the Y chromosome is a risk factor for impaired spermatogenesis in Han Chinese in Taiwan. Hum Mutat 28(5):486–494
Lo Giacco D et al (2014) Clinical relevance of Y-linked CNV screening in male infertility: new insights based on the 8-year experience of a diagnostic genetic laboratory. Eur J Hum Genet 22(6):754–761
Lowe X et al (2001) Frequency of XY sperm increases with age in fathers of boys with Klinefelter syndrome. Am J Hum Genet 69(5):1046–1054
Lu C et al (2009) The b2/b3 subdeletion shows higher risk of spermatogenic failure and higher frequency of complete AZFc deletion than the gr/gr subdeletion in a Chinese population. Hum Mol Genet 18(6):1122–1130. https://doi.org/10.1093/hmg/ddn427
Lu C et al (2014) Gene copy number alterations in the azoospermia-associated AZFc region and their effect on spermatogenic impairment. Mol Hum Reprod 20(9):836–843
Luddi A et al (2009) Spermatogenesis in a man with complete deletion of USP9Y. N Engl J Med 360(9):881–885
Luetjens CM et al (2002) Sperm aneuploidy rates in younger and older men. Hum Reprod 17(7):1826–1832
Maher GJ, Goriely A, Wilkie AOM (2014) Cellular evidence for selfish spermatogonial selection in aged human testes. Andrology 2(3):304–314
Maher GJ et al (2016a) Cellular correlates of selfish spermatogonial selection. Andrology 4(3):550–553
Maher GJ et al (2016b) Visualizing the origins of selfish de novo mutations in individual seminiferous tubules of human testes. Proc Natl Acad Sci U S A 113(9):2454–2459
Maher GJ et al (2018) Selfish mutations dysregulating RAS-MAPK signaling are pervasive in aged human testes. Genome Res 28:1779
Makova KD, Li W-H (2002) Strong male-driven evolution of DNA sequences in humans and apes. Nature 416(6881):624–626
Malaspina D et al (2001) Advancing paternal age and the risk of schizophrenia. Arch Gen Psychiatry 58(4):361–367
Malaspina D et al (2002) Paternal age and sporadic schizophrenia: evidence for de novo mutations. Am J Med Genet 114(3):299–303
Martin RH, Rademaker AW (1987) The effect of age on the frequency of sperm chromosomal abnormalities in normal men. Am J Hum Genet 41(3):484–492
Martin RH et al (1995) The relationship between paternal age, sex ratios, and aneuploidy frequencies in human sperm, as assessed by multicolor FISH. Am J Hum Genet 57(6):1395–1399
McElreavey K, Krausz C (1999) Sex chromosome genetics '99. Male infertility and the Y chromosome. Am J Hum Genet 64(4):928–933
McInnes B et al (1998) Abnormalities for chromosomes 13 and 21 detected in spermatozoa from infertile men. Hum Reprod 13(1O):2787–2790
Michaelson JJ et al (2012) Whole-genome sequencing in autism identifies hot spots for de novo germline mutation. Cell 151(7):1431–1442
Morin SJ et al (2017) Translocations, inversions and other chromosome rearrangements. Fertil Steril 107(1):19–26
Morris JK et al (2008) Is the prevalence of Klinefelter syndrome increasing? Eur J Hum Genet 16(2):163–170
Navarro-Costa P, Gonçalves J, Plancha CE (2010) The AZFc region of the Y chromosome: at the crossroads between genetic diversity and male infertility. Hum Reprod Update 16(5):525–542

Noordam MJ et al (2011) Gene copy number reduction in the azoospermia factor c (AZFc) region and its effect on total motile sperm count. Hum Mol Genet 20(12):2457–2463

O'Roak BJ et al (2011) Exome sequencing in sporadic autism spectrum disorders identifies severe de novo mutations. Nat Genet 43(6):585–589

Ohye T et al (2010) Paternal origin of the de novo constitutional t(11;22)(q23;q11). Eur J Hum Genet 18(7):783–787

Olson SB, Magenis RE (1988) Preferential paternal origin of de novo structural chromosome rearrangments. In: Daniels A (ed) Progress and topics in cytogenetics. The cytogenetics of mammalian autosomal rearrangements, vol 8. Liss, New York. pp 585–599

Paul C, Robaire B (2013) Ageing of the male germ line. Nat Rev Urol 10(4):227–234

Penrose LS (1955) Parental age and mutation. Lancet (London, England) 269(6885):312–313

Pinto D et al (2010) Functional impact of global rare copy number variation in autism spectrum disorders. Nature 466(7304):368–372

Plotton I et al (2010) Transmissible microdeletion of the Y-chromosome encompassing two DAZ copies, four RBMY1 copies, and both PRY copies. Fertil Steril 94(7):2770.e11–2770.e16

Qin J et al (2007) The molecular anatomy of spontaneous germline mutations in human testes. PLoS Biol. Crow J (ed) 5(9):e224

Rahbari R et al (2016) Timing, rates and spectra of human germline mutation. Nat Genet 48(2):126–133

Rajpert-De Meyts E (2007) Recent advances and future directions in research on testicular germ cell cancer. Int J Androl 30(4):192–197

Rannan-Eliya SV et al (2004) Paternal origin of FGFR3 mutations in Muenke-type craniosynostosis. Hum Genet 115(3):200–207

Rauch A et al (2012) Range of genetic mutations associated with severe non-syndromic sporadic intellectual disability: an exome sequencing study. Lancet (London, England) 380(9854):1674–1682

Raue F, Frank-Raue K (2010) Update multiple endocrine neoplasia type 2. Fam Cancer 9(3):449–457

Repping S et al (2002) Recombination between palindromes P5 and P1 on the human Y chromosome causes massive deletions and spermatogenic failure. Am J Hum Genet 71(4):906–922

Repping S et al (2003) Polymorphism for a 1.6-Mb deletion of the human Y chromosome persists through balance between recurrent mutation and haploid selection. Nat Genet 35(3):247–251

Repping S et al (2006) High mutation rates have driven extensive structural polymorphism among human Y chromosomes. Nat Genet 38(4):463–467

Risch N et al (1987) Spontaneous mutation and parental age in humans. Am J Hum Genet 41(2):218–248

Roach JC et al (2010) Analysis of genetic inheritance in a family quartet by whole-genome sequencing. Science 328(5978):636–639

Robbins WA et al (1997) Use of fluorescence in situ hybridization (FISH) to assess effects of smoking, caffeine, and alcohol on aneuploidy load in sperm of healthy men. Environ Mol Mutagen 30(2):175–183

Rolf C et al (2002) Natural transmission of a partial AZFb deletion of the Y chromosome over three generations: case report. Hum Reprod 17(9):2267–2271

Rousseau F et al (1994) Mutations in the gene encoding fibroblast growth factor receptor-3 in achondroplasia. Nature 371(6494):252–254. https://doi.org/10.1038/371252a0

Rousseaux S et al (1998) Disomy rates for chromosomes 14 and 21 studied by fluorescent in-situ hybridization in spermatozoa from three men over 60 years of age. Mol Hum Reprod 4(7):695–699

Rozen SG et al (2012) AZFc deletions and spermatogenic failure: a population-based survey of 20,000 Y chromosomes. Am J Hum Genet 91(5):890–896. https://doi.org/10.1016/j.ajhg.2012.09.003

Rubes J et al (1998) Smoking cigarettes is associated with increased sperm disomy in teenage men. Fertil Steril 70(4):715–723

Samuels IS, Saitta SC, Landreth GE (2009) MAP'ing CNS development and cognition: an ERKsome process. Neuron 61(2):160–167

Sartorelli EM, Mazzucatto LF, de Pina-Neto JM (2001) Effect of paternal age on human sperm chromosomes. Fertil Steril 76(6):1119–1123

Shankar RK, Backeljauw PF (2018) Current best practice in the management of Turner syndrome. Ther Adv Endocrinol Metab. SAGE Publications 9(1):33

Shendure J, Akey JM (2015) The origins, determinants, and consequences of human mutations. Science (New York, NY) 349(6255):1478–1483

Shinde DN et al (2013) New evidence for positive selection helps explain the paternal age effect observed in achondroplasia. Hum Mol Genet 22(20):4117–4126

Sin H-S et al (2010) Features of constitutive gr/gr deletion in a Japanese population. Hum Reprod 25(9):2396–2403

Sloter E et al (2004) Effects of male age on the frequencies of germinal and heritable chromosomal abnormalities in humans and rodents. Fertil Steril 81(4):925–943

Sloter ED et al (2007) Frequency of human sperm carrying structural aberrations of chromosome 1 increases with advancing age. Fertil Steril 87(5):1077–1086

Soares AR et al (2012) AZFb microdeletions and oligozoospermia--which mechanisms? Fertil Steril 97(4):858–863

Stouffs K et al (2011) What about gr/gr deletions and male infertility? Systematic review and meta-analysis. Hum Reprod Update 17(2):197–209

Stouffs K et al (2017) Are AZFb deletions always incompatible with sperm production? Andrology 5(4):691–694

Sun C et al (2000) Deletion of azoospermia factor a (AZFa) region of human Y chromosome caused by recombination between HERV15 proviruses. Hum Mol Genet 9(15):2291–2296

Tartaglia M, Zampino G, Gelb BD (2010) Noonan syndrome: clinical aspects and molecular pathogenesis. Mol Syndromol 1(1):2–26

Taylor J et al (2006) Strong and weak male mutation bias at different sites in the primate genomes: insights from the human-chimpanzee comparison. Mol Biol Evol 23(3):565–573

Templado C et al (2011) Advanced age increases chromosome structural abnormalities in human spermatozoa. Eur J Hum Genet 19(2):145–151

Thomas NS et al (2006) Parental and chromosomal origin of unbalanced de novo structural chromosome abnormalities in man. Hum Genet 119(4):444–450

Thomas NS et al (2010) De novo apparently balanced translocations in man are predominantly paternal in origin and associated with a significant increase in paternal age. J Med Genet 47(2):112–115

Tiemann-Boege I et al (2002) The observed human sperm mutation frequency cannot explain the achondroplasia paternal age effect. Proc Natl Acad Sci U S A 99(23):14952–14957

Tsuchiya KJ et al (2008) Paternal age at birth and high-functioning autistic-spectrum disorder in offspring. Br J Psychiatry 193(4):316–321

Turner DJ et al (2008) Germline rates of de novo meiotic deletions and duplications causing several genomic disorders. Nat Genet 40(1):90–95

Tüttelmann F et al (2007) Gene polymorphisms and male infertility--a meta-analysis and literature review. Reprod Biomed Online 15(6):643–658

Tyler-Smith C, Krausz C (2009) The will-o'-the-wisp of genetics — hunting for the azoospermia factor gene. N Engl J Med 360(9):925–927

Vajo Z, Francomano CA, Wilkin DJ (2000) The molecular and genetic basis of fibroblast growth factor receptor 3 disorders: the achondroplasia family of skeletal dysplasias, Muenke craniosynostosis, and Crouzon syndrome with acanthosis nigricans. Endocr Rev 21(1):23–39

Vijesh VV et al (2015) Screening for AZFc partial deletions in Dravidian men with nonobstructive azoospermia and oligozoospermia. Genet Test Mol Biomarkers 19(3):150–155

Visser L et al (2009) Y chromosome gr/gr deletions are a risk factor for low semen quality. Hum Reprod 24(10):2667–2673

Vissers LELM et al (2010) A de novo paradigm for mental retardation. Nat Genet 42(12):1109–1112

Wapner RJ et al (2012) Chromosomal microarray versus karyotyping for prenatal diagnosis. N Engl J Med 367(23):2175–2184

Weckselblatt B, Hermetz KE, Rudd MK (2015) Unbalanced translocations arise from diverse mutational mechanisms including chromothripsis. Genome Res 25(7):937–947

Wilkie AOM et al (1995) Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. Nat Genet 9(2):165–172

Wilson Sayres MA, Makova KD (2011) Genome analyses substantiate male mutation bias in many species. Bioessays 33(12):938–945

Wu B et al (2007) A frequent Y chromosome b2/b3 subdeletion shows strong association with male infertility in Han-Chinese population. Hum Reprod 22(4):1107–1113

Yanagimachi R, Yanagimachi H, Rogers BJ (1976) The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. Biol Reprod 15(4):471–476

Yang Y et al (2010) Differential effect of specific gr/gr deletion subtypes on spermatogenesis in the Chinese Han population. Int J Androl 33(5):745–754

Yang B et al (2015) Common AZFc structure may possess the optimal spermatogenesis efficiency relative to the rearranged structures mediated by non-allele homologous recombination. Sci Rep 5(1):10551

Ye J et al (2013) Partial AZFc duplications not deletions are associated with male infertility in the Yi population of Yunnan Province, China. J Zhejiang Univ Sci B 14(9):807–815

Yoon S-R et al (2009) The ups and downs of mutation frequencies during aging can account for the Apert syndrome paternal age effect. PLoS Genet.. Walsh B (ed) 5(7):e1000558

Yoon S-R et al (2013) Age-dependent germline mosaicism of the most common noonan syndrome mutation shows the signature of germline selection. Am J Hum Genet 92(6):917–926

Zhang Y-S et al (2017) Complete azoospermia factor b deletion of Y chromosome in an infertile male with severe Oligoasthenozoospermia: case report and literature review. Urology 102:111–115

3 The Sperm Epigenome: Implications for Assisted Reproductive Technologies

Douglas T. Carrell

Abstract

Compared to other cells, sperm undergo dramatic remodeling of their chromatin during late spermiogenesis in which approximately 95% of histones are removed and replaced with protamines. Despite this large-scale remodeling, key developmental genes, some miRNA genes, and imprinted genes retain their association with histone. The developmental genes have a unique epigenetic signature, termed bivalency, that poises the genes for embryonic activation. Anomalies in that epigenetic poising signature, either in the form of DNA methylation aberrations, improper protamination, or altered histone modifications, are associated with infertility and reduced embryogenesis capability. Additionally, some small noncoding RNAs are retained, while others are actively added to the sperm and appear to affect embryogenesis. Therefore, initial studies have begun to formulate pathways by which the sperm epigenome can be used as a diagnostic tool in the clinic. While in their infancy, these assays likely portend improved diagnostics and added information for patients and clinicians. Recent studies also highlight the possibility that the sperm epigenome can be used to evaluate lifestyle and environmental risks to the patient and potentially to the offspring.

Keywords

ART · Embryogenesis · Epigenetics · DNA methylation · Histones · Small RNAs · Environment

Introduction

Epigenetics, a term first coined by Conrad Waddington in the 1940s, is generally defined as having three major components. First, epigenetic "marks" are stable, non-DNA coding (polymorphisms or mutations) changes that, second, affect gene expression. Third, epigenetic alterations are heritable, generally defined as being passed at least to the F3 generation (Waddington 1942; Holliday 2006). Epigenetics is both practically and historically linked to the field of reproductive biology, since such gene expression changes are the key to cellular differentiation during embryonic development and since the term "epigenetics" is derived from "epigenesis," the embryological concept of "stepwise" development of the embryo that has been discussed since the times of Aristotle, but particularly debated in the seventeenth and eighteenth centuries during

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the debates of the "preformation theory" of development versus the epigenesis theory of development (Harvey 1651, 1653). Since the term epigenetics was first coined, epigenetics has moved into a much broader realm, including the study of environmental influences on gene expression and disease etiologies (Jirtle and Skinner 2007; Deans and Maggert 2015).

Numerous studies have now demonstrated the powerful effects of alterations of the epigenome due to environmental or lifestyle factors on subsequent health. Among the best examples are studies evaluating famine or diet alterations at specific developmental time periods, such as the prenatal, perinatal, and peri-pubertal periods. For example, children born during the 1944–1945 Dutch famine period have been shown to have an increased risk of heart disease and obesity if their mother was exposed to the famine, apparently due to a change in DNA methylation to the insulin-like growth factor 2 (ILGF2) gene (Painter et al. 2005; Heijmans et al. 2008). In another example, paternal grandsons of prepubertal boys exposed to famine in the Overkalix region of Sweden have been shown to have increased mortality, although no effects were seen for the paternal grandmother or maternal grandparents (Pembrey et al. 2006). These studies highlight the ability of environmental changes, diet in these cases, to alter the epigenome through sperm, as well as highlighting the potential of abnormal epigenetics to affect the health of offspring and progeny.

As is often the case, the first studies of the sperm epigenome were not aimed at defining the normal sperm epigenome in normal development, rather the result of a possible associated disease risk. The earliest studies of the sperm epigenome were the result of a reported increase in the incidence of imprinting diseases, a form of epigenetic abnormality, in the offspring of individuals conceived using intracytoplasmic sperm injection (ICSI) during assisted reproductive therapy (ART) (Cox et al. 2002; Kobayashi et al. 2007; Le Bouc et al. 2010). Imprinting diseases are the result of improper sex-determined allelic methylation, and some studies demonstrated that the sperm of men undergoing ICSI had increased levels of abnormal DNA methylation, associated with decreased sperm counts. These studies opened the door to a deeper evaluation of the sperm epigenome and the role it may play in embryogenesis and the health of the offspring. While still in its infancy, the role of the sperm epigenome in embryogenesis is becoming better understood, as well as the role of certain lifestyle and environmental factors in altering it. This chapter explores these advances in terms of the potential to offer improved diagnosis and treatment of infertility through ART and in terms of ultimately better understanding the transmission of health risk to offspring through the sperm epigenome.

The Sperm Epigenome: Protamination and Histone Modifications

During late spermiogenesis, sperm undergo a dramatic remodeling of the sperm chromatin in which approximately 95% of the histones are sequentially replaced, first by transition proteins and then by protamines (P1 and P2) (see Fig. 3.1). The replacement of histones facilitates a higher order of DNA packaging, up to 20 times more than DNA in somatic cells, and is useful in providing a compact sperm head consistent with sperm motility requirements. Lastly, it is believed that the compaction of the DNA into toroidal structures also protects the DNA from oxidative stress while the sperm traverse the female reproductive tract. In fertile men, P1 and P2 replace histones with an approximate ratio of 1:1 (Carrell and Liu 2001), and alterations in the P1/P2 ratio reflect abnormal protamination and are associated with reduced semen quality, increased sperm DNA fragmentation, and reduced fertilization capabilities and embryo implantation in couples undergoing IVF (Aoki et al. 2005, 2006a; Carrell et al. 2008; Hammoud et al. 2009a; Carrell 2012).

Using a mouse model, intracytoplasmic sperm injection (ICSI) of sperm containing altered histone/protamine ratios and high DNA fragmentation resulted in embryos with a lower competency (Cho et al. 2003). Generally, there is a consensus

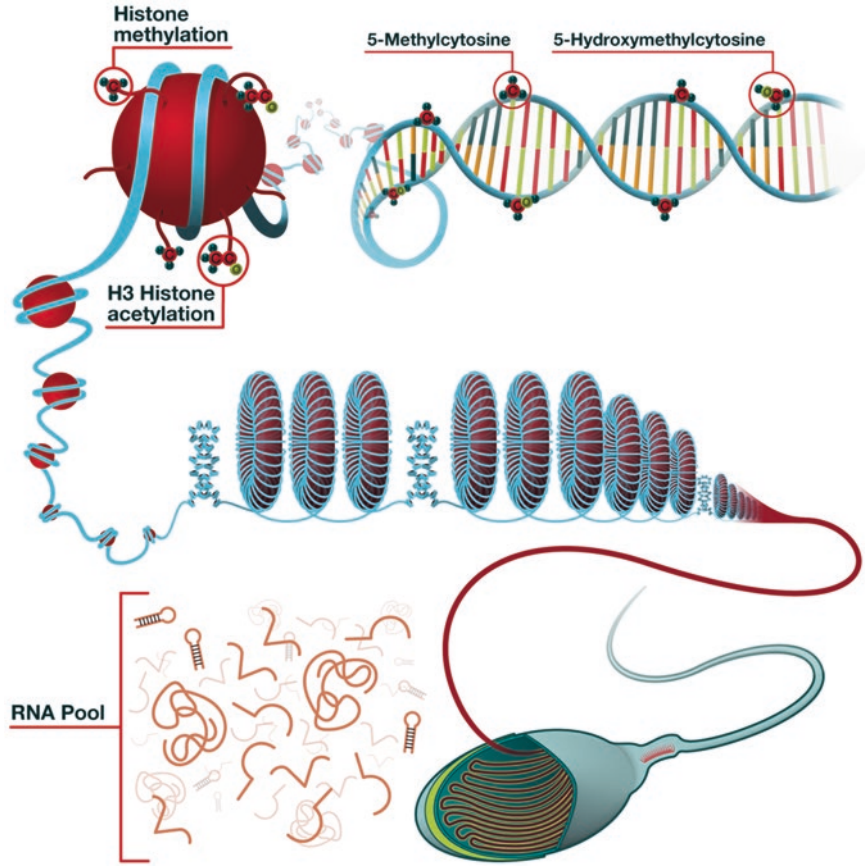

Fig. 3.1 An overview of the sperm epigenome. This figure highlights the repackaging of the sperm chromatin with protamines, with interspersed histones at key loci, including developmental gene promoters. The packaging of the genome with protamines facilitates higher-order chromatin compaction, including the formation of toroids. The figure shows the three key components of the sperm epigenome: histone modifications, DNA methylation, and the pool of RNAs, some of which are miRNAs and tRFs

that aberrant protamination is associated with increased DNA fragmentation and reduced embryo quality (Carrell and Liu 2001; Aoki et al. 2006a, b, c; Cho et al. 2003).

In addition to the role of protamination on protecting sperm DNA, the replacement of 95% of histones begs the question of if there is a role for the remaining histones—perhaps an epigenetic role? Stated differently, for such an evolutionary important aspect of reproduction, why would protamination be so inefficient as to leave 5% of the genome non-protaminated? Lastly, one would also hypothesize that if there were a role for retained histones, their loci of retention would be consistent and may suggest a biological role. Studies by Hammoud et al. were initially undertaken to help answer those questions and included genome-wide analysis of the loci of histone retention, specific histone modification analysis, and evaluation of DNA methylation status genome-wide and found that retained histones are found at consistent and deliberate locations throughout the sperm genome, including key developmental genes, poising these genes for activation during early embryogenesis (Hammoud et al. 2009b). These findings imply that proper protamination, and likewise normal retention of specific histones, is not only important in regard to a reflection of male-factor (MF) infertility status, as a reflection of abnormal spermatogenesis, and in regard to protecting the genome from DNA damage but also suggested a

role in paternal contributions to normal embryogenesis.

The hypothesis that retention of sperm histones at key developmental loci is of biological significance is also dependent on proper histone modifications, since specific modifications can either facilitate or preclude transcription by making gene promoters accessible or inaccessible to transcription factors. Histone tail modifications are a major class of epigenetic regulators in somatic cells. Briefly, acetylation of H3 and H4 as well as methylation of H3K4 results in an "open" state of genes that facilitates transcription. Conversely, methylation of H3K9 and H3K27 and deacetylation of H3 and H4 drive a chromatin state which silences genes at those loci (Jenkins and Carrell 2011; Jenuwein and Allis 2001). Hammoud et al., and subsequently others, demonstrated that in human sperm, the modifications of histones, associated with developmental genes, are unique in that there is bivalency as both marks containing both H3K4me3 activation marks and H3K27 silencing marks are present, similar to what is found in some embryonic stem cell gene loci (Hammoud et al. 2009b). This arrangement suggests a "gene poising" of key genes involved in embryonic development. Interestingly, many IVF patients with altered embryogenesis capability have been shown to exhibit defects in this poising pattern (Hammoud et al. 2011). Furthermore, this unique poising pattern of embryonic developmental genes has been confirmed in zebrafish, an evolutionarily distant species (Murphy et al. 2018a).

The Sperm Epigenome: DNA Methylation

DNA methylation is the major regulator of gene transcription and technically easier to evaluate than histone modifications; therefore, more studies have reported DNA methylation status of the human sperm than those evaluating histone modifications. While targeted sequencing studies can be employed following bisulfate conversion, many studies have used arrays to screen many loci and evaluate possible associations between DNA methylation alterations and various phenotypes of male infertility. The arrays offer the advantages of ease of use, as well as screening many possible CpG loci. Hammoud et al. and others have demonstrated that the normal male sperm epigenome has variable methylation at the key developmental loci described above that contain bivalent histone modifications, thus strengthening the poising hypothesis (Hammoud et al. 2009b). Furthermore, several early studies have observed DNA methylation aberrations in sperm with abnormal chromatin packaging, sperm from men who generate embryos of poor quality while undergoing in vitro fertilization, as well as infertile men (Hammoud et al. 2010, 2011; Aston et al. 2012, 2015; Nanassy and Carrell 2011).

Sperm DNA methylation is also important in terms of genomic imprinting, a system in which certain genes are methylated or demethylated based on whether the locus is inherited from the father or mother. Prior to zygotic genome activation in the early embryo, DNA methylation patterns acquired from the sperm and oocyte are actively and passively demethylated and then reset later in the primordial germ cells (Messerschmidt et al. 2014). This process has led some to minimize the potential importance of DNA methylation in epigenetic inheritance; however, it is known that not only imprinted genes but other regions of the genome escape this reprogramming event in early embryos, and at least in the case of imprinted loci, the methylation signature provided to the embryo by sperm is maintained (Messerschmidt et al. 2014; Reik and Walter 2001). Some methylation signatures beyond imprinted regions are retained in the embryo and are involved in modulating development and affecting phenotype transgenerationally. Such signatures have been identified, including methylation patterns inherited via sperm (Guibert et al. 2012; Illum et al. 2018; Seisenberger et al. 2012). One study found that the female offspring of male rats consuming a high-fat diet displayed multiple characteristics consistent with metabolic phenotypes, including reduced birthweight, decreased pancreatic beta-cell mass, and glucose intolerance (Barbosa et al. 2015). DNA methylation analysis was conducted

on the sperm from the F0 high-fat-fed rats and their F1 male offspring, and multiple methylation alterations were observed when compared to control rats, and in fact many differentially methylated regions were concordantly observed in both the F0 and F1 male sperm, suggesting a possible mechanism for transgenerational inheritance of metabolic disease.

Given the early data described above in which methylation errors at imprinted loci were found to be more common in men with abnormal spermatogenesis, and particularly men with low sperm counts, it was imperative to evaluate the methylation status of DNA from sperm of men with broader types of male-associated infertility. In one such early study, Aston et al. evaluated several thousand loci, using an early array system, in sperm from men with either aberrant protamination or men with unexplained poor embryogenesis while undergoing IVF[27]. Interestingly, more than 7% of all loci evaluated were abnormally methylated in these patients, and more than 60% of imprinted loci were aberrantly methylated. This study was supported by numerous other studies and focused attention on the potential use of sperm DNA methylation analysis as a potential screen for IVF embryogenesis outcome, as will be discussed below (Jenkins et al. 2016a; Karaca et al. 2017; Laqqan and Hammadeh 2018; Santi et al. 2017).

The Sperm Epigenome: Small, Noncoding RNAs

Although gene transcription does not occur in a mature sperm, sperm contain many RNA species that are stable in the embryo following fertilization (Ostermeier et al. 2004; Pessot et al. 1989). Sperm RNAs include remnant mRNAs from spermatogenesis (Ostermeier et al. 2002, 2004), mRNAs that may be functionally important to the developing embryo (Ostermeier et al. 2002; Jodar et al. 2015; Sendler et al. 2013), and a variety of noncoding RNAs (Krawetz et al. 2011). Recently, much of the research on sperm epigenetic factors and epigenetic-mediated inheritance has focused on the small, noncoding RNAs (Conine et al. 2018; Liu et al. 2012; Chen et al. 2016; Sharma et al. 2016; Zhang et al. 2018).

Studies in the mouse have shown that there are two major sources of noncoding RNAs in sperm. First, sperm contain a large number of piRNAs that are remnants of spermatogenesis. Second, during epididymal transit, there appears to be a significant remodeling of the RNAs, with some RNAs apparently removed in the caput epididymis and then subsequently replaced, along with other species of RNAs. This replacement and remodeling appears to occur largely through epididymosomes, exosomes that are secreted in the epididymis and attach to sperm and transfer their contents. These epididymosomes transfer a large contingent of miRNAs and tRNA fragments (tRFs), as well as proteins necessary in the acquisition of sperm motility and fertilization ability. Interestingly, the RNA payload of a sperm isolated from the cauda epididymis is similar to a sperm isolated from the testis, but RNAs are removed in the caput epididymis and then are subsequently replaced via the epidiymosomes (Sharma et al. 2018).

In an elegant study by Conine et al., it was shown that some of the RNA species that are lost and subsequently regained by sperm cells transiting the epididymis are associated with improper embryonic implantation as well as gross defects in embryonic development[47]. Using caput and cauda sperm to generate mouse embryos, Conine et al. reported that caput-derived embryos showed significantly reduced rates of successful implantation, gross embryo morphology defects, and a reduced number of viable offspring (Conine et al. 2018). They then showed that the embryos could be "rescued" by injection of the miRNA fraction of epididymosomes. These results suggest that the miRNAs delivered to sperm during epididymal transit are required for proper preimplantation gene expression in the mouse (Conine et al. 2018). The miRNAs injected included miR-34c, which has previously been shown by another group to be essential for the first cleavage division in mouse embryos (Liu et al. 2012). These two studies strongly suggest a role for spermatozoal RNAs, specifically miRNAs, in embryogenesis.

Studies evaluating the effects of diet changes have implicated spermatozoal RNAs in epigenetic inheritance of metabolic disease from fathers. Altered metabolic phenotypes have been observed in the offspring of male mice consuming high-fat or low-protein diets. These offspring display a phenotype characterized by glucose intolerance and impaired insulin secretion (Barbosa et al. 2015; Chen et al. 2016; Sharma et al. 2016; Carone et al. 2010; Ng et al. 2010). Spermatozoal RNAs, especially tRFs whose effects appear to be mediated by DNA methyltransferase 2 (DNMT2), have been implicated in this form of epigenetic inheritance (Chen et al. 2016; Sharma et al. 2016; Zhang et al. 2018). Jodar et al. rescued diet-affected zygotes by microinjection with RNAs isolated from control-diet sperm (Sharma et al. 2016). These studies highlight the ability of spermatozoal RNAs to affect gene expression patterns in the embryo and the possible role of sperm RNAs in epigenetic inheritance.

Using the Sperm Epigenome to Predict Fertility or ART Outcome

The bivalent poising of the human sperm epigenome strongly suggests a role in normal embryogenesis, and similar poising motifs are observed in diverse species, again suggesting an evolutionary role of importance (Hammoud et al. 2009b; Wu et al. 2011). Additionally, numerous studies have reported associations among abnormal sperm DNA methylation, histone replacement, histone modifications, or RNA complements with reduced male fertility, altered spermatogenesis, and altered embryogenesis capability during IVF (Jenkins et al. 2017; Gannon et al. 2014). Therefore, the possible use of the sperm epigenome as a diagnostic tool for couples undergoing infertility evaluation is apparent and has begun to be the focus of some researchers (Carrell 2012).

Aston et al. initially set out to determine the predictive role of sperm methylation patterns in patients who had undergone IVF treatment (Aston et al. 2015). Previous IVF patients were classified on whether their sperm generally generated good-quality embryos (normal blastocyst morphology) and positive pregnancies or generated an unusually high rate of poor morphological quality embryos. Sperm from these two groups were compared to sperm from men of known fertility and analyzed using machine-learning techniques to develop predictive algorithms. Surprisingly, this study found that predictive models based on methylation array data from these groups were highly predictive of male fertility status. In other words, IVF patients, who were not exclusively classified as having male-factor infertility, could with good accuracy be identified from men of known fertility. Interestingly, the most accurate algorithm was able to predict fertility status using a relatively low number of CpG loci, which were enriched for imprinted loci. This finding was surprising but strengthened by a concurrent study in which time to pregnancy was evaluated in young couples not presumed to be experiencing infertility and which also identified DNA methylation markers apparently predictive of fecundity (Jenkins et al. 2016b).

Additionally, hierarchical clustering was capable of identifying clusters containing IVF patients and poor embryo quality samples based on methylation array data, and a predictive algorithm was developed (Aston et al. 2015). While the methylation changes observed between these groups were not biased toward genomic regions of any particular annotation category, such as imprinted regions, these data show that global alterations in sperm methylation can be predictive of male fertility status and potentially embryo quality during IVF treatment (Aston et al. 2015). The data also imply that, as is well understood, embryogenesis includes a broad complement of gene pathways, and it is likely that poor embryogenesis is not the result of a dominant defective pathway, but rather may include a diverse set of defects in a myriad of pathways in a cohort of patients. The initial loci reported by Aston et al. were used by Abbasi et al. to develop a simplified and accurate testing platform for possible patient testing (Abbasi et al. 2018). The utility of such platforms will become apparent with further usage.

Recently, Denomme et al. studied on the epigenetic evaluation of embryos derived from male-factor (MF) infertility patients compared to controls and reported a dysregulation of DNA methylation in the embryos of the MF-derived embryos, including in genes involved in regulation of cellular metabolic processes (Denomme et al. 2018). While the overall pregnancy rates were similar for the two groups, the MF-derived embryos with altered methylation were associated with an increased miscarriage rate. In a separate study that eliminated female-factor confounders by using only couples employing donor oocytes, this group also found differences in sperm DNA methylation and miRNAs, as well as embryonic gene expression, in "good embryo quality" patients versus "poor embryo quality" patients (Denomme et al. 2017). These studies strengthen the potential use of methylation data to predict IVF outcome and fertility.

At present, several studies are underway with the intent of validating the above studies. Similarly, other aspects of reproduction and infertility are also being evaluated. For example, the role of inherited DNA methylation defects in unexplained, recurrent miscarriage is one area of intense interest (Ankolkar et al. 2012; Rotondo et al. 2012; Spinelli et al. 2019). Additionally, studies are beginning to identify abnormalities in sperm DNA methylation associated with environmental exposures, including bisphenol A (Dere et al. 2018), mercury (Lu et al. 2018), pesticides (Pallotta et al. 2019; Skinner et al. 2018), phthalates (Tian et al. 2018), vinclozolin (Beck et al. 2017), tobacco (Murphy et al. 2018b), and other chemicals (Siddeek et al. 2018). Interestingly, studies have also reported changes in the sperm methylome associated with paternal aging, including a stepwise increase in the number of abnormally methylated loci as during aging, beginning in approximately the mid-30s (Jenkins et al. 2013, 2014). This issue is of clinical relevance due to the increasing societal trend of delaying pregnancy until later paternal ages.

The concept that the sperm epigenome is altered by environmental exposures, age, and life events and decisions, coupled with the emerging understanding that epigenetic defects can be transmitted transgenerationally, suggests that in the future it may be able to screen potential fathers for sperm epigenetic abnormalities with the objective of identifying potential risks to offspring and progeny, in addition to infertility risk, and motivating preconception lifestyle changes to mitigate that risk (Jenkins et al. 2018). Such uses of the sperm epigenome are distant but may provide additional benefits to patients and offspring.

Conclusions

This chapter has provided a brief overview of the sperm epigenome and its potential use as a diagnostic tool to predict male infertility, to assess sperm competency in developing normal embryos, and possibly as a means to assess environmental and lifestyle risks. With the growth of the field in the past 10 years, it is likely that the future is bright in regard to meaningful advances that will directly benefit patients and clinicians, as well as aid society at large.

The existing data clearly show an implied mechanism for the sperm epigenome in regulating or facilitating early embryogenesis. Additionally, the studies described above clearly show associations with aberrant sperm epigenomes and diminished embryogenesis capacity. Lastly, early studies have demonstrated an ability to predict embryogenesis ability. At this time, the field awaits independent, large-scale validation studies before these technologies can be implemented in the clinic. Similarly, the sperm epigenome also provides a historical record of spermatogenesis, and numerous studies have shown that altered spermatogenesis is associated with altered sperm DNA methylation, particularly of imprinted genes. However, validation studies are also needed in this regard before sperm epigenetic testing can be used as a screen of fertility status. The use of the sperm epigenome as a toxicology tool and as a means to assess risk to progeny is a more distant goal, but very intriguing. While associations are strong, studies are needed to better understand the biology involved in DNA reprogramming in the embryo and fetus and the means of transmission

to progeny. Most importantly, it will be imperative that such assays quantify risk in a clinically useful manner.

To this point, sperm DNA methylation has been the major focus of studies evaluating the possible development of diagnostic tools. This is due to cost and technical feasibility issues. However, it is important that evaluation of histone modifications and RNAs continue to be a focus, since it is likely that epigenetic pathways using these markers are of high relevance to reproduction.

References

Abbasi M, Smith AD, Swaminathan H, Sangngern P, Douglas A, Horsager A, Carrell DT, Uren PJ (2018) Establishing a stable, repeatable platform for measuring changes in sperm DNA methylation. Clin Epigenetics 10(1):119. https://doi.org/10.1186/s13148-018-0551-7

Ankolkar M et al (2012) Methylation analysis of idiopathic recurrent spontaneous miscarriage cases reveals aberrant imprinting at H19 ICR in normozoospermic individuals. Fertil Steril 98:1186–1192. https://doi.org/10.1016/j.fertnstert.2012.07.1143

Aoki VW, Liu L, Carrell DT (2005) Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. Hum Reprod 20:1298–1306. https://doi.org/10.1093/humrep/deh798

Aoki VW et al (2006a) Sperm protamine 1/protamine 2 ratios are related to in vitro fertilization pregnancy rates and predictive of fertilization ability. Fertil Steril 86:1408–1415. https://doi.org/10.1016/j.fertnstert.2006.04.024

Aoki VW, Emery BR, Liu L, Carrell DT (2006b) Protamine levels vary between individual sperm cells of infertile human males and correlate with viability and DNA integrity. J Androl 27:890–898. https://doi.org/10.2164/jandrol.106.000703

Aoki VW, Liu L, Carrell DT (2006c) A novel mechanism of protamine expression deregulation highlighted by abnormal protamine transcript retention in infertile human males with sperm protamine deficiency. Mol Hum Reprod 12:41–50. https://doi.org/10.1093/molehr/gah258

Aston KI, Punj V, Liu L, Carrell DT (2012) Genome-wide sperm deoxyribonucleic acid methylation is altered in some men with abnormal chromatin packaging or poor in vitro fertilization embryogenesis. Fertil Steril 97:285–292. https://doi.org/10.1016/j.fertnstert.2011.11.008

Aston KI et al (2015) Aberrant sperm DNA methylation predicts male fertility status and embryo quality. Fertil Steril 104:1388–1397 e1381–1385. https://doi.org/10.1016/j.fertnstert.2015.08.019

Barbosa TD et al (2015) Paternal chronic high-fat diet consumption reprogrammes the gametic epigenome and induces transgenerational inheritance of metabolic disorder. Diabetologia 58:S162–S163

Beck D, Sadler-Riggleman I, Skinner MK (2017) Generational comparisons (F1 versus F3) of vinclozolin induced epigenetic transgenerational inheritance of sperm differential DNA methylation regions (epimutations) using MeDIP-Seq. Environ Epigenet 3. https://doi.org/10.1093/eep/dvx016

Carone BR et al (2010) Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. Cell 143:1084–1096. https://doi.org/10.1016/j.cell.2010.12.008

Carrell DT (2012) Epigenetics of the male gamete. Fertil Steril 97:267–274. https://doi.org/10.1016/j.fertnstert.2011.12.036

Carrell DT, Liu L (2001) Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. J Androl 22:604–610

Carrell DT, Emery BR, Hammoud S (2008) The aetiology of sperm protamine abnormalities and their potential impact on the sperm epigenome. Int J Androl 31:537–545. https://doi.org/10.1111/j.1365-2605.2008.00872.x

Chen Q et al (2016) Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. Science 351:397–400. https://doi.org/10.1126/science.aad7977

Cho C et al (2003) Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. Biol Reprod 69:211–217. https://doi.org/10.1095/biolreprod.102.015115

Conine CC, Sun F, Song L, Rivera-Pérez JA, Rando OJ (2018) Small RNAs gained during epididymal transit of sperm are essential for embryonic development in mice. Dev Cell 46:470–480.e473. https://doi.org/10.1016/j.devcel.2018.06.024

Cox GF et al (2002) Intracytoplasmic sperm injection may increase the risk of imprinting defects. Am J Hum Genet 71:162–164. https://doi.org/10.1086/341096

Deans C, Maggert KA (2015) What do you mean, "epigenetic"? Genetics 199:887–896. https://doi.org/10.1534/genetics.114.173492

Denomme MM, McCallie BR, Parks JC, Schoolcraft WB, Katz-Jaffe MG (2017) Alterations in the sperm histone-retained epigenome are associated with unexplained male factor infertility and poor blastocyst development in donor oocyte IVF cycles. Hum Reprod 32:2443–2455. https://doi.org/10.1093/humrep/dex317

Denomme MM et al (2018) Inheritance of epigenetic dysregulation from male factor infertility has a direct impact on reproductive potential. Fertil Steril 110:419–428 e411. https://doi.org/10.1016/j.fertnstert.2018.04.004

Dere E et al (2018) Effects of continuous bisphenol A exposure from early gestation on 90day old rat testes function and sperm molecular profiles: a CLARITY-BPA consortium study. Toxicol Appl Pharmacol 347:1–9. https://doi.org/10.1016/j.taap.2018.03.021

Gannon JR, Emery BR, Jenkins TG, Carrell DT (2014) The sperm epigenome: implications for the embryo. Adv Exp Med Biol 791:53–66. https://doi.org/10.1007/978-1-4614-7783-9_4

Guibert S, Forne T, Weber M (2012) Global profiling of DNA methylation erasure in mouse primordial germ cells. Genome Res 22:633–641. https://doi.org/10.1101/gr.130997.111

Hammoud S, Liu L, Carrell DT (2009a) Protamine ratio and the level of histone retention in sperm selected from a density gradient preparation. Andrologia 41:88–94. https://doi.org/10.1111/j.1439-0272.2008.00890.x

Hammoud SS et al (2009b) Distinctive chromatin in human sperm packages genes for embryo development. Nature 460:473–478. https://doi.org/10.1038/nature08162

Hammoud SS, Purwar J, Pflueger C, Cairns BR, Carrell DT (2010) Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. Fertil Steril 94:1728–1733. https://doi.org/10.1016/j.fertnstert.2009.09.010

Hammoud SS et al (2011) Genome-wide analysis identifies changes in histone retention and epigenetic modifications at developmental and imprinted gene loci in the sperm of infertile men. Hum Reprod 26:2558–2569. https://doi.org/10.1093/humrep/der192

Harvey W (1651) Exercitationes de generatione animalium. Typis, London

Harvey W (1653) Anatomical exercitations concerning the generation of living creatures to which are added particular discourses of births and of conceptions, &c. (James Young, for Octavian Pulleyn)

Heijmans BT et al (2008) Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A 105:17046–17049. https://doi.org/10.1073/pnas.0806560105

Holliday R (2006) Epigenetics: a historical overview. Epigenetics 1:76–80

Illum LRH, Bak ST, Lund S, Nielsen AL (2018) DNA methylation in epigenetic inheritance of metabolic diseases through the male germ line. J Mol Endocrinol 60:R39–R56. https://doi.org/10.1530/JME-17-0189

Jenkins TG, Carrell DT (2011) The paternal epigenome and embryogenesis: poising mechanisms for development. Asian J Androl 13:76–80. https://doi.org/10.1038/aja.2010.61

Jenkins TG, Aston KI, Cairns BR, Carrell DT (2013) Paternal aging and associated intraindividual alterations of global sperm 5-methylcytosine and 5-hydroxymethylcytosine levels. Fertil Steril 100:945–951. https://doi.org/10.1016/j.fertnstert.2013.05.039

Jenkins TG, Aston KI, Pflueger C, Cairns BR, Carrell DT (2014) Age-associated sperm DNA methylation alterations: possible implications in offspring disease susceptibility. PLoS Genet 10:e1004458. https://doi.org/10.1371/journal.pgen.1004458

Jenkins TG et al (2016a) Teratozoospermia and asthenozoospermia are associated with specific epigenetic signatures. Andrology 4:843–849. https://doi.org/10.1111/andr.12231

Jenkins TG et al (2016b) Decreased fecundity and sperm DNA methylation patterns. Fertil Steril 105:51–57 e51–53. https://doi.org/10.1016/j.fertnstert.2015.09.013

Jenkins TG, Aston KI, James ER, Carrell DT (2017) Sperm epigenetics in the study of male fertility, offspring health, and potential clinical applications. Syst Biol Reprod Med 63:69–76. https://doi.org/10.1080/19396368.2016.1274791

Jenkins TG, Aston KI, Carrell DT (2018) Sperm epigenetics and aging. Transl Androl Urol 7:S328–S335. https://doi.org/10.21037/tau.2018.06.10

Jenuwein T, Allis CD (2001) Translating the histone code. Science 293:1074–1080. https://doi.org/10.1126/science.1063127

Jirtle RL, Skinner MK (2007) Environmental epigenomics and disease susceptibility. Nat Rev Genet 8:253–262. https://doi.org/10.1038/nrg2045

Jodar M et al (2015) Absence of sperm RNA elements correlates with idiopathic male infertility. Sci Transl Med 7:295re296. https://doi.org/10.1126/scitranslmed.aab1287

Karaca MZ et al (2017) Association between methylenetetrahydrofolate reductase (MTHFR) gene promoter hypermethylation and the risk of idiopathic male infertility. Andrologia 49(7). https://doi.org/10.1111/and.12698

Kobayashi H et al (2007) Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. Hum Mol Genet 16:2542–2551. https://doi.org/10.1093/hmg/ddm187

Krawetz SA et al (2011) A survey of small RNAs in human sperm. Hum Reprod 26:3401–3412. https://doi.org/10.1093/humrep/der329

Laqqan M, Hammadeh ME (2018) Aberrations in sperm DNA methylation patterns of males suffering from reduced fecundity. Andrologia 50(3). https://doi.org/10.1111/and.12913

Le Bouc Y et al (2010) Epigenetics, genomic imprinting and assisted reproductive technology. Ann Endocrinol (Paris) 71:237–238. https://doi.org/10.1016/j.ando.2010.02.004

Liu WM et al (2012) Sperm-borne microRNA-34c is required for the first cleavage division in mouse. Proc Natl Acad Sci U S A 109:490–494. https://doi.org/10.1073/pnas.1110368109

Lu Z et al (2018) Urine mercury levels correlate with DNA methylation of imprinting gene H19 in the sperm of reproductive-aged men. PLoS One 13:e0196314. https://doi.org/10.1371/journal.pone.0196314

Messerschmidt DM, Knowles BB, Solter D (2014) DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos.

Genes Dev 28:812–828. https://doi.org/10.1101/gad.234294.113

Murphy PJ, Wu SF, James CR, Wike CL, Cairns BR (2018a) Placeholder nucleosomes underlie germline-to-embryo DNA methylation reprogramming. Cell 172:993–1006 e1013. https://doi.org/10.1016/j.cell.2018.01.022

Murphy SK et al (2018b) Cannabinoid exposure and altered DNA methylation in rat and human sperm. Epigenetics 13:1208. https://doi.org/10.1080/15592294.2018.1554521

Nanassy L, Carrell DT (2011) Abnormal methylation of the promoter of CREM is broadly associated with male factor infertility and poor sperm quality but is improved in sperm selected by density gradient centrifugation. Fertil Steril 95:2310–2314. https://doi.org/10.1016/j.fertnstert.2011.03.096

Ng SF et al (2010) Chronic high-fat diet in fathers programs beta-cell dysfunction in female rat offspring. Nature 467:963–966. https://doi.org/10.1038/nature09491

Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA (2002) Spermatozoal RNA profiles of normal fertile men. Lancet 360:772–777. https://doi.org/10.1016/S0140-6736(02)09899-9

Ostermeier GC, Miller D, Huntriss JD, Diamond MP, Krawetz SA (2004) Reproductive biology: delivering spermatozoan RNA to the oocyte. Nature 429:154. https://doi.org/10.1038/429154a

Painter RC, Roseboom TJ, Bleker OP (2005) Prenatal exposure to the Dutch famine and disease in later life: an overview. Reprod Toxicol 20:345–352. https://doi.org/10.1016/j.reprotox.2005.04.005

Pallotta MM et al (2019) In vitro exposure to CPF affects bovine sperm epigenetic gene methylation pattern and the ability of sperm to support fertilization and embryo development. Environ Mol Mutagen 60:85–95. https://doi.org/10.1002/em.22242

Pembrey ME et al (2006) Sex-specific, male-line transgenerational responses in humans. Eur J Hum Genet 14:159–166. https://doi.org/10.1038/sj.ejhg.5201538

Pessot CA et al (1989) Presence of RNA in the sperm nucleus. Biochem Biophys Res Commun 158:272–278

Reik W, Walter J (2001) Genomic imprinting: parental influence on the genome. Nat Rev Genet 2:21–32. https://doi.org/10.1038/35047554

Rotondo JC et al (2012) Methylenetetrahydrofolate reductase gene promoter hypermethylation in semen samples of infertile couples correlates with recurrent spontaneous abortion. Hum Reprod 27:3632–3638. https://doi.org/10.1093/humrep/des319

Santi D, De Vincentis S, Magnani E, Spaggiari G (2017) Impairment of sperm DNA methylation in male infertility: a meta-analytic study. Andrology 5:695–703. https://doi.org/10.1111/andr.12379

Seisenberger S et al (2012) The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. Mol Cell 48:849–862. https://doi.org/10.1016/j.molcel.2012.11.001

Sendler E et al (2013) Stability, delivery and functions of human sperm RNAs at fertilization. Nucleic Acids Res 41:4104–4117. https://doi.org/10.1093/nar/gkt132

Sharma U et al (2016) Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals. Science 351:391–396. https://doi.org/10.1126/science.aad6780

Sharma U et al (2018) Small RNAs are trafficked from the epididymis to developing mammalian sperm. Dev Cell 46:481–494.e486. https://doi.org/10.1016/j.devcel.2018.06.023

Siddeek B, Mauduit C, Simeoni U, Benahmed M (2018) Sperm epigenome as a marker of environmental exposure and lifestyle, at the origin of diseases inheritance. Mutat Res 778:38–44. https://doi.org/10.1016/j.mrrev.2018.09.001

Skinner MK et al (2018) Alterations in sperm DNA methylation, non-coding RNA and histone retention associate with DDT-induced epigenetic transgenerational inheritance of disease. Epigenetics Chromatin 11:8. https://doi.org/10.1186/s13072-018-0178-0

Spinelli P et al (2019) Identification of the novel Ido1 imprinted locus and its potential epigenetic role in pregnancy loss. Hum Mol Genet 28:662. https://doi.org/10.1093/hmg/ddy383

Tian M, Liu L, Zhang J, Huang Q, Shen H (2018) Positive association of low-level environmental phthalate exposure with sperm motility was mediated by DNA methylation: a pilot study. Chemosphere 220:459–467. https://doi.org/10.1016/j.chemosphere.2018.12.155

Waddington CH (1942) The epigenotype. Endeavour 1:18–20

Wu SF, Zhang H, Cairns BR (2011) Genes for embryo development are packaged in blocks of multivalent chromatin in zebrafish sperm. Genome Res 21:578–589. https://doi.org/10.1101/gr.113167.110

Zhang Y et al (2018) Dnmt2 mediates intergenerational transmission of paternally acquired metabolic disorders through sperm small non-coding RNAs. Nat Cell Biol 20:535–540. https://doi.org/10.1038/s41556-018-0087-2

Epigenetic Transgenerational Inheritance

4

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Abstract

Epigenetic information refers to heritable changes in gene expression that occur without modifications at the DNA sequence level. These changes are orchestrated by different epigenetic mechanisms such as DNA methylation, post-translational modifications of histones, and the presence of noncoding RNAs. Epigenetic information regulates chromatin structure to confer cell-specific gene expression.

The sperm epigenome is the result of three periods of global resetting during men's life. Germ cell epigenome reprogramming is designed to allow cell totipotency and to prevent the transmission of epimutations via spermatozoa. At the end of these reprogramming events, the sperm epigenome has a very specific epigenetic pattern that is a footprint of past reprogramming events and has an influence on embryo development.

Several data demonstrate that not all regions of the epigenome are erased during the reprogramming periods, suggesting the transmission of epigenetic information from fathers to offspring via spermatozoa. Moreover, it is becoming increasingly clear that the sperm epigenome is sensitive to environmental factors during the process of gamete differentiation, suggesting the plasticity of the sperm epigenetic signature according to the circumstances of the individual's life.

In this chapter, we provided strong evidences about the association between variations of the sperm epigenome and the exposure to environmental factors. Moreover, we will present data about how epigenetic mechanisms are candidates for transferring paternal environmental information to offspring.

Keywords

Spermatozoa · Epigenome · Chromatin · Transgenerational inheritance · DNA methylation · Noncoding RNA

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Layers of Epigenetic Information in Sperm

Spermatozoa are highly differentiated cells that play an essential role in reproduction by providing the haploid paternal genome to the embryo. Nevertheless, the biological relevance of sperm cells is not merely based on DNA sequence, but also on a wide range of epigenetic information such as DNA methylation, posttranslational modifications of histones, and the cargo of a specific set of RNA molecules. The orchestrated action of the different epigenetic mechanisms is essential for modulating sperm chromatin structure and gene expression, creating functional sperm able to achieve the processes of fertilization and early embryogenesis successfully.

DNA Methylation

DNA methylation mainly occurs at position 5 of cytosines (5-methylcytosine, 5mC) in 5'-CpG-3′ dinucleotides. It has been called the "fifth base" of the human genome since 4% of the cytosines are methylated. The CpG dinucleotides are present throughout the genome but concentrated in genomic regions called CpG islands (CG islands, CGI). CGI are normally found within gene promoters, being unmethylated in the case of genes that are actively transcribed and methylated in the case of inactive genes. The significance of CpG dinucleotide methylation along the transcription unit (exons, introns, and 5′ and 3′ untranslated regions) is less known.

The sperm methylome is the result of different waves of genome-wide DNA reprogramming during the differentiation of primordial germ cells (PGCs) into spermatozoa. PGCs arise from the epiblast and migrate to colonize the genital ridge (Chuva de Sousa Lopes and Roelen 2010). They initiated their differentiation as cells with a somatic epigenetic signature exhibiting high levels of 5mC, which are passively removed during PGC migration (Guibert et al. 2012; Kagiwada et al. 2013; Seisenberger et al. 2012). PGCs enter a second stage of active DNA demethylation in the genital ridge, resulting in an almost complete loss of 5mC (Hackett et al. 2013; Tang et al. 2015). The demethylation process in PGCs also affects imprinted genes (Hackett et al. 2013; Hajkova et al. 2002; Sasaki and Matsui 2008). Although the global loss of methylation affects all methylation levels, some retrotransposon-associated and single copy regions of the genome are resistant to reprogramming (Tang et al. 2015). The establishment of new methylation marks starts in type A spermatogonia (Kota and Feil 2010) and is completed before the onset of meiosis (Davis et al. 2000; Kerjean et al. 2000).

The sperm methylome is the consequence of this process of DNA methylation erasure and reestablishment. The result is a marked hypomethylated state with a high homogeneity among sperm samples from different individuals (Camprubí et al. 2017; Krausz et al. 2012). Some authors have demonstrated that genes with hypomethylated promoter regions are functionally associated with biological processes related to embryonic development (Camprubí et al. 2017; Hammoud et al. 2009; Krausz et al. 2012; Molaro et al. 2011). In contrast, genomic regions containing repetitive DNA sequences appear to be significantly hypermethylated, probably to prevent the activation of transposable elements (Molaro et al. 2011). Authors agree that these features reflect the reprogramming phenomena occurred during spermatogenesis, a process designed to confer a pluripotent state to the sperm, which will facilitate the epigenetic reprogramming that will take place during the early stages of embryo development.

Sperm Chromatin

During the postmeiotic differentiation of round spermatids into spermatozoa, chromatin is extensively remodeled resulting in nucleoprotamine structure in 85% of the nucleus (Gatewood et al. 1987). This process allows the establishment of highly ordered and compacted toroid-chromatin structures. The remaining 15% of the sperm chromatin retain a nucleohistone structure (Gatewood et al. 1987).

In human spermatozoa, residual nucleosomes are programmatically retained in gene regulatory regions, including the promoters of developmental genes, microRNA genes, and imprinted loci (Hammoud et al. 2009). Moreover, these histones carry multiple posttranslational modifications, suggesting some degree of retained regulatory competence through histone tail modifications (Arpanahi et al. 2009; Hammoud et al. 2009). The fact that sperm histone modifications are transmitted to the embryo and are resistant to protein oocyte replacement (Van Der Heijden et al. 2008) argues in favor of an effect beyond fertilization.

Like histones, protamines also exhibited posttranslational modifications (Brunner et al. 2014; Oliva et al. 2015). Nevertheless, protamines are exchanged by the histones provided by the oocyte (Van Der Heijden et al. 2008), which argues against an effect of posttranslational protamine modifications beyond fertilization.

Noncoding RNAs

Sperm RNAs have emerged as a field of interest because of their high complexity and diversity. Beyond the relevance of coding RNAs, different populations of sperm noncoding RNAs (ncRNAs) have been characterized in the last decade, revealing their strong contribution in processes related to cellular spermatogenesis, fertilization, and embryogenesis (Corral-Vazquez and Anton 2018). Sperm RNA transcripts mainly originated from the two transcriptional waves that take place during spermatogenesis generating specific transcripts for the correct development of spermatogenesis (de Mateo and Sassone-Corsi 2014). Moreover, some sperm ncRNAs remain intact after being released into the oocyte (Boerke et al. 2007) regulating the expression of specific oocytes transcripts (Amanai et al. 2006), which suggest their ability to introduce epigenetic modifications in the early embryo.

ncRNAs are classified, depending on their length, into long noncoding RNA (lncRNA) and small noncoding RNA (sncRNA). The biological functions of lncRNAs mainly comprise epigenetic regulation of single mRNA transcription or whole chromosomes (Bao et al. 2013). There are specific lncRNAs that are especially abundant in the sperm transcriptome, suggesting their role in male fertility (Jodar et al. 2013).

The sperm sncRNA family includes microRNA (miRNAs), Piwi-interacting RNA (piRNAs), and endogenous small interfering RNAs (endo-siRNAs). MicroRNAs are a family of functional RNA molecules of 22–24 nucleotides (nt) that form complementary stem-loop structures in the 3′ untranslated region (3’ UTR) of their target messenger RNAs (mRNAs). Usually, this association leads to mRNA degradation and/or translational repression. It is known that each miRNA has hundreds of potential mRNA targets, and it has been estimated that they can regulate up to 60% of protein-coding genes (Luo et al. 2015). Human spermatozoa show homogeneous and stable expression patterns of miRNAs, which have a significant ontological relation with processes involved in embryogenesis and spermatogenesis (Salas-Huetos et al. 2014). PiRNAs are 24–30 nt monocatenary RNA molecules. They are the most abundant sncRNA in both human and mice sperm transcriptomes (Pantano et al. 2015; Röther and Meister 2011). Their functionality is based on their attachment to PIWI proteins, which are exclusive of germ cells, to allow the posttranscriptional silencing of retrotransposons (Chuma and Nakano 2012). Accordingly, their biological function is in connection with a protective mechanism against genome modifications produced by transposable elements. Endo-siRNAs are 22 nt RNA molecules highly expressed in male germ cells (Song et al. 2011). The posttranscriptional gene regulatory function of endo-siRNAs is similar to the gene-silencing pathway of miRNAs. It is based on their attachment to 3’ UTR regions of target mRNAs (Song et al. 2011), which lead to the silencing or degradation of the mRNA sequences (Luo et al. 2015). In spermatozoa, these molecules control the expression of epigenetic regulators, such as histone methyltransferases, and promote the modification of chromatin conformation (Song et al. 2011). Additionally, some studies suggest that endo-siRNAs are

necessary in postfertilization processes for the correct development of preimplantational embryos (Suh et al. 2010).

An Overview into the Concept of Transgenerational Inheritance

In human and animal models, several studies have demonstrated that the exposure to certain environmental factors in specific windows of the epigenome reprogramming affects the mechanisms that lead to the establishment of the sperm epigenome. Since the sperm epigenome is crucial for the proper fertility of the individuals, these variations have been related to male infertility (Camprubí et al. 2016). Moreover, it is becoming clear that some epimutations could be also transmitted via spermatozoa to offspring, which introduce the concept of epigenetic inheritance.

Inheritance of environmental-induced epigenetic changes is associated to the permanent transmission of epigenetic variations through the germline (Skinner 2008). In the case of exposure of a gestating F0 female, only the transmission of a phenotypic alteration until the third generation (F3) could be considered true transgenerational inheritance (Fig. 4.1). In this case, the germ cells of the F1 generation also carry the epigenetic variation induced in the gestating female (F0), which could also affect F1 gametes. Accordingly, F2 individuals could inherit the trait from an F0 gestating female. Therefore, in this model, the transmission until the F3 generation is required to assure that the results are the consequence of epigenetic transmission between cells unrelated to previous exposure effects (Fig. 4.1). When the exposure occurs in an adult male (F0), germ cells of the F1 generation could inherit the variation from the F0 spermatozoa. Thus, in this case, the first nonexposed generation involved would be F2 (Fig. 4.2). If the transmission of an epigenetic treat does not reach F3 (from a gestating female exposure) or F2 (from an adult male exposure), we talk about intergenerational or multigenerational inheritance.

In this context, it is important to remark that the only way to explain the transmission of any epigenetic variation induced by any agent between generations is the permanent reprogramming of germ cells. That is, the variation must be resistant to the resetting periods in which the epigenome is involved during the man's life. This would guarantee stable transmission across generations.

From these premises, posttranslational modifications of histones and sncRNAs signature are epigenetic mechanisms that can hardly be associated with transgenerational epigenetic

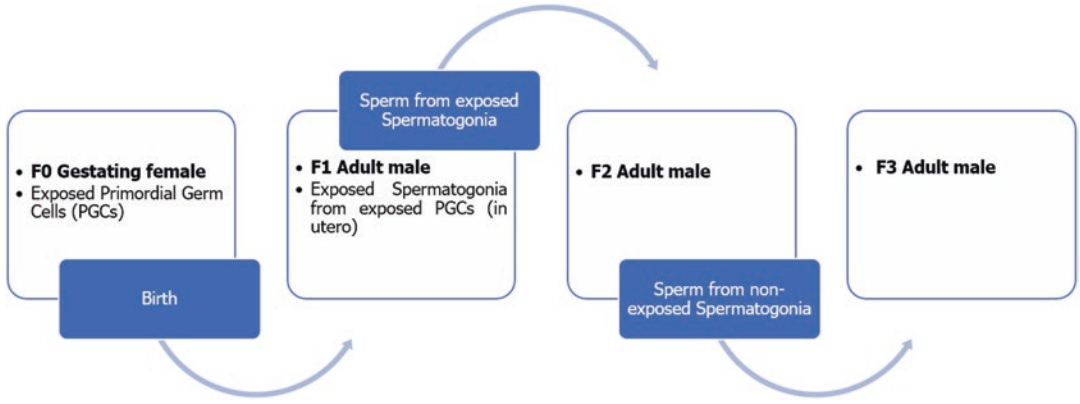

Fig. 4.1 Epigenetic inheritance of environmental-induced changes through the male germline. Transgenerational epigenetic inheritance from an exposed gestating female (F0) occurs when the transmission of a phenotypic alteration via spermatozoa reaches the third generation (F3); otherwise the mode of inheritance is classified as intergenerational

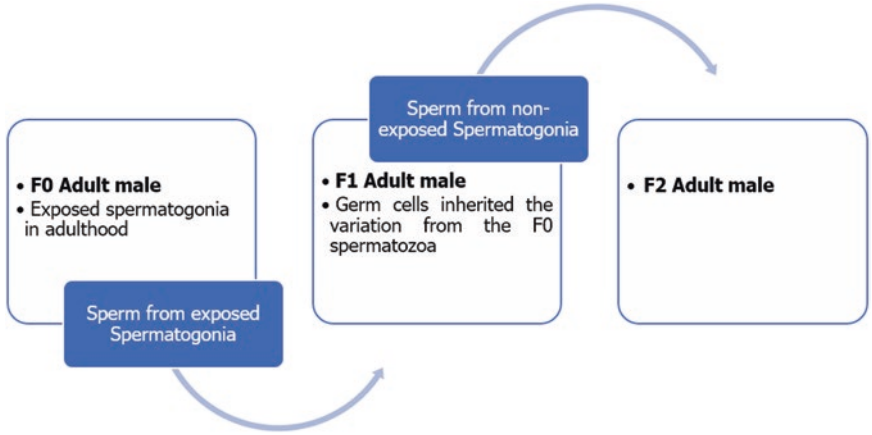

Fig. 4.2 Epigenetic inheritance of environmental-induced changes through the male germline. Transgenerational epigenetic inheritance from the exposure in an adult individual (F0) occurs when the transmission of a phenotypic alteration via spermatozoa reaches the second generation (F2); otherwise the mode of inheritance is classified as intergenerational

transmission. Concerning DNA methylation, it was assumed until a few years ago that the only regions that escaped from the global demethylation during epigenetic reprogramming were those regulated by genomic imprinting (Branco et al. 2008) and some repetitive noncoding DNA (Lane et al. 2003). Nevertheless, several pieces of data suggest that the number of regions is more extensive, affecting non-imprinted coding regions of the genome. For instance, it has been identified a group of CGI (Hackett et al. 2013; Seisenberger et al. 2012) and non-imprinted promoter sequences (Borgel et al. 2010) that resist the global DNA methylation reprogramming in the embryo. In human and mouse embryonic cells, it has been demonstrated the existence of single copy non-imprinted sequences resistant to reprogramming. Interestingly, these regions seem to be enriched in genes particularly active in the brain during adult life development (McGraw et al. 2015; Tang et al. 2015).

The existence of coding regions that escape DNA methylation epigenetic reprogramming points to the possibility of the existence of transgenerational epigenetic inheritance. That is, a part of the genome could be involved in the transgenerational epigenetic transmission of adult-onset disease phenotypes.

Environmental Factors Affect the Human Sperm Epigenome

There are several pieces of evidence demonstrating the influence of environmental factors over the sperm epigenome. Nevertheless, the molecular basis of this phenomenon is poorly understood and appears to be variable between inductor factors. In overall terms, the alteration of the sperm epigenetic signature has been associated to epigenetic insults in the development of PGCs that ultimately affects spermatozoa. Moreover, environmental factors could also disturb testis microenvironment that is crucial to accomplish the epigenetic mechanisms in germ cells during spermatogenesis. It is important to mention that epigenetic modifications associated to environmental factors mainly affect germ cells rather than spermatozoa since the sperm chromatin is a highly condensed structure and, therefore, highly resistant to environmental-induced perturbations.

Overall, the information provided in this section suggest that fetal, perinatal, or adult exposure of male germ cells to environmental factors has a detrimental effect on the sperm epigenome. Therefore, the fertility of the exposed individuals could be compromised. Furthermore, since some of the epimutations appear to be permanent,

which is resistant to the reprogramming events, they could be transmitted to upcoming generations.

Age

Some authors have found a general increase of sperm DNA methylation with age (Camprubí et al. 2016; Jenkins et al. 2014). Since the negative influence of age on the testicular function and seminogram is well documented (Eisenberg and Meldrum 2017), it has been suggested that advanced age could alter the methylation marks of genes associated with male fertility. Actually, the influence of age over DNA methylation goes beyond male fertility. It has been described that DNA from blood of old individuals is more heterogeneous and hypomethylated in comparison with newborn DNA (Heyn et al. 2012).

It is interesting to remark that age-associated epigenome variations observed in human spermatozoa are specially associated to genes involved in neuropsychiatric disease in adult life (Jenkins et al. 2014). In a mouse model, a genome-wide DNA methylation study comparing sperm from young and old mice has revealed that the offspring of older fathers exhibited similar brain DNA methylation abnormalities than that observed in the paternal sperm (Milekic et al. 2014). Moreover, these methylation abnormalities are related to transcriptional dysregulation of developmental genes implicated in autism and schizophrenia (Milekic et al. 2014). These results suggest the possibility of transmission to the next generation of epimutations associated with brain disorders via spermatozoa.

Although the mechanisms that drive age-related methylation alterations in the sperm remain elusive, it appears that the rate of cell proliferation has a direct influence. It has been reported that highly proliferative cells exhibited a greater magnitude of age-associated DNA methylation changes (Thompson et al. 2010), while nondividing cells are less prone to these age effects (Chu et al. 2007). The high proliferation rate of spermatogonial germ cells along reproductive man lifespan made this cell type especially susceptible to age-related epigenetic alterations. It is possible that dividing cells are more prone to the accumulation of epimutations over time since they are exposed to errors during the transmission of the methylation marks in the S-phase of the cell cycle. As stated by other authors, further studies are required to determine whether the observed age-associated effects in spermatozoa are a consequence of the accumulation of epimutations in primordial germ cells or whether they are a consequence of testicular microenvironment perturbations related to advanced age (Oakes et al. 2003).

Obesity

Obesity may induce male infertility by a combination of different factors including endocrine abnormalities that ultimately affects the process of spermatogenesis and early embryogenesis (Du Plessis et al. 2010).

It is well documented that obese men had an increased incidence of sperm epimutations, which is interpreted by some authors as a contributing factor for male infertility. For instance, it has been described sperm DNA methylation differences at specific CpG of imprinted genes between overweight men and normal weight men (Soubry et al. 2016). In a sperm epigenome study from lean and obese men, a difference in small noncoding RNA expression and DNA methylation pattern was observed (Donkin et al. 2016). Moreover, morbidly obese men submitted to surgery-induced weight loss modifies the sperm epigenetic pattern (Donkin et al. 2016). In this regard, in an obesity mouse model, it has been demonstrated the differential abundance of different molecules of sperm microRNAs that have been ontologically associated with embryo development and metabolic and reproductive dysregulations in adulthood (Fullston et al. 2016).

The reason why obesity induces sperm epigenetic alterations has been related to different causes. Endocrine disruptions appear to be one of the most significant. Obesity has been associated with hypogonadism, leading to alterations of the

testicular microenvironment that could interfere with the normal development of the sperm epigenome. In rat models, tamoxifen (estrogen receptor modulator) has been shown to reduce sperm DNA methylation at specific loci (Igf2/H19 differentially methylated region) through DNA methyltransferase 1 (Dnmt1) functional alterations in the testis. Hence, it is likely that this alteration could influence the proliferative phase of spermatogonial germ cells where Dnmt1 proteins are expressed abundantly, resulting in methylation errors in spermatozoa leading to male infertility (Pathak et al. 2009).

Other authors have related the presence of obesity-related epigenetic variations with an increased scrotal temperature, which led to testis hyperthermia and the subsequent reactive oxygen species (ROS) production. It has been described that DNA damage induced by oxidative stress could disturb the functionality of DNA methyltransferases (DNMTs), resulting in methylome variations. DNA lesions affect the ability of DNA to function as a substrate for the DNMTs resulting in hypomethylation (Franco et al. 2008). Moreover, oxidative DNA damage leads to mutations preferably at methylated CpGs that would result in loss of epigenetic marks (Lee 2002). In this regard, ROS production has also been associated to hypermethylation of promoter regions of tumor suppression genes promoting carcinogenesis (Lim et al. 2008). Moreover, DNA damage induced by oxidative stress has also been implicated in the regulation of miRNA expression (Mateescu et al. 2011; Simone et al. 2009).

In animal models, it has been demonstrated a perturbed methylation pattern in the paternal pronuclei derived from heat-stressed spermatozoa (Rahman et al. 2014). In humans, it has been described that varicocele, which has been related to the exposure of sperm to heat, is associated with alterations of the sperm methylome (Bahreinian et al. 2015). Since an increased scrotal temperature is expected in obese men (because of sedentarism), testis heat stress and their detrimental effects on the sperm methylome are expected in obese men.

Endocrine Disruptors

Endocrine disruptors (ER) are a heterogeneous set of exogenous chemical substances capable of altering the regulation of the hormonal system. In reproduction, ER can disturb the regulation of the hypothalamic-pituitary-gonads axis and therefore alter the gonadal sex differentiation and gametogenesis, which ultimately lead to infertility.

In animal models, prenatal or perinatal exposure to relevant doses of ER leads to testis disease, ovarian disease, and pubertal abnormalities in adult individuals (Manikkam et al. 2013; Salian et al. 2011). The exposure to ER in mice causes changes in spermatogonia that result in meiotic alterations in the spermatogenesis of the adult male (Vrooman et al. 2015) that could result in a disruption in the progression of meiosis I and decreased sperm counts (Liu et al. 2013; Tiwari and Vanage 2013).

Since ER act at the time of the germ cell epigenome reprogramming, some authors have associated the exposure to ER to perturbation of the sperm epigenome, mainly by means of alterations of DNA methylation (Consales et al. 2016; Miao et al. 2014). ER would induce alterations of the testicular microenvironment and increase sperm DNA damage (Tiwari and Vanage 2013) that ultimately would perturb the epigenetic marks by affecting DNA methylation patterns.

Diet

It is well known that dietary compounds, such as phytochemicals, minerals and vitamins, can promote changes in epigenetic mechanisms of somatic as well as germ cells by influencing enzymes and other proteins responsible for epigenetic modifications (Schagdarsurengin and Steger 2016).

For instance, B vitamins must be provided by diet or supplementation and modulate the availability of methyl groups provided by the 1 Carbon Cycle, which is essential to ensure the availability of activated methyl groups for the methylation

reactions of the cell. Methyl groups needed by methyltransferases are provided by S-adenosyl-L-methionine (SAM) through the 1 Carbon Cycle. Thus, diet can influence the levels of DNA methylation and consequently affect gene expression. Other authors have reported an association between vitamin D deficiency and global dysregulation of the methylome via overexpression of DNA methyltransferase 3b (Dnmt3b) transcripts (Xue et al. 2016).

In this regard, the influence of diet on the sperm epigenome has been demonstrated in several studies including humans (Schagdarsurengin et al. 2012), mainly through alteration of sperm DNA methylation (Aarabi et al. 2015; Lambrot et al. 2013). These variations have been associated with negative effects on the sperm quality that would affect the reproduction success of the couple.

Metabolic Disorders: Diabetes

Glucose metabolism is of great importance for sperm cell functionality. Diabetic disease has been associated with detrimental effects on male fertility, especially on sperm quality, sperm DNA integrity, and sperm epigenome dysregulations (Ding et al. 2015). In particular, alterations of the sperm methylome in paternal prediabetes individuals have been described (Wei et al. 2014).

Diabetes-induced testicular impairment due to its detrimental effect over testis microcirculation (Long et al. 2018). This detrimental effect increases the susceptibility of spermatogenic germ cells to generate ROS (Long et al. 2015). ROS generation in diabetic patients has been also associated with increased testicular temperature resulting from fat accumulation, which leads to testis hyperthermia (Wei et al. 2014). Among the collateral damage on male fertility induced by ROS, aberrant sperm DNA methylation is one of the most significant.

Chemotherapy

Those agents used to treat cancer that interfere with the process of DNA methylation or DNA replication have a severe impact over spermatogenesis (Chan et al. 2012; Doerksen et al. 2000; Doerksen and Trasler 1996; Kelly et al. 2003) and early embryo development (Doerksen et al. 2000; Kelly et al. 2003). When these treatments are of sufficient duration to affect the spermatogonia, the alterations of the sperm epigenome are permanent (Chan et al. 2012; Doerksen et al. 2000; Kelly et al. 2003). In this regard, adolescent chemotherapy exposure in patients with osteoblastoma has been related with sperm epimutations in adult life (Shnorhavorian et al. 2017). These results suggest that chemotherapy exposure causes permanent epigenetic alterations in the spermatogonial epigenome.

Alcohol

Although the association between alcohol intake and male infertility remains controversial (Martini et al. 2004), there is no doubt about the detrimental effect of alcohol consumption on DNA integrity due to the oxidative damage induced by consumption (Ellegaard and Poulsen 2016). This effect has been also found in male germ cell line (Aboulmaouahib et al. 2018). Since ROS is connected to alterations of DNA methylation, some authors have found sperm methylome variations in alcohol-exposed individuals (Liang et al. 2014; Ouko et al. 2009).

The alteration of the sperm methylome induced by alcohol intake has been also associated with decreases in the activity of DNA methyltransferase 1 (Dnmt1) (Bielawski et al. 2002; Garro et al. 1991) or reduced production of the methyl donor SAM (Sultana et al. 2015).

Smoking

Like alcohol intake, seminal quality is not clearly altered by cigarette consumption, although subtle modifications have been described suggesting an effect on male reproductive function (Martini et al. 2004).

There is a clear connection between tobacco and DNA oxidative damage as a consequence of

the production of ROS (Ellegaard and Poulsen 2016). Moreover, smoking is known to cause ROS throughout spermatogenesis, which would affect the sperm DNA integrity (Aboulmaouahib et al. 2018), including some marginal effects on sperm DNA methylation (Al Khaled et al. 2018; Hamad et al. 2018; Laqqan et al. 2017).

Although the reason why smoking causes sperm DNA methylation variations deserves further investigation, some authors have identified a detrimental effect of nicotine on DNA methyltransferase expression (Satta et al. 2008). Moreover, cigarette smoke may alter DNA methylation via the interference of hypoxia (which is usual in smoker individuals) with the availability of SAM (Liu et al. 2011).

Epigenetic Mechanisms Are Strong Candidates for Transferring Paternal Environmental Information

In the last decade, several studies have addressed the analysis of the sperm epigenome as a vehicle for the transmission to offspring of epimutations induced by environmental factors (Table 4.1). Among the different epigenetic mechanisms, DNA methylation has been the most studied, probably because it has been proved that some sperm DNA methylation signatures escape the reprogramming events in the early embryo. Accordingly, at least a portion of the sperm DNA methylation variations induced by environmental factor has the potential to be retained in germ cells and be transmitted to the next generation.

Sperm Epimutations Affect Embryo Development

Several pieces of data suggest that sperm epigenome variations have a detrimental effect on embryo development, suggesting their fundamental role in postfertilization events. In humans, some authors have associated the presence of sperm DNA methylation variations and low pregnancy rate (Benchaib et al. 2005). Recently, Denommme et al. have described sperm DNA methylation differences at CGI contained in retained histone regions between good and poor blastocyst development groups (Denomme et al. 2017). In the case of histones, the fact that histones are retained in the promoters of developmental genes (Hammoud et al. 2009), and the fact that sperm histone modifications are transmitted to the embryo and are resistant to protein oocyte replacement (Van Der Heijden et al. 2008), argues in favor of an effect beyond fertilization. Finally, some data from sncRNAs demonstrated the importance of some sperm-borne miRNAs for early embryo development, suggesting that alteration of the sperm RNA cargo could be critical for the first cleavage events (Liu et al. 2012).

Sperm Epimutations Affect the Health of the Exposed Men and Their Offspring

Environmentally induced epigenetic inheritance refers to the transmission of epigenetic information through sperm cells in the absence of continuous exposure to the inductor agent. A great number of studies have addressed the issue of the transmission of epigenetic changes via spermatozoa through epigenetic perturbations of the germ line (Table 4.1). Most of the studies have analyzed this phenomenon using animal models, whereas in humans this phenomenon has been poorly studied. Several factors may induce epigenetic variations among which are endocrine disruptors, diet, exercise training, diabetes, alcohol, obesity, stress, smoking, dioxin, pesticide, hydrocarbon, and age.

As we stated before, the only way to explain the transmission of an induced epigenetic variation across generations is the permanent reprogramming of germ cells. That is, the variation must be resistant to the different reprogramming periods. This situation hardly will occur in the case of posttranslational modifications of histones and sncRNA signature, but it is possible for DNA methylation. The discovery of coding regions that escape DNA methylation epigenetic

Table 4.1 Summary of the 26 studies that investigated the spermatozoa as a vehicle for the transmission to offspring of epimutations induced by environmental factors

	Agent	Exposure	Epigenetic mechanisms	Sperm variation	Adult phenotypic affectation	Sperm vs somatic[c]	Inheritance
Anway et al. (2005)	Endocrine disruptor	Gestating rat female	DNA methylation	F2, F3	Male infertility (from F1 to F4)	NA	Transgenerational
Carone et al. (2010)	Diet	Adult mouse male	DNA methylation/ miRNAs	F0[b]	Metabolic disorders (F1)	No	Intergenerational
de Castro Barbosa et al. (2016)	Diet	Adult rat male	DNA methylation/ sncRNA	F0, F1	Body weight and metabolic disorders (F1 and F2)	Yes (miRNA)	Transgenerational
Denham et al. (2015)	Exercise	Adult human male	DNA methylation	F0	NA	NA	NA
Ding et al. (2012)	Diabetes	Gestating mouse female	DNA methylation	F1	Insulin secretion (F1 and F2) Body weight (F2)	Yes	Intergenerational
Finegersh and Homanics (2014)	Alcohol	Adult mouse male	DNA methylation	F0, F1	Behavioral changes to alcohol (F1)	NA	Intergenerational
Fullston et al. (2013)	Obesity	Adult mouse male	DNA methylation/ miRNAs/mRNAs	F0	Obesity (F1 and F2)	NA	NA
Fullston et al. (2016)	Obesity	Adult mouse male	miRNAs	F0, F1[b]	Suboptimal metabolic and reproductive outcomes (F1)	NA	Intergenerational
Gapp et al. (2014)	Stress	Adult mouse male	sncRNA	F0, F1, F2[b]	Abnormal behavior (F1 to F3)	Yes	Transgenerational
Ge et al. (2014)	Obesity/ diabetes	Gestating mouse female	DNA methylation	F1	NA	NA	NA
Guerrero-Bosagna et al. (2010)	Endocrine disruptor	Gestating rat female	DNA methylation	F3	NA	NA	NA
Iqbal et al. (2015)	Endocrine disruptor	Gestating mouse female	DNA methylation	F1, F2[b]	NA	NA	NA
Jenkins et al. (2017)	Smoking	Adult human male	DNA methylation	F0	NA	NA	NA
Lambrot et al. (2013)	Diet	Gestating mouse female	DNA methylation/H3 methylation	F0	Birth defects (F1)	Yes (2 out of 300 genes)	Intergenerational
Liang et al. (2014)	Alcohol	Adult mouse male	DNA methylation	F0	Brain disorders (F1)	Yes	Intergenerational
Manikkam et al. (2012a)	Dioxin	Gestating rat female	DNA methylation	F3	Multiple disorders (F1 and F3)	NA	Transgenerational
Manikkam et al. (2012b)	Various[a]	Gestating rat female	DNA methylation	F3	Multiple disorders (F1 and F3)	NA	Transgenerational

Manikkam et al. (2013)	Endocrine disruptor	Gestating rat female	DNA methylation	F3	Multiple disorders (F1 and F3)	NA	Transgenerational
Martínez et al. (2014)	Diet	Gestating mouse female	DNA methylation	F1	Metabolic disorders (F2)	Yes	Intergenerational
Milekic et al. (2014)	Age	Adult mouse male	DNA methylation	F0	Autism and schizophrenia (F1)	Yes	Intergenerational
Radford et al. (2014)	Diet	Gestating mouse female	DNA methylation	F1	Metabolic disorders (F2)	No	Intergenerational
Soubry et al. (2016)	Obesity	Adult human male	DNA methylation	F0	NA	NA	NA
Stouder and Paoloni-Giacobino (2010)	Endocrine disruptor	Gestating mouse female	DNA methylation	F1-F2-F3	NA	Yes	NA
Tracey et al. (2013)	Hydrocarbon	Gestating rat female	DNA methylation	F3	Multiple disorders (F1 and F3)	NA	Transgenerational
Wei et al. (2014)	Diabetes	Adult mouse male	DNA methylation	F1	Metabolic disorders (F1 and F2)	Yes	Transgenerational
Xue et al. (2016)	Diet	Gestating mouse female	DNA methylation	F1-F2	Body and testes weight (F1 and F2)	No	Intergenerational

[a]Pesticide, endocrine disruptor, dioxin, hydrocarbon
[b]Lack of sperm epigenome variations
[c]Sperm differential DNA methylation is maintained in the next generation of cells from somatic tissues

reprogramming points out the possibility of the participation of this mechanism in transgenerational epigenetic inheritance events. Therefore, DNA methylation is, by far, the most studied epigenetic mechanisms in transgenerational studies (Table 4.1).

It is important to mention again that only the transmission of a phenotypic alteration via spermatozoa until the third generation (in the case of exposure of a gestating F0 female; Fig. 4.1), or the second generation (when the exposure occurs in an adult individual; Fig. 4.2), could be considered true transgenerational inheritance (Skinner 2008).

Intergenerational Inheritance

Intergenerational analysis has been performed in 10 different studies, 5 from a gestating female (Ding et al. 2012; Lambrot et al. 2013; Martínez et al. 2014; Radford et al. 2014; Xue et al. 2016) and 5 from the exposure of an adult male (Carone et al. 2010; Finegersh and Homanics 2014; Fullston et al. 2016; Liang et al. 2014; Milekic et al. 2014).

In the studies from a gestating female, authors demonstrated the transmission of phenotypic alterations to the F2 generation, including metabolic and body weight alterations which in all cases were related with the inducing factor (diabetes and diet) (Ding et al. 2012; Lambrot et al. 2013; Martínez et al. 2014; Radford et al. 2014; Xue et al. 2016). In three studies, authors found that the same epimutations observed in spermatozoa were maintained, at least in part, in somatic tissues of the following generation, reinforcing the interpretation of epigenetic inheritance (Ding et al. 2012; Lambrot et al. 2013; Martínez et al. 2014).

The remaining five studies were designed from exposures of adult males (Carone et al. 2010; Finegersh and Homanics 2014; Fullston et al. 2016; Liang et al. 2014; Milekic et al. 2014). Again, the authors observed phenotypic effects in offspring related to the inducing agent. Concerning the postulation of the sperm cell as a vehicle for transmission, all studies except one (Carone et al. 2010) demonstrated sperm epigenome variations. Two works demonstrated the presence of the same sperm methylome variation in sperm and somatic tissues from next generation (Liang et al. 2014; Milekic et al. 2014).

Transgenerational Inheritance

Eight transgenerational studies have been published so far, five from the exposure to a gestating female (Anway et al. 2005; Manikkam et al. 2012a, b, Manikkam et al. 2013; Tracey et al. 2013) and three from adult male exposure (de Castro Barbosa et al. 2016; Gapp et al. 2014; Wei et al. 2014).

In the cases of gestational female exposure (Anway et al. 2005; Manikkam et al. 2012a, b, Manikkam et al. 2013; Tracey et al. 2013), authors demonstrated the transmission of phenotypic alterations (including alterations of the reproductive system, kidney disease, and obesity) until the F3 generation. Moreover, all these studies found sperm DNA methylation variations in F3 spermatozoa, suggesting that the transmission of the epigenetic phenotypic alteration is associated to sperm methylome variations that are not reprogrammed across generations. It is important to mention that, in none of them, the authors analyzed if the epimutations observed in spermatozoa were also present in somatic tissues of the subsequent generation.

Three more studies demonstrated transgenerational inheritance in adult male exposures (F2 phenotypic alterations related to the inducing agent) (de Castro Barbosa et al. 2016; Gapp et al. 2014; Wei et al. 2014). In all the cases, authors identify sperm and somatic epigenome variations associated with the inducing factor that would explain the phenotypic alteration observed in F2 individuals. These results are highly indicative of true transgenerational inheritance.

Overall, the revision of the literature performed in the present manuscript (Table 4.1) demonstrated the existence of strong evidences about the presence of epigenetic inheritance via

spermatozoa. Nevertheless, we must be cautious in the interpretation of the results. It is important to mention that most of the studies only provide partial evidences about this phenomenon. In this sense, a study demonstrating unequivocally the presence of transgenerational inheritance via spermatozoa is currently lacking. This study must accomplish the following requirements: (i) to identify sperm epigenome variations induced by environmental factors, (ii) to demonstrate the transmission of epigenome variations from sperm to somatic tissues, (iii) to identify phenotypic effects associated to the presence of epimutations, and iv) to demonstrate the presence of the same epimutations in sperm and somatic tissues at least until the first nonexposed generation.

Is Multigenerational Disease Prevention a New Paradigm?

From the information provided in the preceding paragraphs, it becomes clear that the exposure to certain environmental factors in specific windows of sperm development influences the risk of developing chronic diseases and behavior disorders in adulthood. These studies support the intriguing idea that human beings could adapt the expression of genes to environmental signals. That is, epigenetic plasticity would provide the ability for adaptation to the current environment in individuals of equal genotype. Accordingly, an area of research that could be crucial in the near future regards the possibility to prevent the onset of epigenetic-based diseases through the modulation of the sperm epigenome in the previous generation. That is, the modification of lifestyle factors driving to sperm epimutations could be a powerful tool to normalize the sperm epigenome and avoid their negative consequences.

Some evidence suggests the veracity of this possibility. For instance, in a mouse model, it has been demonstrated that diet or exercise training in obese males restores insulin sensitivity and normalized adiposity in female offspring. These modifications are associated with the normalization of sperm microRNA pattern, suggesting that diet and/or exercise normalize aberrant epigenetic signals in sperm and improve the metabolic health of offspring (McPherson et al. 2015). In humans, it has been demonstrated that exercise training modified the sperm DNA methylation mark of genes related to schizophrenia and Parkinson's disease (Denham et al. 2015). Also, surgery-induced weight loss has been associated with a remodeling of sperm DNA methylation, especially at genetic locations implicated in the central control of appetite (Donkin et al. 2016).

Concluding Remarks

The sperm epigenome is the result of the different periods of epigenome reprogramming in germ cells. These reprogramming events have the main function to develop totipotent cells and to prevent the transmission of epimutations via spermatozoa. At the end of these reprogramming events, spermatozoa carry a distinctive epigenome, which is a footprint of spermatogenesis events and is programmed to allow embryogenesis and to influence in adult life.

Since the sperm epigenome is sensitive to numerous environmental factors, it is clearly susceptible to variations. The discovery of coding regions that escape DNA methylation epigenetic reprogramming points to the possibility of the transmission of epigenetic variation between generations (induced by environmental factors) and hence, to the existence of transgenerational epigenetic inheritance. In animal models, there are strong evidences about the presence of transgenerational epigenetic inheritance via spermatozoa. Nevertheless, a complete study unequivocally demonstrating this kind of transmission is currently lacking.

The high plasticity of the sperm epigenome opens the possibility of its modulation through the modification of lifestyle factors. This is a very promising area in the field of reproductive epigenetics, that is, the analysis of the normalizer effect of changes in lifestyle factors on the sperm epigenome as a tool to overcome some types of male infertility.

References

Aarabi M, San Gabriel MC, Chan D, Behan NA, Caron M, Pastinen T, Bourque G, MacFarlane AJ, Zini A, Trasler J (2015) High-dose folic acid supplementation alters the human sperm methylome and is influenced by the *MTHFR* C677T polymorphism. Hum Mol Genet 24:6301–6313

Aboulmaouahib S, Madkour A, Kaarouch I, Sefrioui O, Saadani B, Copin H, Benkhalifa M, Louanjli N, Cadi R (2018) Impact of alcohol and cigarette smoking consumption in male fertility potential: looks at lipid peroxidation, enzymatic antioxidant activities and sperm DNA damage. Andrologia 50:e12926

Al Khaled Y, Tierling S, Laqqan M, Lo Porto C, Hammadeh ME (2018) Cigarette smoking induces only marginal changes in sperm DNA methylation levels of patients undergoing intracytoplasmic sperm injection treatment. Andrologia 50:e12818

Amanai M, Brahmajosyula M, Perry ACF (2006) A restricted role for sperm-borne MicroRNAs in mammalian fertilization. Biol Reprod 75:877–884

Anway MD, Cupp AS, Uzumcu M, Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308:1466–1469

Arpanahi A, Brinkworth M, Iles D, Krawetz SA, Paradowska A, Platts AE, Saida M, Steger K, Tedder P, Miller D (2009) Endonuclease-sensitive regions of human spermatozoal chromatin are highly enriched in promoter and CTCF binding sequences. Genome Res 19:1338–1349

Bahreinian M, Tavalaee M, Abbasi H, Kiani-Esfahani A, Shiravi AH, Nasr-Esfahani MH (2015) DNA hypomethylation predisposes sperm to DNA damage in individuals with varicocele. Syst Biol Reprod Med 61:179–186

Bao J, Wu J, Schuster AS, Hennig GW, Yan W (2013) Expression profiling reveals developmentally regulated lncRNA repertoire in the mouse male germline. Biol Reprod 89:107

Benchaib M, Braun V, Ressnikof D, Lornage J, Durand P, Niveleau A, Guérin JF (2005) Influence of global sperm DNA methylation on IVF results. Hum Reprod 20:768–773

Bielawski DM, Zaher FM, Svinarich DM, Abel EL (2002) Paternal alcohol exposure affects sperm cytosine methyltransferase messenger RNA levels. Alcohol Clin Exp Res 26:347–351

Boerke A, Dieleman SJ, Gadella BM (2007) A possible role for sperm RNA in early embryo development. Theriogenology 68(Suppl 1):S147–S155

Borgel J, Guibert S, Li Y, Chiba H, Schübeler D, Sasaki H, Forné T, Weber M (2010) Targets and dynamics of promoter DNA methylation during early mouse development. Nat Genet 42:1093–1100

Branco MR, Oda M, Reik W (2008) Safeguarding parental identity: Dnmt1 maintains imprints during epigenetic reprogramming in early embryogenesis. Genes Dev 22:1567–1571

Brunner AM, Nanni P, Mansuy IM (2014) Epigenetic marking of sperm by post-translational modification of histones and protamines. Epigenetics Chromatin 7(2)

Camprubí C, Salas-Huetos A, Aiese-Cigliano R, Godo A, Pons MC, Castellano G, Grossmann M, Sanseverino W, Martin-Subero JI, Garrido N, Blanco J (2016) Spermatozoa from infertile patients exhibit differences of DNA methylation associated with spermatogenesis-related processes: an array-based analysis. Reprod Biomed Online 33:709–719

Camprubí C, Aiese-Cigliano R, Salas-Huetos A, Garrido N, Blanco J (2017) What the human sperm methylome tells us. Epigenomics 9:1299–1315

Carone BR, Fauquier L, Habib N, Shea JM, Hart CE, Li R, Bock C, Li C, Gu H, Zamore PD, Meissner A, Weng Z, Hofmann HA, Friedman N, Rando OJ (2010) Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. Cell 143:1084–1096

Chan D, Delbe's G, Landry M, Robaire B, Trasler JM (2012) Epigenetic alterations in sperm DNA associated with testicular cancer treatment. Toxicol Sci 125:532–543

Chu MW, Siegmund KD, Eckstam CL, Kim JY, Yang AS, Kanel GC, Tavaré S, Shibata D (2007) Lack of increases in methylation at three CpG-rich genomic loci in non-mitotic adult tissues during aging. BMC Med Genet 8:50

Chuma S, Nakano T (2012) piRNA and spermatogenesis in mice. Philos Trans R Soc London B Biol Sci 368:20110338

Chuva de Sousa Lopes SM, Roelen BAJ (2010) On the formation of germ cells: the good, the bad and the ugly. Differentiation 79:131–140

Consales C, Toft G, Leter G, Bonde JPE, Uccelli R, Pacchierotti F, Eleuteri P, Jönsson BAG, Giwercman A, Pedersen HS, Struciński P, Góralczyk K, Zviezdai V, Spanò M (2016) Exposure to persistent organic pollutants and sperm DNA methylation changes in Arctic and European populations. Environ Mol Mutagen 57:200–209

Corral-Vazquez C, Anton E (2018) Epigenetic and assisted reproduction experimental studies: sperm ncRNAs. In: Blanco J, Camprubí C (eds) Epigenetics and assisted reproduction an introductory guide. Taylor & Francis, Boca Raton

Davis TL, Yang GJ, McCarrey JR, Bartolomei MS (2000) The H19 methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development. Hum Mol Genet 9:2885–2894

de Castro Barbosa T, Ingerslev LR, Alm PS, Versteyhe S, Massart J, Rasmussen M, Donkin I, Sjögren R, Mudry JM, Vetterli L, Gupta S, Krook A, Zierath JR, Barrès R (2016) High-fat diet reprograms the epigenome of rat spermatozoa and transgenerationally affects metabolism of the offspring. Mol Metab 5:184–197

de Mateo S, Sassone-Corsi P (2014) Regulation of spermatogenesis by small non-coding RNAs: role of the germ granule. Semin Cell Dev Biol 29:84–92

Denham J, O'Brien BJ, Harvey JT, Charchar FJ (2015) Genome-wide sperm DNA methylation changes after 3 months of exercise training in humans. Epigenomics 7:1–15

Denomme MM, McCallie BR, Parks JC, Schoolcraft WB, Katz-Jaffe MG (2017) Alterations in the sperm histone-retained epigenome are associated with unexplained male factor infertility and poor blastocyst development in donor oocyte IVF cycles. Hum Reprod 32:1–13

Ding GL, Wang FF, Shu J, Tian S, Jiang Y, Zhang D, Wang N, Luo Q, Zhang Y, Jin F, Leung PCK, Sheng JZ, Huang HF (2012) Transgenerational glucose intolerance with Igf2/H19 epigenetic alterations in mouse islet induced by intrauterine hyperglycemia. Diabetes 61:1133–1142

Ding G-L, Liu Y, Liu M-E, Pan J-X, Guo M-X, Sheng J-Z, Huang H-F (2015) The effects of diabetes on male fertility and epigenetic regulation during spermatogenesis. Asian J Androl 17:948

Doerksen T, Trasler JM (1996) Developmental exposure of male germ cells to 5-Azacytidine results in abnormal preimplantation development in rats. Biol Reprod 55:1155–1162

Doerksen T, Benoit G, Trasler JM (2000) Deoxyribonucleic acid hypomethylation of male germ cells by mitotic and meiotic exposure to 5-azacytidine is associated with altered testicular histology. Endocrinology 141:3235–3244

Donkin I, Versteyhe S, Ingerslev LR, Qian K, Mechta M, Nordkap L, Mortensen B, Appel EVR, Jørgensen N, Kristiansen VB, Hansen T, Workman CT, Zierath JR, Barrès R (2016) Obesity and bariatric surgery drive epigenetic variation of spermatozoa in humans. Cell Metab 23:369–378

Du Plessis SS, Cabler S, McAlister DA, Sabanegh E, Agarwal A (2010) The effect of obesity on sperm disorders and male infertility. Nat Rev Urol 7:153–161

Eisenberg ML, Meldrum D (2017) Effects of age on fertility and sexual function. Fertil Steril 107:301–304

Ellegaard PK, Poulsen HE (2016) Tobacco smoking and oxidative stress to DNA: a meta-analysis of studies using chromatographic and immunological methods. Scand J Clin Lab Invest 76:151–158

Finegersh A, Homanics GE (2014) Paternal alcohol exposure reduces alcohol drinking and increases behavioral sensitivity to alcohol selectively in male offspring. PLoS One 9:e99078

Franco R, Schoneveld O, Georgakilas AG, Panayiotidis MI (2008) Oxidative stress, DNA methylation and carcinogenesis. Cancer Lett 266:6–11

Fullston T, Ohlsson Teague EM, Palmer NO, DeBlasio MJ, Mitchell M, Corbett M, Print CG, Owens JA, Lane M (2013) Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content. FASEB J 27:4226–4243

Fullston T, Ohlsson-Teague EMC, Print CG, Sandeman LY, Lane M (2016) Sperm microRNA content is altered in a mouse model of male obesity, but the same suite of microRNAs are not altered in offspring's sperm. PLoS One 11:e0166076

Gapp K, Jawaid A, Sarkies P, Bohacek J, Pelczar P, Prados J, Farinelli L, Miska E, Mansuy IM (2014) Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. Nat Neurosci 17:667–669

Garro AJ, McBeth DL, Lima V, Lieber CS (1991) Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. Alcohol Clin Exp Res 15:395–398

Gatewood JM, Cook GR, Balhorn R, Bradbury EM, Schmid CW (1987) Sequence-specific packaging of DNA in human sperm chromatin. Science 236:962–964

Ge ZJ, Liang QX, Hou Y, Han ZM, Schatten H, Sun QY, Zhang CL (2014) Maternal obesity and diabetes may cause DNA methylation alteration in the spermatozoa of offspring in mice. Reprod Biol Endocrinol 12:29

Guerrero-Bosagna C, Settles M, Lucker B, Skinner MK (2010) Epigenetic transgenerational actions of vinclozolin on promoter regions of the sperm epigenome. PLoS One 5:e13100

Guibert S, Forne T, Weber M (2012) Global profiling of DNA methylation erasure in mouse primordial germ cells. Genome Res 22:633–641

Hackett JA, Sengupta R, Zylicz JJ, Murakami K, Lee C, Down TA, Surani MA (2013) Germline DNA demethylation dynamics and imprint erasure through 5-hydroxymethylcytosine. Science 339:448–452

Hajkova P, Erhardt S, Lane N, Haaf T, El-Maarri O, Reik W, Walter J, Surani MA (2002) Epigenetic reprogramming in mouse primordial germ cells. Mech Dev 117:15–23

Hamad MF, Dayyih WAA, Laqqan M, AlKhaled Y, Montenarh M, Hammadeh ME (2018) The status of global DNA methylation in the spermatozoa of smokers and non-smokers. Reprod Biomed Online 37:581–589

Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR (2009) Distinctive chromatin in human sperm packages genes for embryo development. Nature 460:473–478

Heyn H, Li N, Ferreira HJ, Moran S, Pisano DG, Gomez A, Diez J, Sanchez-Mut JV, Setien F, Carmona FJ, Puca AA, Sayols S, Pujana MA, Serra-Musach J, Iglesias-Platas I, Formiga F, Fernandez AF, Fraga MF, Heath SC, Valencia A, Gut IG, Wang J, Esteller M (2012) Distinct DNA methylomes of newborns and centenarians. Proc Natl Acad Sci 109:10522–10527

Iqbal K, Tran DA, Li AX, Warden C, Bai AY, Singh P, Wu X, Pfeifer GP, Szabó PE (2015) Deleterious effects of endocrine disruptors are corrected in the mammalian germline by epigenome reprogramming. Genom Biol 16:59

Jenkins TG, Aston KI, Pflueger C, Cairns BR, Carrell DT (2014) Age-associated sperm DNA methylation alterations: possible implications in offspring disease susceptibility. PLoS Genet 10:e1004458

Jenkins TG, James ER, Alonso DF, Hoidal JR, Murphy PJ, Hotaling JM, Cairns BR, Carrell DT, Aston KI (2017) Cigarette smoking significantly alters sperm DNA methylation patterns. Andrology 5:1089–1099

Jodar M, Selvaraju S, Sendler E, Diamond MP, Krawetz SA (2013) The presence, role and clinical use of spermatozoal RNAs. Hum Reprod Update 19:604–624

Kagiwada S, Kurimoto K, Hirota T, Yamaji M, Saitou M (2013) Replication-coupled passive DNA demethylation for the erasure of genome imprints in mice. EMBO J 32:340–353

Kelly TLJ, Li E, Trasler JM (2003) 5-Aza-2′-Deoxycytidine induces alterations in murine spermatogenesis and pregnancy outcome. J Androl 24:822–830

Kerjean A, Dupont JM, Vasseur C, Le Tessier D, Cuisset L, Pàldi A, Jouannet P, Jeanpierre M (2000) Establishment of the paternal methylation imprint of the human H19 and MEST/PEG1 genes during spermatogenesis. Hum Mol Genet 9:2183–2187

Kota SK, Feil R (2010) Epigenetic transitions in germ cell development and meiosis. Dev Cell 19:675–686

Krausz C, Sandoval J, Sayols S, Chianese C, Giachini C, Heyn H, Esteller M (2012) Novel insights into DNA methylation features in spermatozoa: stability and peculiarities. PLoS One 7:e44479

Lambrot R, Xu C, Saint-Phar S, Chountalos G, Cohen T, Paquet M, Suderman M, Hallett M, Kimmins S (2013) Low paternal dietary folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes. Nat Commun 4:2889

Lane N, Dean W, Erhardt S, Hajkova P, Surani A, Walter J, Reik W (2003) Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. Genesis 35:88–93

Laqqan M, Tierling S, Alkhaled Y, Porto CL, Solomayer EF, Hammadeh ME (2017) Aberrant DNA methylation patterns of human spermatozoa in current smoker males. Reprod Toxicol 71:126–133

Lee DH (2002) Oxidative DNA damage induced by copper and hydrogen peroxide promotes CG -->TT tandem mutations at methylated CpG dinucleotides in nucleotide excision repair-deficient cells. Nucleic Acids Res 30:3566–3573

Liang F, Diao L, Liu J, Jiang N, Zhang J, Wang H, Zhou W, Huang G, Ma D (2014) Paternal ethanol exposure and behavioral abnormities in offspring: associated alterations in imprinted gene methylation. Neuropharmacology 81:126–133

Lim SO, Gu JM, Kim MS, Kim HS, Park YN, Park CK, Cho JW, Park YM, Jung G (2008) Epigenetic changes induced by reactive oxygen species in hepatocellular carcinoma: methylation of the E-cadherin promoter. Gastroenterology 135:2128–2140

Liu Q, Liu L, Zhao Y, Zhang J, Wang D, Chen J, He Y, Wu J, Zhang Z, Liu Z (2011) Hypoxia induces genomic DNA demethylation through the activation of HIF-1 and transcriptional upregulation of MAT2A in hepatoma cells. Mol Cancer Ther 10:1113–1123

Liu WM, Pang RTK, Chiu PCN, Wong BPC, Lao K, Lee KF, Yeung WSB (2012) Sperm-borne microRNA-34c is required for the first cleavage division in mouse. Proc Natl Acad Sci 109:490–494

Liu C, Duan W, Li R, Xu S, Zhang L, Chen C, He M, Lu Y, Wu H, Pi H, Luo X, Zhang Y, Zhong M, Yu Z, Zhou Z (2013) Exposure to bisphenol a disrupts meiotic progression during spermatogenesis in adult rats through estrogen-like activity. Cell Death Dis 4:e676

Long L, Wang J, Lu X, Xu Y, Zheng S, Luo C, Li Y (2015) Protective effects of scutellarin on type II diabetes mellitus-induced testicular damages related to reactive oxygen species/Bcl-2/Bax and reactive oxygen species/microcirculation/staving pathway in diabetic rat. J Diabetes Res 2015:252530

Long L, Qiu H, Cai B, Chen N, Lu X, Zheng S, Ye X, Li Y (2018) Hyperglycemia induced testicular damage in type 2 diabetes mellitus rats exhibiting microcirculation impairments associated with vascular endothelial growth factor decreased via PI3K/Akt pathway. Oncotarget 9:5321–5336

Luo LF, Hou CC, Yang WX (2015) Small non-coding RNAs and their associated proteins in spermatogenesis. Gene 578:141–157

Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK (2012a) Dioxin (TCDD) induces epigenetic transgenerational inheritance of adult onset disease and sperm epimutations. PLoS One 7:e46249

Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK (2012b) Pesticide and insect repellent mixture (permethrin and DEET) induces epigenetic transgenerational inheritance of disease and sperm epimutations. Reprod Toxicol 34:708–719

Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK, Gounon P (2013) Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. PLoS One 8:e55387

Martínez D, Pentinat T, Ribó S, Daviaud C, Bloks VW, Cebrià J, Villalmanzo N, Kalko SG, Ramón-Krauel M, Díaz R, Plösch T, Tost J, Jiménez-Chillarón JC (2014) In utero undernutrition in male mice programs liver lipid metabolism in the second-generation offspring involving altered Lxra DNA methylation. Cell Metab 19:941–951

Martini AC, Molina RI, Estofán D, Senestrari D, Fiol De Cuneo M, Ruiz RD (2004) Effects of alcohol and cigarette consumption on human seminal quality. Fertil Steril 82:374–377

Mateescu B, Batista L, Cardon M, Gruosso T, De Feraudy Y, Mariani O, Nicolas A, Meyniel JP, Cottu P, Sastre-Garau X, Mechta-Grigoriou F (2011) miR-141 and miR-200a act on ovarian tumorigenesis by controlling oxidative stress response. Nat Med 17:1627–1635

McGraw S, Zhang JX, Farag M, Chan D, Caron M, Konermann C, Oakes CC, Mohan KN, Plass C, Pastinen T, Bourque G, Chaillet JR, Trasler JM (2015) Transient DNMT1 suppression reveals hidden

heritable marks in the genome. Nucleic Acids Res 43:1485–1497

McPherson NO, Owens JA, Fullston T, Lane M (2015) Preconception diet or exercise intervention in obese fathers normalizes sperm microRNA profile and metabolic syndrome in female offspring. Am J Physiol Endocrinol Metab 308:E805–E821

Miao M, Zhou X, Li Y, Zhang O, Zhou Z, Li T, Yuan W, Li R, Li DKK (2014) LINE-1 hypomethylation in spermatozoa is associated with Bisphenol A exposure. Andrology 2:138–144

Milekic MH, Xin Y, O'Donnell A, Kumar KK, Bradley-Moore M, Malaspina D, Moore H, Brunner D, Ge Y, Edwards J, Paul S, Haghighi FG, Gingrich J a (2014) Age-related sperm DNA methylation changes are transmitted to offspring and associated with abnormal behavior and dysregulated gene expression. Mol Psychiatry 20:1–7

Molaro A, Hodges E, Fang F, Song Q, McCombie WR, Hannon GJ, Smith AD (2011) Sperm methylation profiles reveal features of epigenetic inheritance and evolution in primates. Cell 146:1029–1041

Oakes CC, Smiraglia DJ, Plass C, Trasler JM, Robaire B (2003) Aging results in hypermethylation of ribosomal DNA in sperm and liver of male rats. Proc Natl Acad Sci U S A 100:1775–1780

Oliva R, Castillo J, Estanyol J, Ballescà J (2015) Human sperm chromatin epigenetic potential: genomics, proteomics, and male infertility. Asian J Androl 17:601

Ouko LA, Shantikumar K, Knezovich J, Haycock P, Schnugh DJ, Ramsay M (2009) Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male gametes – implications for fetal alcohol spectrum disorders. Alcohol Clin Exp Res 33:1615–1627

Pantano L, Jodar M, Bak M, Ballescà JL, Tommerup N, Oliva R, Vavouri T (2015) The small RNA content of human sperm reveals pseudogene-derived piRNAs complementary to protein-coding genes. RNA 21:1085–1095

Pathak S, Kedia-Mokashi N, Saxena M, D'Souza R, Maitra A, Parte P, Gill-Sharma M, Balasinor N (2009) Effect of tamoxifen treatment on global and insulin-like growth factor 2-H19 locus-specific DNA methylation in rat spermatozoa and its association with embryo loss. Fertil Steril 91:2253–2263

Radford EJ, Ito M, Shi H, Corish JA, Yamazawa K, Isganaitis E, Seisenberger S, Hore TA, Reik W, Erkek S, Peters AH, Patti ME, Ferguson-Smith AC (2014) In utero undernourishment perturbs the adult sperm methylome and intergenerational metabolism. Science 345:1255903

Rahman MB, Kamal MM, Rijsselaere T, Vandaele L, Shamsuddin M, Van Soom A (2014) Altered chromatin condensation of heat-stressed spermatozoa perturbs the dynamics of DNA methylation reprogramming in the paternal genome after in vitro fertilisation in cattle. Reprod Fertil Dev 26:1107–1116

Röther S, Meister G (2011) Small RNAs derived from longer non-coding RNAs. Biochimie 93:1905–1915

Salas-Huetos A, Blanco J, Vidal F, Mercader JM, Garrido N, Anton E (2014) New insights into the expression profile and function of micro-ribonucleic acid in human spermatozoa. Fertil Steril 102:213–222

Salian S, Doshi T, Vanage G (2011) Perinatal exposure of rats to Bisphenol A affects fertility of male offspring-an overview. Reprod Toxicol 31:359–362

Sasaki H, Matsui Y (2008) Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet 2008:129–140

Satta R, Maloku E, Zhubi A, Pibiri F, Hajos M, Costa E, Guidotti A (2008) Nicotine decreases DNA methyltransferase 1 expression and glutamic acid decarboxylase 67 promoter methylation in GABAergic interneurons. Proc Natl Acad Sci 105:16356–16361

Schagdarsurengin U, Steger K (2016) Epigenetics in male reproduction: effect of paternal diet on sperm quality and offspring health. Nat Rev Urol 13:584–595

Schagdarsurengin U, Paradowska A, Steger K (2012) Analysing the sperm epigenome: roles in early embryogenesis and assisted reproduction. Nat Rev Urol:1–11

Seisenberger S, Andrews S, Krueger F, Arand J, Walter JJJ, Santos FF, Popp C, Thienpont B, Dean W, Reik W (2012) The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. Mol Cell 48:849–862

Shnorhavorian M, Schwartz SM, Stansfeld B, Sadler-Riggleman I, Beck D, Skinner MK (2017) Differential DNA methylation regions in adult human sperm following adolescent chemotherapy: potential for epigenetic inheritance. PLoS One 12:e0170085

Simone NL, Soule BP, Ly D, Saleh AD, Savage JE, DeGraff W, Cook J, Harris CC, Gius D, Mitchell JB (2009) Ionizing radiation-induced oxidative stress alters miRNA expression. PLoS One 4:e6377

Skinner MK (2008) What is an epigenetic transgenerational phenotype?. F3 or F2. Reprod Toxicol 25:2–6

Song R, Hennig GW, Wu Q, Jose C, Zheng H, Yan W (2011) Male germ cells express abundant endogenous siRNAs. Proc Natl Acad Sci U S A 108:13159–13164

Soubry A, Guo L, Huang Z, Hoyo C, Romanus S, Price T, Murphy SK (2016) Obesity-related DNA methylation at imprinted genes in human sperm: results from the TIEGER study. Clin Epigenetics 8:51

Stouder C, Paoloni-Giacobino A (2010) Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. Reproduction 139:373–379

Suh N, Baehner L, Moltzahn F, Melton C, Shenoy A, Chen J, Blelloch R (2010) MicroRNA function is globally suppressed in mouse oocytes and early embryos. Curr Biol 20:271–277

Sultana S, Zulkifle M, Ansari A, Shahnawaz (2015) Efficacy of local application of an Unani formulation in acne vulgaris. Anc Sci Life 35:124

Tang WWC, Dietmann S, Irie N, Leitch HG, Floros VI, Bradshaw CR, Hackett JA, Chinnery PF, Surani MA (2015) A unique gene regulatory network resets the

human germline epigenome for development. Cell 161:1453–1467

Thompson RF, Atzmon G, Gheorghe C, Liang HQ, Lowes C, Greally JM, Barzilai N (2010) Tissue-specific dysregulation of DNA methylation in aging. Aging Cell 9:506–518

Tiwari D, Vanage G (2013) Mutagenic effect of Bisphenol A on adult rat male germ cells and their fertility. Reprod Toxicol 40:60–68

Tracey R, Manikkam M, Guerrero-Bosagna C, Skinner MK (2013) Hydrocarbons (jet fuel JP-8) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. Reprod Toxicol 36:104–116

Van Der Heijden GW, Ramos L, Baart EB, Van Den Berg IM, Derijck AA, Van Der Vlag J, Martini E, De Boer P (2008) Sperm-derived histones contribute to zygotic chromatin in humans. BMC Dev Biol 8:34

Vrooman LA, Oatley JM, Griswold JE, Hassold TJ, Hunt PA (2015) Estrogenic exposure alters the spermatogonial stem cells in the developing testis, permanently reducing crossover levels in the adult. PLoS Genet 11:e1004949

Wei Y, Yang CR, Wei YP, Zhao ZA, Hou Y, Schatten H, Sun QY (2014) Paternally induced transgenerational inheritance of susceptibility to diabetes in mammals. Proc Natl Acad Sci U S A 111:1873–1878

Xue J, Schoenrock SA, Valdar W, Tarantino LM, Ideraabdullah FY (2016) Maternal vitamin D depletion alters DNA methylation at imprinted loci in multiple generations. Clin Epigenetics 8:107

Sperm DNA Fragmentation: Mechanisms of Origin

5

Monica Muratori, Sara Marchiani, Lara Tamburrino, and Elisabetta Baldi

Abstract

Spermatozoa have the task to deliver an intact paternal genome to the oocyte and to support a successful embryo development. The high levels of sperm DNA fragmentation (sDF) found in sub-/infertile men threat human reproduction and health of the offspring. Strategies to prevent the onset of this type of sperm damage are extensively sought.

sDF can be induced by factors like lifestyle-related habits, diseases, drugs, aging, infections and exposure to pollutants. At the cell level, all these factors induce sperm DNA breaks by three main mechanisms: apoptosis, impairment of sperm chromatin maturation and oxidative stress. Apoptosis and defects in maturation of sperm chromatin appear to act in the testis and account for DNA breaks found in dead ejaculated spermatozoa, whereas oxidative stress is likely inducing sDF during the transit through the male genital tracts and accounting for DNA breaks observed in viable spermatozoa of the ejaculate. Oxidative stress appears to be also the main mechanism responsible for induction of sDF after ejaculation, during in vitro manipulation of spermatozoa. Whether or not mature spermatozoa are able to trigger a cell death program is not yet clarified. In particular, it is not clear whether apoptotic nucleases or reactive oxygen species are responsible for producing DNA breaks in ejaculated mature spermatozoa. Knowledge of the mechanisms inducing sDF is a valuable starting point to define possible therapeutic options that however are still far to be established.

Keywords

Sperm DNA fragmentation · Chromatin maturation · Abortive apoptosis · Oxidative stress · Reactive oxygen species · Male infertility

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Introduction

Spermatozoa are highly specialised cells with the task to deliver an intact paternal genome to the oocyte and to support a successful embryo development. Both genetic and epigenetic alterations

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can impair these tasks. In the last two decades, research attention has been focused on sperm DNA fragmentation, as the presence of DNA breaks represents the most frequent genetic anomaly found in ejaculated human spermatozoa. In particular, the discovery that high levels of sperm DNA fragmentation (sDF) can be found in spermatozoa of sub/infertile men (Gorczyca et al. 1993) raised concerns regarding the reproductive functions of these men and, most importantly, the health of the offspring. We know now that sDF affects both natural (Evenson et al. 1999; Giwercman et al. 2010; Muratori et al. 2015a) and assisted reproduction (Simon et al. 2017; Robinson et al. 2012) and thus represents a serious problem in our society where couple infertility has become a top priority for the public health. In addition, studies in animal models have shown important health effects (such as development of tumours) on the health of offspring, after ICSI with spermatozoa where DNA fragmentation has been induced by freeze-thawing (Fernández-Gonzalez et al. 2008). In this scenario, it appears of importance to develop strategies to prevent the onset or decrease the amount of sDF in the ejaculate. To this aim, research activity should be devoted to identify the conditions inducing and the mechanisms involved in generation of this type of sperm damage.

Increase of sDF can be produced by a variety of factors, including lifestyle-related habits, diseases, drugs, aging, infections and exposure to pollutants. All these factors ultimately provoke sDF mainly by inducing testis apoptosis or increasing production of reactive oxygen species (ROS), two mechanisms believed responsible for the onset of sperm DNA breaks. A third mechanism, however, strictly connected to testis apoptosis and/or to oxidative damage, regards an impairment of sperm chromatin maturation. In particular, the failure to re-ligate the DNA nicks previously produced to promote histone to protamine replacement during the process of chromatin maturation can result in the presence of DNA breaks in the ejaculated spermatozoa and/or can trigger the apoptotic program. In addition, an impaired compaction of sperm chromatin makes sperm nuclei more susceptible to ROS attack.

In this chapter, we review the literature on the factors known to induce an increase of sDF and on the mechanisms which are believed responsible for the in vivo and in vitro origin of sperm DNA breaks.

Factors Inducing sDF

Evidence has been accumulating indicating that several lifestyle factors influence the levels of sDF. Nutrition appears to be one of these factors, as western diet (high intake of processed red meats, high fat dairy, French fries, refined grains, sweets and high-energy drinks) has been reported to increase sperm DNA damage both in animal (Ferramosca et al. 2016) and in human (Jurewicz et al. 2018; Vujkovic et al. 2009). However, whether or not obesity, a diet-related disease, is associated with increased levels of sDF remains controversial as both increases and no change in this type of sperm damage in obese subjects have been reported (Campbell et al. 2015; Bandel et al. 2015; Rybar et al. 2011). Consumption of alcohol is another detrimental lifestyle factor for sperm chromatin and DNA integrity (Anifandis et al. 2014; Robbins et al. 1997). In men affected by diabetes or with smoking habit, other conditions which may be associated with elevated sDF (see below), alcohol consumption may further increase sperm DNA damage (Martini et al. 2004). However, the relationship between smoking and sperm DNA damage is still unclear. Indeed, although several in vitro studies demonstrated that toxic substances contained in cigarettes, including nicotine, cadmium and lead, induce damage to sperm DNA (Condorelli et al. 2013; Oliveira et al. 2009; Gomes et al. 2015; Cui et al. 2016), in vivo studies in humans reported controversial results (Antoniassi et al. 2016; Mitra et al. 2012; Bounartzi et al. 2016; Bojar et al. 2013) possibly due to lack of consideration of confounders (such as age, polymorphisms in checkpoint and DNA repair genes, alcohol assumption, occurrence of varicocele), known to affect the association between smoking and sperm DNA damage.

Another important inducer of sperm DNA damage is corpuscular and non-corpuscolar radiation. In animals, in vivo radiation provokes increases of DNA damage in spermatogonia, which ultimately results in generation of spermatozoa with high levels of DNA breaks (Cordelli et al. 2012; Haines et al. 2001). Men exposed to ionising radiation for occupational reasons show higher levels of sDF with respect to control group (Kumar et al. 2013). The low amount of radiation generated by cell phone is dangerous for sperm DNA integrity as well. In vitro exposure of spermatozoa with similar radiation to cell phones provokes damage to sperm DNA (Gorpinchenko et al. 2014) in amounts increasing in subjects with poor semen parameters (Zalata et al. 2015). DNA damage from in vivo exposure to cell phone in men has been demonstrated in a retrospective study reporting that the use of cell phone for more than 10 years is associated with higher levels of sDF (Radwan et al. 2016).

Beside lifestyles and radiation, some diseases are known to increase sDF. Not surprisingly, diabetes, causing damage to almost all the systems in the body, impacts several reproductive districts and results detrimental for sperm DNA quality (Condorelli et al. 2018; Roessner et al. 2012; Rama Raju et al. 2012). Varicocele is another disease associated with high sDF (Agarwal et al. 2016), albeit some authors failed to find such association (Lotti et al. 2017), and varicocelectomy not always restores sperm DNA quality (Roque and Esteves 2014). Most studies investigating the effect of cancer on male fertility reported that cancer patients show higher levels of sperm DNA damage (Kumar et al. 2018; O'Flaherty et al. 2008; O'Donovan 2005; Meseguer et al. 2008; Ståhl et al. 2009; Rives et al. 2012; Bujan et al. 2013, 2014). In addition, as replicating pre-meiotic cells are extremely sensitive to genotoxic agents, treatment with chemo- and radio-therapies impairs spermatogenesis and sperm DNA quality (O'Flaherty 2014) at extents depending on the type and intensity of the therapy (Paoli et al. 2015).

The increase of age at which men conceive their first child is well documented (Martin et al. 2011), whereas the effect of advanced male age on reproductive outcomes is still controversial (Humm and Sakkas 2013). The increase of sDF with age is reported in many studies (Wyrobek et al. 2006; Moskovtsev et al. 2006; Schmid et al. 2007), but whether such increase may be involved in increased risk of offspring health is debated (Yatsenko and Turek 2018; Johnson et al. 2015). Interestingly, it has also been reported that the nonhomologous end joining (NHEJ), considered the most likely DNA repair system in haploid spermatids (see below), declines with age (Vaidya et al. 2014). This finding could explain not only the increase of male-transmitted de novo mutations (Kong et al. 2012), but also the increased presence of sperm DNA breaks in old men. Indeed, NHEJ is considered the system that more likely repairs the DNA nicks (Gregoire et al. 2013) formed during sperm chromatin maturation to favour nucleoprotein replacement. Another link between age and levels of sDF could be the incremental exposure to environmental toxicants with age. Indeed, the spread contamination of water, soil, air, food, beverages and household exposes humans to many environmental toxicants that may accumulate in tissues provoking increasing cell damages with age. Although results are often conflicting, evidence exists indicating increases of sDF after occupational exposure to some toxicants, including styrene, lead and boron, or environmental exposure to air pollutants such as polychlorinated biphenyls (PCBs), phthalates and pesticides (Giwercman and Spanò 2014).

Finally, it is well known that infections of the male genital tract induce leukocytospermia and increase semen levels of oxidative stress, in turn provoking sperm DNA breaks. Many studies reported the deleterious effect on sperm DNA integrity of infection by bacteria and fungi (Sasikumar et al. 2013), including *Escherichia coli*, *Staphylococus aureus* (Villegas et al. 2005), *Ureaplasma urealyticum* (Sellami et al. 2014), *Pseudomonas aeruginosa*, *Candida albicans*, *Chlamydia trachomatis* and *Mycoplasma* (Gallegos et al. 2008). Exposure to both Hepatitis B Virus (Kang et al. 2012) and human papillomavirus (Connelly et al. 2001) decreases sperm DNA quality, albeit controversy exists regarding

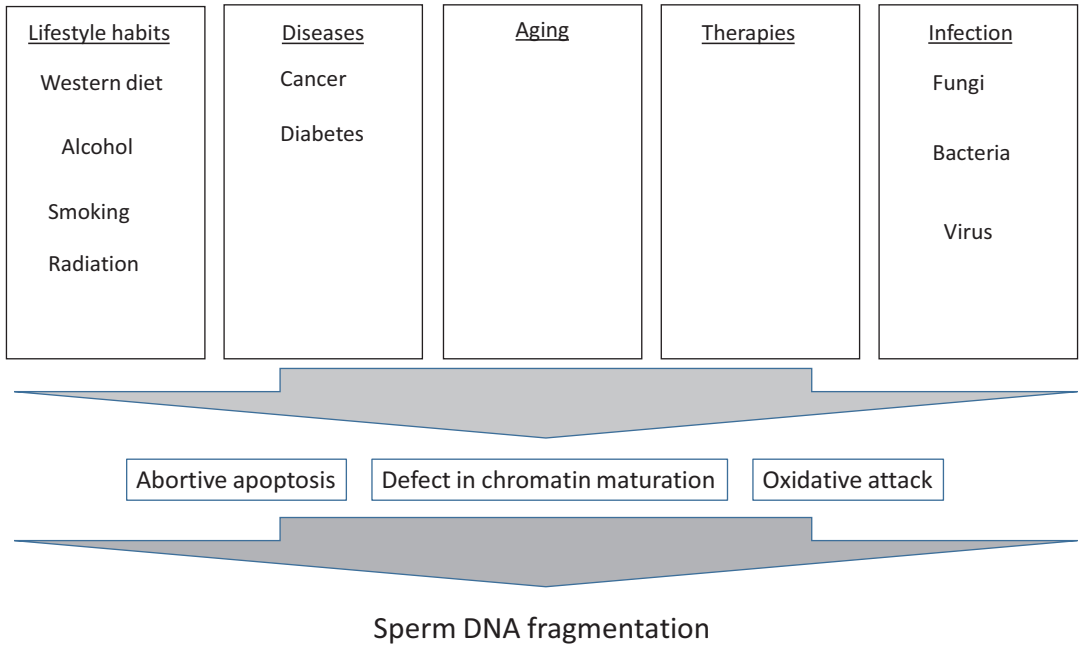

Fig. 5.1 Scheme of the main known factors inducing increased levels of sDF. These factors act through the indicated cell mechanisms

the latter (Cortés-Gutiérrez et al. 2017; Kaspersen et al. 2013).

Figure 5.1 summarises the main factors known to increase sDF. Whatever factor involved in inducing sDF, at the cell level it may act through one or more of the three main mechanisms so far hypothesised: induction of apoptosis, disturbance of the chromatin maturation process and induction of oxidative attack (Fig. 5.1).

Mechanisms Inducing sDF

The occurrence of abortive apoptosis in the testis was first hypothesised after observing, in ejaculated spermatozoa of subfertile men, signs of apoptosis like as FAS receptor expression (Sakkas et al. 1999) and presence of apoptosis-like ultrastructural features (Baccetti et al. 1996). Apoptosis begins in germ cells but fails to complete (abortive), producing spermatozoa with apoptotic signs including DNA breaks, and semen apoptotic bodies (Marchiani et al. 2007). In the testis, sDF may also originate during the elongation phases of spermatids, when transient double-strand breaks arise, likely due to the action of topoisomerase enzymes (Mc Pherson and Longo 1993) to promote the transient relief of torsional stress of DNA and thus favour histone to protamine substitution (Sakkas et al. 1995; Marcon and Boissonneault 2004). As spermatids are haploid, repair of such DNA nicks based on homologous recombination, depending on sister chromatids, is unlikely to occur and it has been hypothesised that their re-ligation relies on the NHEJ repair system (Gregoire et al. 2013). According to this mechanism, sDF would be produced by the failure in the re-ligation of the DNA nicks, producing spermatozoa with persistent DNA breakage. The third mechanism consists in the attack to sperm DNA by ROS and, in principle, can occur both in the testis and after spermiation, during the transit in the male genital tracts.

Several studies support the role of each of these mechanisms in generating sDF; however, it is expected that they are not alternative but can concur together in generating sperm DNA breaks. For instance, the impairment of sperm chromatin maturation may trigger the apoptotic pathway or may produce spermatozoa with a less condensed

nucleus, and thus, be more susceptible to the oxidative attack; similarly, ROS can induce an apoptotic program as reported in somatic cells (Sinha et al. 2013).

Recently, our groups investigated the role of each of the above-described mechanisms in generating sDF, by detecting by flow cytometry the concomitant presence, in native semen samples, of sperm DNA nicks (detected by TUNEL) and of markers of apoptosis, sperm immaturity and oxidative attack (Muratori et al. 2015b). We found a clear overlapping between sperm DNA breaks and the signs of both apoptosis (caspase activity and cleaved PARP) and chromatin maturation defect (staining with aniline blue); a high overlapping between apoptotic markers and signs of chromatin immaturity also occurred (Muratori et al. 2015b). Conversely, sDF was scarcely concomitant with markers of oxidative damage (8-hydroxyguanosine, 8-OHdG, a hallmark of DNA oxidation and malondialdeyde, MDA, indicative of oxidative damage to sperm membranes) (Muratori et al. 2015b). Surprisingly, these data appear to reappraise the role of oxidative attack in producing sDF, contrary to many previous studies demonstrating the role of oxidative attack in inducing sperm DNA breaks during in vitro incubations (Aitken et al. 2016). However, in native semen, most DNA-fragmented spermatozoa are not viable (Mitchell et al. 2010), whereas most in vitro studies used selected sperm populations where virtually all cells are viable. Focusing on the viable sperm fraction of the ejaculate, we could unmask the high overlapping between DNA breaks and both nuclear and membrane oxidative damage, indicating that oxidative stress may generate DNA breaks mainly in viable spermatozoa (Muratori et al. 2015b). Overall, our study proposed a model (Fig. 5.2) where apoptosis and chromatin maturation impairment are the main mechanisms originating sDF in the bulk of spermatozoa of native semen samples where most DNA-fragmented spermatozoa are unviable. In these spermatozoa, the onset of sDF is likely occurring in the testis due to different local (such as the impairment of chromatin maturation process (Muratori et al. 2015b) or of hormonal modulation of cell survival (Correia et al. 2015)) or external stimuli triggering the apoptotic program. Conversely, in viable DNA-fragmented spermatozoa, the onset of sDF appears to be recent, during the transit through the genital tracts and due mainly to oxidative attack (Muratori et al. 2015b). ROS could induce DNA breaks by a direct attack to the DNA backbone or triggering an apoptotic program ultimately leading to enzymatic DNA cleavage. The finding that only a low fraction of the live spermatozoa with residues of 8-OHdG concomitantly expresses caspase activity and, vice versa, all caspase positive (apoptotic) spermatozoa exhibited oxidative damage signs is somehow consistent with the idea that oxidative assault precedes the apoptotic program (Fig. 5.2) (Muratori et al. 2015b). The enzymatic sperm DNA cleavage after spermiation has been questioned because of the high compaction of mature sperm chromatin, hindering the access of the endonucleases into DNA. However, the occurrence of a nuclease cleaving DNA into 50 kb fragments (about 50 kb) has been reported in mature spermatozoa (Sotolongo et al. 2005) and it has to be considered that full chromatin maturation occurs during epididymal transit possibly permitting the action of the apoptotic endonucleases after spermiation.

Intriguingly, a later paper from our group supported the proposed model, using a different strategy which evaluated the association between sDF in sperm populations containing viable or unviable DNA-fragmented spermatozoa and clinical and ultrasound features of the patients (Lotti et al. 2017). The results of this study showed that the amount of DNA fragmented unviable spermatozoa is associated mainly with ultrasound signs of testicular abnormalities, whereas the sperm population containing DNA fragmented viable spermatozoa was mostly associated with clinical and ultrasound alterations of the prostate and of seminal vesicles (Lotti et al. 2017), likely due to inflammatory statuses, known to promote an oxidative environment.

As mentioned, our model proposes that the DNA fragmented unviable spermatozoa are cells where the onset of DNA breakage is located in testis due to apoptotic nucleases. In these

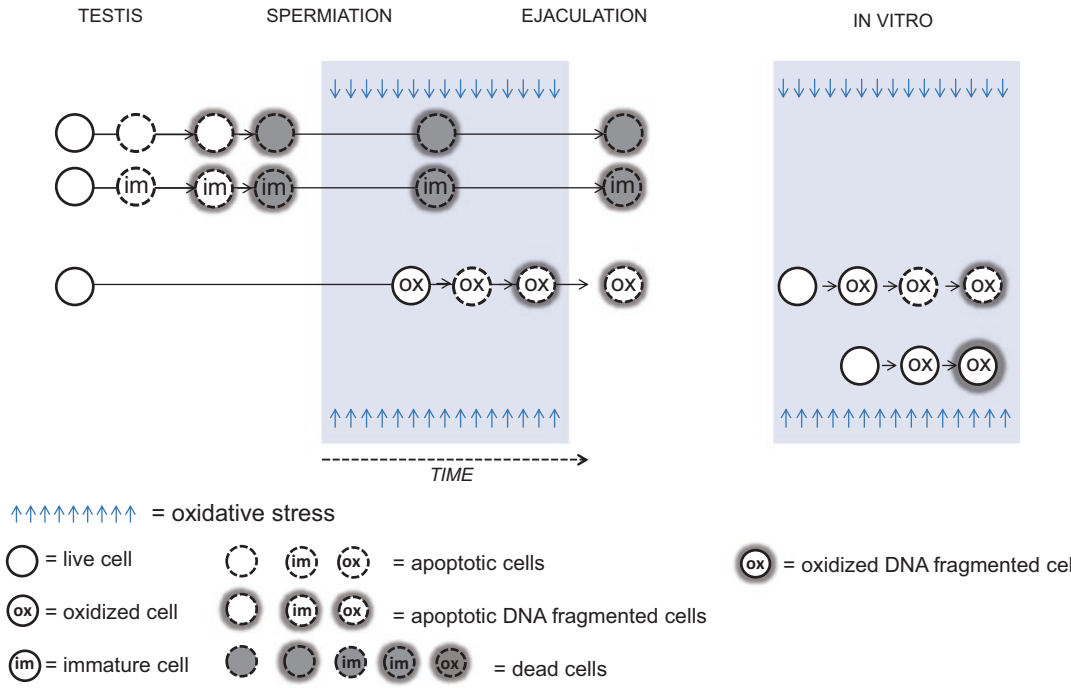

Fig. 5.2 A proposed model for the in vivo and in vitro origin of sDF (Muratori et al. 2015b). In vivo – in ejaculated DNA fragmented, dead spermatozoa, sDF originates in testis due to an apoptotic program triggered by stimuli like chromatin maturation impairment. These cells arrive already dead in the genital male tract and thus are not injured by the possible presence of an oxidative environment. Conversely, viable cells are oxidated, prime apoptosis and fragment their DNA. In vitro – a similar route as the latter might occur for sDF developed in in vitro condition. It is also possible that oxidation breaks directly sperm DNA, without involving endonuclease activation

spermatozoa, the amount of oxidative damage to DNA is low and not concomitant with DNA breaks (Muratori et al. 2015b), suggesting that ROS do not play a relevant role in generating sDF in testis, possibly because of occurrence of effective antioxidant defence systems. We cannot exclude, however, that in patients with high level of testicular oxidative stress due, for instance, to varicocele (Ishikawa et al. 2007), the onset of sDF in testis may be due to oxidative attack.

Oxidative attack appears to be also the main mechanism responsible for induction of sDF after ejaculation, during in vitro incubation (Muratori et al. 2003) or manipulation of spermatozoa (Muratori et al. 2016; Aitken et al. 2014) (Fig. 5.2). We cannot even exclude that the DNA breaks detected in the viable spermatozoa might originate after ejaculation because of manipulation of semen necessary for the detection procedure. However, the finding that the amount of DNA damage is higher in the ejaculated spermatozoa than in spermatozoa extracted from the testis (Esteves et al. 2017), where similar procedures as for ejaculated spermatozoa are used to detect DNA fragmentation, indicates that this is not the case, suggesting that at least a fraction of sDF is induced during the transit in the male genital tracts.

Many questions about the mechanisms originating sDF are still open. For instance, as mentioned above, it is not clear whether mature spermatozoa retain the ability to undergo a full apoptotic program and, in case that apoptosis does occur in these cells, which stimuli trigger it. In mature ejaculated spermatozoa, it has been proposed that apoptosis is a default condition, continuously prevented by the activity of phosphatidylinositol 3-kinase (PIK3) (Koppers et al.

2011). Only the inhibition of such enzyme could trigger the apoptotic pathway, in turn inducing loss of sperm motility, mitochondrial ROS production and activation of caspase (Koppers et al. 2011). However, many authors reported the induction of apoptotic markers by treating spermatozoa with oxidant and non-oxidant agents (Lozano et al. 2009; Grizard et al. 2007; Martínez-Pastor et al. 2009; Kang et al. 2012; Fujita et al. 2011; Villegas et al. 2005) suggesting the existence of different routes to prime the apoptotic program. Another open issue is what provokes sperm DNA breaks in ejaculated, mature spermatozoa. As mentioned, although the induction of caspase activity has been extensively reported also after in vitro incubation, it is not clear whether such induction in turn activates apoptotic endonucleases. In somatic cells, early steps of apoptosis lead to the activation of cytoplasmic or mitochondrial endonucleases which then translocate into the nuclei and cut DNA in the internucleosomal spaces. This pathway could be hindered in spermatozoa because of the compartmentalisation of mitochondria and most cytoplasm in the mid-piece, which is physically separated from the nucleus. The latter, being highly compacted, further would hinder the action of apoptotic endonucleases, unless compaction is poor or the histone/protamine ratio altered. Conversely, according to recent studies, the small molecules of ROS could readily reach DNA and produce in it oxidised base adducts (like as 8-OHdG) later converted in abasic sites, as a part of the base excision repair (Aitken et al. 2015, 2016). Since spermatozoa lack apurinic endonucleases I, such abasic sites do not develop in DNA breaks, which appear only when the cells are near to die, because of the action of DNAases possibly activated by a rise in the intracellular calcium accompanying cell death (Smith et al. 2013). This scenario, however, is not consistent with the finding of large percentage of sDF in the highly motile sperm populations prepared by selection with density gradient centrifugation (Muratori et al. 2016; Aitken et al. 2014) and swim up (Muratori et al., unpublished results), suggesting that sDF does occur in live spermatozoa with a mechanism independent from death-associated processes. In addition, induction of apoptosis without increasing sperm ROS has been reported after incubation with bacteria (Villegas et al. 2005). Therefore, other mechanisms should be considered to explain the appearance of sDF in live spermatozoa. It has to be also considered that most studies on mechanisms generating sperm DNA breaks were conducted in in vitro conditions, far from the in vivo ones. In addition, such studies were conducted with selected sperm populations that are not representative of the whole ejaculate.

In conclusion, sDF represents a threat for human reproduction and for the health of the offspring. There are many conditions known to provoke or favour the increase of this type of sperm damage, including lifestyles factors, diseases, drugs, aging and infections. All these conditions determine the onset of sDF by acting through three main mechanisms, i.e. inducing an apoptotic process in the testis, altering the sperm chromatin maturation process and inducing oxidative stress. These three mechanisms represent a valuable starting point to define possible therapeutic options to prevent or reduce sDF in the ejaculate. At present, existing therapies are mostly based on antioxidants, however, although some results are encouraging, whether antioxidants can solve the problem is questioned (Showell et al. 2014). Although targeting apoptosis appears unfeasible, treatment with Follicle-stimulating hormone, which has specific anti-apoptotic action in the testis, appears promising (Santi et al. 2018; Muratori and Baldi 2018). However, further research is needed before the origin of sDF will be completely clarified and more specific therapeutic agents developed.

References

Agarwal A, Majzoub A, Esteves SC, Ko E, Ramasamy R, Zini A (2016) Clinical utility of sperm DNA fragmentation testing: practice recommendations based on clinical scenarios. Transl Androl Urol 5:935–950

Aitken RJ, Baker MA, Nixon B (2015) Are sperm capacitation and apoptosis the opposite ends of a continuum driven by oxidative stress? Asian J Androl 17:633–639

Aitken RJ, Finnie JM, Muscio L, Whiting S, Connaughton HS, Kuczera L, Rothkirch TB, De Iuliis GN (2014) Potential importance of transition metals in the induction of DNA damage by sperm preparation media. Hum Reprod 29:2136–2147

Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P (2016) Causes and consequences of oxidative stress in spermatozoa. Reprod Fertil Dev 28:1–10

Anifandis G, Bounartzi T, Messini CI, Dafopoulos K, Sotiriou S, Messinis IE (2014) The impact of cigarette smoking and alcohol consumption on sperm parameters and sperm DNA fragmentation (SDF) measured by Halosperm(®). Arch Gynecol Obstet 290:777–782

Antoniassi MP, Intasqui Lopes P, Camargo M, Zylbersztejn DS, Carvalho VM, Cardozo KH, Bertolla RP (2016) Analysis of the sperm functional aspects and seminal plasma proteomic profile from male smokers. BJU Int 118:814–822

Baccetti B, Collodel G, Piomboni P (1996) Apoptosis in human ejaculated sperm cells. (Notulae seminologicae 9). J Submicrosc Cytol Pathol 28:587–596

Bandel I, Bungum M, Richtoff J, Malm J, Axelsson J, Pedersen HS, Ludwicki JK, Czaja K, Hernik A, Toft G, Bonde JP, Spanò M, Malm G, Haugen TB, Giwercman A (2015) No association between body mass index and sperm DNA integrity. Hum Reprod 30:1704–1713

Bojar I, Witczak M, Wdowiak A (2013) Biological and environmental conditionings for a sperm DNA fragmentation. Ann Agric Environ Med 20:865–868

Bounartzi T, Dafopoulos K, Anifandis G, Messini CI, Koutsonikou C, Kouris S, Satra M, Sotiriou S, Vamvakopoulos N, Messinis IE (2016) Pregnancy prediction by free sperm DNA and sperm DNA fragmentation in semen specimens of IVF/ICSI-ET patients. Hum Fertil (Camb) 19:56–62

Bujan L, Walschaerts M, Brugnon F, Daudin M, Berthaut I, Auger J, Saias J, Szerman E, Moinard N, Rives N, Hennebicq S (2014) Impact of lymphoma treatments on spermatogenesis and sperm deoxyribonucleic acid: a multicenter prospective study from the CECOS network. Fertil Steril 102:667–674

Bujan L, Walschaerts M, Moinard N, Hennebicq S, Saias J, Brugnon F, Auger J, Berthaut I, Szerman E, Daudin M, Rives N (2013) Impact of chemotherapy and radiotherapy for testicular germ cell tumors on spermatogenesis and sperm DNA: a multicenter prospective study from the CECOS network. Fertil Steril 100:673–680

Campbell JM, Lane M, Owens JA, Bakos HW (2015) Paternal obesity negatively affects male fertility and assisted reproduction outcomes: a systematic review and meta-analysis. Reprod Biomed Online 31:593–604

Condorelli RA, La Vignera S, Giacone F, Iacoviello L, Vicari E, Mongioi' L, Calogero AE (2013) In vitro effects of nicotine on sperm motility and bio-functional flow cytometry sperm parameters. Int J Immunopathol Pharmacol 26:739–746

Condorelli RA, La Vignera S, Mongioì LM, Alamo A, Calogero AE (2018) Diabetes mellitus and infertility: different pathophysiological effects in type 1 and type 2 on sperm function. Front Endocrinol (Lausanne) 9:268

Connelly DA, Chan PJ, Patton WC, King A (2001) Human sperm deoxyribonucleic acid fragmentation by specific types of papillomavirus. Am J Obstet Gynecol 184:1068–1070

Cordelli E, Eleuteri P, Grollino MG, Benassi B, Blandino G, Bartoleschi C, Pardini MC, Di Caprio EV, Spanò M, Pacchierotti F, Villani P (2012) Direct and delayed X-ray-induced DNA damage in male mouse germ cells. Environ Mol Mutagen 53:429–439

Correia S, Cardoso HJ, Cavaco JE, Socorro S (2015) Oestrogens as apoptosis regulators in mammalian testis: angels or devils? Expert Rev Mol Med 17:e2

Cortés-Gutiérrez EI, Dávila-Rodríguez MI, Fernández JL, de la O-Pérez LO, Garza-Flores ME, Eguren-Garza R, Gosálvez J (2017) The presence of human papillomavirus in semen does not affect the integrity of sperm DNA. Andrologia 49(10)

Cui X, Jing X, Wu X, Wang Z, Li Q (2016) Potential effect of smoking on semen quality through DNA damage and the downregulation of Chk1 in sperm. Mol Med Rep 14:753–761

Esteves SC, Roque M, Bradley CK, Garrido N (2017) Reproductive outcomes of testicular versus ejaculated sperm for intracytoplasmic sperm injection among men with high levels of DNA fragmentation in semen: systematic review and meta-analysis. Fertil Steril 108:456–467

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP (1999) Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 14:1039–1049

Fernández-Gonzalez R, Moreira PN, Pérez-Crespo M, Sánchez-Martín M, Ramirez MA, Pericuesta E, Bilbao A, Bermejo-Alvarez P, de Dios Hourcade J, de Fonseca FR, Gutiérrez-Adán A (2008) Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. Biol Reprod 78:761–772

Ferramosca A, Conte A, Moscatelli N, Zara V (2016) A high-fat diet negatively affects rat sperm mitochondrial respiration. Andrology 4:520–525

Fujita Y, Mihara T, Okazaki T, Shitanaka M, Kushino R, Ikeda C, Negishi H, Liu Z, Richards JS, Shimada M (2011) Toll-like receptors (TLR) 2 and 4 on human sperm recognize bacterial endotoxins and mediate apoptosis. Hum Reprod 26:2799–2806

Gallegos G, Ramos B, Santiso R, Goyanes V, Gosálvez J, Fernández JL (2008) Sperm DNA fragmentation in infertile men with genitourinary infection by Chlamydia trachomatis and Mycoplasma. Fertil Steril 90:328–334

Giwercman A, Spanò M (2014) Sperm chromatin and environmental factors. In: Zini A, Agarwal A (eds) A clinician's guide to sperm DNA and chromatin damage. Springer, New York

Giwercman A, Lindstedt L, Larsson M, Bungum M, Spano M, Levine RJ, Rylander L (2010) Sperm chromatin structure assay as an independent predictor of fertility in vivo: a case-control study. Int J Androl 33:e221–e227

Gomes M, Gonçalves A, Rocha E, Sá R, Alves A, Silva J, Barros A, Pereira ML, Sousa M (2015) Effect of in vitro exposure to lead chloride on semen quality and sperm DNA fragmentation. Zygote 23:384–393

Gorczyca W, Traganos F, Jesionowska H, Darzynkiewicz Z (1993) Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. Exp Cell Res 207:202–205

Gorpinchenko I, Nikitin O, Banyra O, Shulyak A (2014) The influence of direct mobile phone radiation on sperm quality. Cent Eur J Urol 67:65–71

Gregoire M-C, Massonneau J, Simard O, Gouraud A, Brazeau M-A, Arguin M et al (2013) Male-driven de novo mutations in haploid germ cells. Mol Hum Reprod 19:495–499

Grizard G, Roddier H, Artonne C, Sion B, Vasson MP, Janny L (2007) In vitro alachlor effects on reactive oxygen species generation, motility patterns and apoptosis markers in human spermatozoa. Reprod Toxicol 23:55–62

Haines GA, Hendry JH, Daniel CP, Morris ID (2001) Increased levels of comet-detected spermatozoa DNA damage following in vivo isotopic- or X-irradiation of spermatogonia. Mutat Res 495:21–32

Humm KC, Sakkas D (2013) Role of increased male age in IVF and egg donation: is sperm DNA fragmentation responsible? Fertil Steril 99:30–36

Ishikawa T, Fujioka H, Ishimura T, Takenaka A, Fujisawa M (2007) Increased testicular 8-hydroxy-2′-deoxyguanosine in patients with varicocele. BJU Int 100:863–866

Johnson SL, Dunleavy J, Gemmell NJ, Nakagawa S (2015) Consistent age-dependent declines in human semen quality: a systematic review and meta-analysis. Ageing Res Rev 19:22–33

Jurewicz J, Radwan M, Sobala W, Radwan P, Bochenek M, Hanke W (2018) Dietary patterns and their relationship with semen quality. Am J Mens Health 12:575–583

Kang X, Xie Q, Zhou X, Li F, Huang J, Liu D, Huang T (2012) Effects of hepatitis B virus S protein exposure on sperm membrane integrity and functions. PLoS One 7:e33471

Kaspersen MD, Bungum M, Fedder J, Bonde J, Larsen PB, J Ingerslev H, Höllsberg P (2013) No increased sperm DNA fragmentation index in semen containing human papillomavirus or herpesvirus. Andrology 1:361–364

Kong A, Frigge ML, Masson G, Besenbacher S, Sulem P, Magnusson G, Gudjonsson SA, Sigurdsson A, Jonasdottir A, Jonasdottir A, Wong WS, Sigurdsson G, Walters GB, Steinberg S, Helgason H, Thorleifsson G, Gudbjartsson DF, Helgason A, Magnusson OT, Thorsteinsdottir U, Stefansson K (2012) Rate of de novo mutations and the importance of father's age to disease risk. Nature 488:471–475

Koppers AJ, Mitchell LA, Wang P, Lin M, Aitken RJ (2011) Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxidative DNA damage in human spermatozoa. Biochem J 436:687–698

Kumar D, Salian SR, Kalthur G, Uppangala S, Kumari S, Challapalli S, Chandraguthi SG, Krishnamurthy H, Jain N, Kumar P, Adiga SK (2013) Semen abnormalities, sperm DNA damage and global hypermethylation in health workers occupationally exposed to ionizing radiation. PLoS One 8:e69927

Kumar K, Lewis S, Vinci S, Riera-Escamilla A, Fino MG, Tamburrino L, Muratori M, Larsen P, Krausz C (2018) Evaluation of sperm DNA quality in men presenting with testicular cancer and lymphoma using alkaline and neutral Comet assays. Andrology 6:230–235

Lotti F, Tamburrino L, Marchiani S, Maseroli E, Vitale P, Forti G, Muratori M, Maggi M, Baldi E (2017) DNA fragmentation in two cytometric sperm populations: relationship with clinical and ultrasound characteristics of the male genital tract. Asian J Androl 19:272–279

Lozano GM, Bejarano I, Espino J, González D, Ortiz A, García JF, Rodríguez AB, Pariente JA (2009) Relationship between caspase activity and apoptotic markers in human sperm in response to hydrogen peroxide and progesterone. J Reprod Dev 55:615–621

Marchiani S, Tamburrino L, Maoggi A, Vannelli GB, Forti G, Baldi E, Muratori M (2007) Characterization of M540 bodies in human semen: evidence that they are apoptotic bodies. Mol Hum Reprod 13:621–631

Marcon L, Boissonneault G (2004) Transient DNA strand breaks during mouse and human spermiogenesis new insights in stage specificity and link to chromatin remodeling. Biol Reprod 70:910–918

Martin JA, Hamilton BE, Ventura SJ, Osterman MJ, Kirmeyer S, Mathews TJ, Wilson EC (2011) Births: final data for 2009. Natl Vital Stat Rep 60:1–70

Martínez-Pastor F, Aisen E, Fernández-Santos MR, Esteso MC, Maroto-Morales A, García-Alvarez O, Garde JJ (2009) Reactive oxygen species generators affect quality parameters and apoptosis markers differently in red deer spermatozoa. Reproduction 137:225–235

Martini AC, Molina RI, Estofán D, Senestrari D, Fiol de Cuneo M, Ruiz RD (2004) Effects of alcohol and cigarette consumption on human seminal quality. Fertil Steril 82:374–377

Mc Pherson SMG, Longo FJ (1993) Chromatin structurefunction alterations during mammalian spermatogenesis: origin and significance of sperm DNA fragmentation 1497 DNA nicking and repair in elongating spermatids. Eur J Histochem 37:109–128

Meseguer M, Santiso R, Garrido N, Fernandez JL (2008) The effect of cancer on sperm DNA fragmentation as measured by the sperm chromatin dispersion test. Fertil Steril 90:225–227

Mitchell LA, De Iuliis GN, Aitken RJ (2010) The TUNEL assay consistently underestimates DNA damage in

human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. Int J Androl 34:2–13

Mitra A, Chakraborty B, Mukhopadhay D, Pal M, Mukherjee S, Banerjee S, Chaudhuri K (2012) Effect of smoking on semen quality, FSH, testosterone level, and CAG repeat length in androgen receptor gene of infertile men in an Indian city. Syst Biol Reprod Med 58:255–262

Moskovtsev SI, Willis J, Mullen JB (2006) Age-related decline in sperm deoxyribonucleic acid integrity in patients evaluated for male infertility. Fertil Steril 85:496–499

Muratori M, Baldi E (2018) Effects of FSH on sperm DNA fragmentation: review of clinical studies and possible mechanisms of action. Front Endocrinol (Lausanne) 9:734

Muratori M, Maggi M, Spinelli S, Filimberti E, Forti G, Baldi E (2003) Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. J Androl 24:253–262

Muratori M, Marchiani S, Tamburrino L, Cambi M, Lotti F, Natali I et al (2015a) DNA fragmentation in brighter sperm predicts male fertility independently from age and semen parameters. Fertil Steril 104:582–590. e4

Muratori M, Tamburrino L, Marchiani S, Cambi M, Olivito B, Azzari C, Forti G, Baldi E (2015b) Investigation on the origin of sperm DNA fragmentation: role of apoptosis, immaturity and oxidative stress. Mol Med 21:109–122

Muratori M, Tarozzi N, Cambi M, Boni L, Iorio AL, Passaro C, Luppino B, Nadalini M, Marchiani S, Tamburrino L, Forti G, Maggi M, Baldi E, Borini A (2016) Variation of DNA fragmentation levels during density gradient sperm selection for assisted reproduction techniques: a possible new male predictive parameter of pregnancy? Medicine (Baltimore) 95:e3624

O'Donovan M (2005) An evaluation of chromatin condensation and DNA integrity in the spermatozoa of men with cancer before and after therapy. Andrologia 37:83–90

O'Flaherty C, Vaisheva F, Hales BF, Chan P, Robaire B (2008) Characterization of sperm chromatin quality in testicular cancer and Hodgkin's lymphoma patients prior to chemotherapy. Hum Reprod 23:1044–1052

O'Flaherty C (2014) Iatrogenic genetic damage of spermatozoa. Adv Exp Med Biol 791:117–135

Oliveira H, Spanò M, Santos C (2009) Pereira MeL. Adverse effects of cadmium exposure on mouse sperm. Reprod Toxicol 28:550–555

Paoli D, Gallo M, Rizzo F, Spanò M, Leter G, Lombardo F, Lenzi A, Gandini L (2015) Testicular cancer and sperm DNA damage: short- and long-term effects of antineoplastic treatment. Andrology 3:122–128

Radwan M, Jurewicz J, Merecz-Kot D et al (2016) Sperm DNA damage-the effect of stress and everyday life factors. Int J Impot Res 28:148–154

Rama Raju GA, Jaya Prakash G, Murali Krishna K, Madan K, Siva Narayana T, Ravi Krishna CH (2012) Noninsulin-dependent diabetes mellitus: effects on sperm morphological and functional characteristics, nuclear DNA integrity and outcome of assisted reproductive technique. Andrologia 44 Suppl 1:490–498

Rives N, Perdrix A, Hennebicq S, Saïas-Magnan J, Melin MC, Berthaut I et al (2012) The semen quality of 1158 men with testicular cancer at the time of cryopreservation: results of the French National CECOS Network. J Androl 33:1394–1401

Robbins WA, Vine MF, Truong KY, Everson RB (1997) Use of fluorescence in situ hybridization (FISH) to assess effects of smoking, caffeine, and alcohol on aneuploidy load in sperm of healthy men. Environ Mol Mutagen 30:175–183

Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S et al (2012) The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod 27:2908–2917

Roessner C, Paasch U, Kratzsch J, Glander HJ, Grunewald S (2012) Sperm apoptosis signalling in diabetic men. Reprod Biomed Online 25:292–299

Roque M, Esteves SC (2014) Varicocelectomy. In: Zini A, Agarwal A (eds) A clinician's guide to sperm DNA and chromatin damage. Springer, New York

Rybar R, Kopecka V, Prinosilova P, Markova P, Rubes J (2011) Male obesity and age in relationship to semen parameters and sperm chromatin integrity. Andrologia 43:286–291

Sakkas D, Mariethoz E, St John JC (1999) Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. Exp Cell Res 251:350–355

Sakkas D, Manicardi G, Bianchi PG, Bizzaro D, Bianchi U (1995) Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilizing mouse spermatozoa. Biol Reprod 52:1149–1155

Santi D, Spaggiari G, Simoni M (2018) Sperm DNA fragmentation index as a promising predictive tool for male infertility diagnosis and treatment management - meta-analyses. Reprod Biomed Online 37:315–326

Sasikumar S, Dakshayani D, Sarasa D (2013) An investigation of DNA fragmentation and morphological changes caused by bacteria and fungi in human spermatozoa. Int J Curr Microbiol App Sci 2:84–96

Schmid TE, Eskenazi B, Baumgartner A, Marchetti F, Young S, Weldon R et al (2007) The effects of male age on sperm DNA damage in healthy nonsmokers. Hum Reprod 22:180–187

Sellami H, Znazen A, Sellami A, Mnif H, Louati N, Ben Zarrouk S, Keskes L, Rebai T, Gdoura R, Hammami A (2014) Molecular detection of Chlamydia trachomatis and other sexually transmitted bacteria in semen of male partners of infertile couples in Tunisia: the effect on semen parameters and spermatozoa apoptosis markers. PLoS One 9:e98903

Showell MG, Mackenzie-Proctor R, Brown J, Yazdani A, Stankiewicz MT, Hart RJ (2014) Antioxidants for male subfertility. Cochrane Database Syst Rev:CD007411

Simon L, Zini A, Dyachenko A, Ciampi A, Carrell DT (2017) A systematic review and meta-analysis to

determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome. Asian J Androl 19:80–90

Sinha K, Das J, Pal PB, Sil PC (2013) Oxidative stress: the mitochondria-dependent and mitochondria independent pathways of apoptosis. Arch Toxicol 87:1157–1180

Smith TB, Dun MD, Smith ND, Curry BJ, Connaughton HS, Aitken RJ (2013) The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. J Cell Sci 126:1488–1497

Sotolongo B, Huang TT, Isenberger E, Ward WS (2005) An endogenous nuclease in hamster, mouse, and human spermatozoa cleaves DNA into loop-sized fragments. J Androl 26:272–280

Ståhl O, Eberhard J, Cavallin-Ståhl E, Jepson K, Friberg B, Tingsmark C et al (2009) Sperm DNA integrity in cancer patients: the effect of disease and treatment. Int J Androl 32:695–703

Vaidya A, Mao Z, Tian X, Spencer B, Seluanov A, Gorbunova V (2014) Knock-in reporter mice demonstrate that DNA repair by non-homologous end joining declines with age. Hasty P, editor. PLoS Genet 10:e1004511

Villegas J, Schulz M, Soto L, Sanchez R (2005) Bacteria induce expression of apoptosis in human spermatozoa. Apoptosis 10:105–110

Vujkovic M, de Vries JH, Dohle GR et al (2009) Associations between dietary patterns and semen quality in men undergoing IVF/ICSI treatment. Hum Reprod 24:1304–1312

Wyrobek AJ, Eskenazi B, Young S, Arnheim N, Tiemann-Boege I, Jabs EW et al (2006) Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. Proc Natl Acad Sci U S A 103:9601–9606

Yatsenko AN, Turek PJ (2018) Reproductive genetics and the aging male. J Assist Reprod Genet 35:933–941

Zalata A, El-Samanoudy AZ, Shaalan D, El-Baiomy Y, Mostafa T (2015) In vitro effect of cell phone radiation on motility, DNA fragmentation and clusterin gene expression in human sperm. Int J Fertil Steril 9:129–136

Sperm DNA Fragmentation: Consequences for Reproduction

6

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Abstract

DNA fragmentation, or the accumulation of single- and double-strand DNA breaks, is a common property of sperm, and an increase in the level of sperm DNA fragmentation is known to influence natural reproduction. The effect of sperm DNA fragmentation on male infertility and assisted reproductive treatment (ART) outcomes remains controversial and is one of the most frequently debated topics of reproductive medicine. For the past 30 years, a number of assays have been developed to quantify the level of sperm DNA fragmentation. In this chapter, we review the causes of sperm DNA fragmentation, describe the commonly used tests to evaluate these abnormalities, and perform a systematic review of existing studies to determine the impact of sperm DNA fragmentation on male fertility and ART outcomes.

Keywords

Sperm DNA fragmentation · Comet assay · SCSA · TUNEL assay · SCD assay · Male infertility · ART outcomes

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Introduction

Sperm are a well-designed vehicle that facilitate the transfer of a haploid genome from the father to the oocyte (Aitken and De Iuliis 2010). To perform such a function, the spermatogonial stem cell must undergo a series of meiotic divisions and morphological and biochemical alterations resulting in the formation of a mature sperm, and this process is known as spermatogenesis. As a result of spermatogenesis, millions of sperm are produced every day. Clearly, normal embryonic development is dependent on the delivery of intact and complete genetic material to the oocyte (Simon et al. 2014a). Therefore, the sperm nucleus has adopted a unique structural architecture in which the DNA is tightly packaged with small and positively charged proteins, termed protamines, resulting in the formation of compact nuclear structure (Oliva 2006). During this process, the sperm loses its cytoplasmic content, resulting in the formation of streamline sperm structure that facilitates the motility and protection of the genetic material.

The removal of the cytoplasm leaves the sperm nucleus vulnerable to the potential negative effects of free radicals; however, the compact, toroidal organization of sperm chromatin in normally protaminated sperm is known to protect sperm DNA from most free radical-mediated damage (Aitken 2012). Additionally, the seminal plasma not only acts as a medium for the sperm

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to swim, but consists of high concentrations of antioxidants that can scavenge the free radicals to minimize the effect of oxidative stress-mediated DNA damage (Koca et al. 2009). Despite these preventive mechanisms, oxidative stress is generated in sperm when the concentration of free radicals produced exceeds the level of antioxidant activity, resulting in sperm "DNA fragmentation", or the accumulation of DNA strand breaks (Saleh et al. 2002). Recently, it has been shown that DNA fragmentation is a common property of all sperm and the level of DNA damage may vary from one sperm to another (Simon et al. 2017a).

Sperm DNA fragmentation can also occur as a result of intrinsic factors where poor structural organization of sperm chromatin leaves the sperm vulnerable to oxidative stress-mediated DNA damage (Aoki et al. 2005). Studies have suggested that there may be a cascade of events that start with seminal oxidative stress leading to apoptosis of sperm (Aitken and Koppers 2011). Other factors such as medication, heat, radiation, etc. are some of the extrinsic factors also known to cause sperm DNA fragmentation (Agarwal and Allamaneni 2005; Aitken et al. 2005; Morris 2002). Regardless of the cause, DNA fragmentation occurring in sperm is permanent, as sperm lack any ability to repair damaged DNA.

Methods of Sperm DNA Fragmentation Analysis

A number of assays are now available to measure the level of sperm DNA fragmentation. Of these methods, single-cell gel electrophoresis (commonly called as the Comet assay), in situ nick translation assay, and the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assays directly measure the level of DNA fragmentation, whereas the Sperm Chromatin Dispersion (SCD) assay (commonly called as the Halo test) and Sperm Chromatin Structure Assay (SCSA) are known to indirectly measure the level of DNA fragmentation in sperm. These assays differ in their ease of use, cost, and the type of DNA strand breakage measured (Fig. 6.1).

Comet Assay

The Comet assay is one of the simplest methods to measure sperm DNA fragmentation and quantifies single- and double-strand breaks (McKelvey-Martin et al. 1997). The principle of the assay is that the sperm nuclear DNA is separated in an electric field based on charge and size, which can be viewed by using a fluorescent dye. The resulting image resembles a comet, with an intact head and tail based on the amount of DNA fragmentation. The intensity of staining determines the extent of DNA fragmentation (Ostling and Johanson 1984). Additional quantitative parameters have been used to increase the efficiency of the test, such as diameter of the nucleus, olive tail moment, and the comet length (Singh et al. 1988).

One of the principles of the Comet assay is that the double-stranded DNA remains in the comet head, whereas short fragments of double- and single-stranded DNA migrate into the tail area (Klaude et al. 1996). Therefore, sperm with high levels of DNA strand breaks would show an increased comet tail which can be measured by its intensity of fluorescence (Hughes et al. 1999) and comet tail length (Singh and Stephens 1998). The Comet assay can be performed in a neutral or alkaline environment. In neutral pH buffer, only DNA with double-strand breaks are measured, while in the alkaline buffer single- and double-strand breaks, and alkali-labile sites, are detectable due to unwinding of the strands (Tarozzi et al. 2009). This is the only technique that can measure the direct level of DNA fragmentation in individual cells. The Comet assay is relatively inexpensive and one of the most sensitive techniques available to measure DNA fragmentation. However, the assay is relatively labor intensive. According to published results, Comet assay results are correlated to the results obtained from the TUNEL assay (Aravindan et al. 1997). The alkaline Comet assay can be used in all cell types and also in the sperm. The assay requires only a

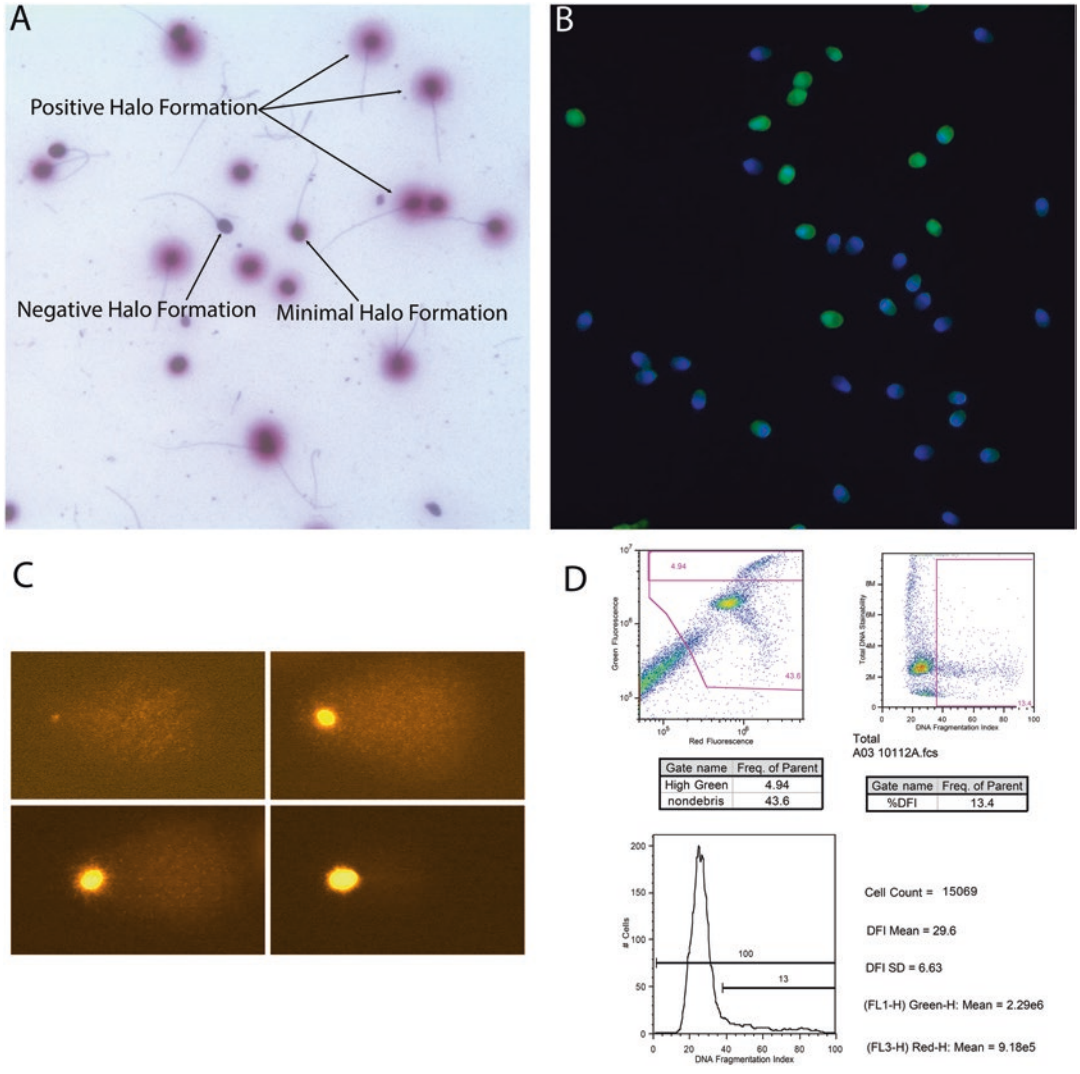

Fig. 6.1 Image of the four major assays for DNA fragmentation detection. (**a**) Micrograph of sperm analyzed using the SCD assay. (**b**) TUNEL assay micrograph identifying sperm with DNA damage (green) and non-damaged (blue). (**c**) Comet assay showing varying degrees of damage evidenced by tail length and intensity. (**d**) Printout of the data derived using the SCSA

few cells, of benefit for analysis of sperm from severely oligozoospermic men, and data can be collected at the level of individual cells.

The clinical importance of the Comet assay in assessing male infertility has been demonstrated by a number of authors (Simon et al. 2010, 2011a, 2017a, b; Irvine et al. 2000; Donnelly et al. 2001; Lewis and Agbaje 2008). The disadvantage of the assay is that it still lacks standardized protocols, which makes it difficult to fully understand and relate the results of different authors (Tarozzi et al. 2007). It is known to damage the alkaline labile sites and therefore makes it difficult to discriminate between endogenous and induced DNA breaks. The assay is also criticized for underestimation of DNA fragmentation due to entangling of DNA strands. Additionally, incomplete chromatin decondensation, in the case of sperm DNA, will not allow breaks to be revealed. Overlapping

comet tails decrease the accuracy of the assay and some extremely small tail fragments are lost or too small fragments are difficult to be visualized. The assay is laborious, has high level of inter-laboratory variation, and hence is not routinely recommended for routine clinical screening, but has been shown to be valuable in research applications (Olive et al. 2001).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay

The TUNEL assay quantifies the incorporation of deoxyuridine triphosphate (dUTP) at single- and double-strand DNA breaks in a reaction catalyzed by the template-independent enzyme, terminal deoxynucleotidyl transferase (Gorczyca et al. 1993). The incorporated dUTP which is labeling the breaks can be quantified by flow cytometry, fluorescent microscopy, or even light microscopy (Tarozzi et al. 2007). The TUNEL assay is widely known to measure direct sperm DNA fragmentation. The TUNEL assay resembles the nick translation in situ in a number of technical aspects, but can reveal both single- and double-strand breaks (Tarozzi et al. 2007). The sperm DNA fragmentation measured by TUNEL assay has good stability over time, so it is possible to measure and monitor baseline damage in both fertile and subfertile men (Sergerie et al. 2005a). The assay is broadly used to assess sperm DNA fragmentation as an indicator of male fertility (Sergerie et al. 2005b) and has been demonstrated to predict assisted reproduction outcome (Sun et al. 1997; Lopes et al. 1998; Duran et al. 2002; Benchaib et al. 2003; Borini et al. 2006).

The TUNEL assay can simultaneously detect single- and double-strand breaks, unlike other assays that either simply measure sperm susceptibility to DNA damage or require elaborate protocols to study both types of strand breakages (Lopes et al. 1998; Fraser 2004). Freezing raw or washed semen samples does not affect the results of the TUNEL assay (Sailer et al. 1995). The TUNEL assay is highly sophisticated and expensive; however, its popularity is justified by good quality control parameters, such as a low intra- and inter-observer variability (Barroso et al. 2000). This fluorescence labeling technique eliminates the problems associated with dye fading in the conventional microscopic method, thereby giving technicians more time to analyze a greater number of cells (Host et al. 1999). Due to the unique chromatin packaging of sperm, staining can be limited to the periphery of the cell; therefore, it is necessary to include techniques for relaxation of sperm DNA prior to labeling (Fraser 2004).

The use of flow cytometry protocols within the TUNEL assay makes it possible to evaluate a very high number of cells, thus enhancing reproducibility and accuracy of the technique. However, the TUNEL assay does not quantify the magnitude of DNA fragmentation within a given cell unless the measurement is conducted by flow cytometry as it only counts the number of cells within a population with DNA fragmentation as TUNEL-positive cells (Shamsi et al. 2008). The assay can be simplified to analyze cells using light microscopy, in which stained cells (with DNA fragmentation) and unstained cells (without fragmentation) are manually counted. However, in this case background staining can decrease the accuracy of the assay.

In Situ Nick Translation

The in situ nick translation (ISNT) assay is a modified form of the TUNEL assay that quantifies the incorporation of biotinylated deoxyuridine triphosphate (dUTP) at single-stranded DNA breaks in a reaction that is catalyzed by the template-dependent enzyme, DNA polymerase I (Shamsi et al. 2008). Unlike the TUNEL assay, which utilizes template-independent end labeling, nick translation can only be used for single-strand breaks, not for both single-strand and double-strand breaks as in the TUNEL assay (Irvine et al. 2000). This assay identifies variable levels of DNA strand breaks in each sperm (Manicardi et al. 1995) and is positively associated with protamine deficiency

(Bianchi et al. 1993). The clinical value of the nick translation assay is severely limited because no correlation has been proven with fertilization capacity during in vivo studies (Irvine et al. 2000), and it lacks sensitivity compared with other assays (Twigg et al. 1998). Furthermore, the assay may be less biologically relevant given that single-strand breaks can be more easily repaired by the embryo than the double-strand break (Twigg et al. 1998).

The accuracy of the DNA polymerase enzyme used in the assay is high and hence single-strand nicks are efficiently incorporated with labeled dUTP, resulting in identification of sperm with very low levels of DNA strand breaks. The assay is capable to identify a variable level (low to high) of DNA damage in individual sperm within an ejaculate (Shamsi et al. 2008). The clinical value of the nick translation assay is severely limited because no correlation has been proven with fertilization in in vivo studies (Irvine et al. 2000). When the ISNT is compared with other tests, TUNEL and Comet assays show better correlations with ART outcomes as they measure both single-strand and double-strand breaks present in the sperm DNA (Irvine et al. 2000).

Sperm Chromatin Structure Assay

The Sperm Chromatin Structure Assay (SCSA) is the most commonly used commercial test to characterize male infertility. It is a flow cytometric method to determine abnormal sperm chromatin which is highly susceptible to chemically induced in situ partial DNA denaturation. The extent of DNA denaturation is determined by measuring the metachromatic shift from green fluorescence to red fluorescence after heat or acid treatment (Evenson et al. 1980). The most important parameter of this test is the DNA fragmentation index (%), which represents the population of cells with DNA fragmentation (Evenson and Jost 2000). It also measures the High DNA stainability (%). The SCSA measures the susceptibility of sperm DNA to heat- or acid-induced DNA denaturation in situ, followed by staining with acridine orange stain where the double-strand DNA fluoresce green and the single-strand DNA fluoresce red (Evenson and Jost 2000). The use of flow cytometry makes it possible to measure a large number of spermatozoa per sample making the technique therefore simple and highly reproducible (Evenson and Jost 2000). DNA fragmentation index (DFI) represents the sperm population with detectable denaturable single-stranded DNA and the highly DNA stainable (HSD) cells describe the sperm population with increased accessibility of double-stranded DNA to the dye, mainly due to impaired replacement of histones with protamines (Tarozzi et al. 2007).

Sperm DNA fragmentation measured by the SCSA is known to be more constant over a longer period of time when compared with the traditional sperm evaluation parameters (Zini et al. 2001). The consistency of the test makes it useful in epidemiological studies (Spanò et al. 1998). Freezing of semen does not affect the test, allowing samples to be batched for convenience or used in multi-center trials and analyzed at a later date in a central facility. The assay determines the percentage of sperm with DNA fragmentation. Several clinical studies have shown its usefulness in evaluating male fertility (Evenson et al. 2002; Spano et al. 2000; Virro et al. 2004). It is simple and rapid for the analysis of thousands of human sperm (Fraser 2004). Generally, most users have defined that a threshold value above 30% DFI and 15% HSD predicts couples who are likely to be infertile. Several clinical studies have shown its usefulness in evaluating male fertility in relation with fertilization, blastocyst development, ongoing pregnancy in IVF, and ICSI (Evenson and Jost 2000; Evenson et al. 2002; Spano et al. 2000; Virro et al. 2004).

The SCSA does not give information about the extent of DNA fragmentation in individual sperm (Fraser 2004). The assay requires expensive equipment for analysis. Laboratory factors affect the test giving high variation between replicates (Boe-Hansen et al. 2005a, 2006). There is conflicting data as its usefulness in predicting fertilization rates, embryo quality, or pregnancy outcomes (Larson et al. 2000; Payne et al. 2005; Erenpreiss et al. 2006).

Sperm Chromatin Dispersion Assay

The Sperm Chromatin Dispersion (SCD) assay has been described as a simple and inexpensive method for the analysis of sperm DNA fragmentation. It is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo that is seen when sperm are mixed with agarose following acid denaturation and removal of nuclear proteins (Fernandez et al. 2003). The methodology of the test includes the following steps. Sperm are immersed in an agarose matrix on a slide, treated with an acid solution to denature DNA that contains breaks, and then treated with lysis buffer to remove membranes and proteins. The agarose matrix allows working with unfixed sperm on a slide in a suspension-like environment. Removal of nuclear proteins results in nucleoids with a central core and a peripheral halo of dispersed DNA loops. Following fluorescent staining, sperm nuclei with elevated DNA fragmentation produce very small or no halos of DNA dispersion, whereas those sperm with low levels of DNA fragmentation release their DNA loops forming large halos. These results have been confirmed by DNA breakage detection-fluorescence in situ hybridization, a procedure in which the restricted single-stranded DNA motifs generated from DNA breaks can be detected and quantified (Fernández and Gosálvez 2002).

The test does not rely on fluorescence intensity, hence it is simple to analyze with light microscopy. The test does not require the use of complex instrumentation; it can be carried out with equipment normally available in andrology laboratories (microscope). The test endpoints (non-dispersed and dispersed nuclei) can be easily obtained without extensive training of laboratory technicians.

Despite its ease of use, some limitations of the assay are well known. The assay has been reported to have low-density nucleoids, which are fainter with less contrasting images. Thus, the peripheral limit of the halo, where the chromatin is even less dense, may not be accurately discriminated from the background. Furthermore, all of the halos are not necessarily in the same visual plane of the agarose; hence, the use of software to analyze can result in misreading due to unfocused halos. Lastly, sperm tails are not preserved; therefore, discriminating sperm from other contaminant cells is problematic.

Consequence of Sperm DNA Fragmentation on Male Reproductive Health

During the past few decades, a number of studies have associated DNA fragmentation with male infertility (Host et al. 1999; Zini et al. 2001; Hughes et al. 1996; Evenson et al. 1999; Saleh et al. 2003a; Simon et al. 2011b; Castillo et al. 2011). Most of these studies suggest that sperm DNA fragmentation is associated with male infertility. Additionally, DNA fragmentation in the sperm of men from the general population planning their first pregnancy, with no previous knowledge of their fertility capability, was associated with diminished fecundity associated with an increase in sperm DNA fragmentation, indicating the necessity of normal sperm chromatin for the expression of male fertility potential (Spano et al. 2000).

Men with unexplained or idiopathic infertility have been shown to have increased levels of oxidative stress in the seminal plasma compared to controls (Pasqualotto et al. 2001), resulting in sperm DNA fragmentation (Sikka et al. 1995; Alkan et al. 1997). Increased levels of sperm DNA fragmentation has also been observed in men diagnosed with idiopathic male infertility (Saleh et al. 2003a). Leukocytospermia is common in patients with infections in the male genital tract, and resulting oxidative stress can result in sperm DNA fragmentation (Agarwal et al. 2014; Erenpreiss et al. 2002) and have a negative impact on ART outcomes (Lackner et al. 2008).

Varicoceles are a common cause of diminished sperm production and/or decreased sperm quality. In patients with a varicocele, an increased level of oxidants and reduced antioxidants is observed (Abd-Elmoaty et al. 2010). The level of oxidants in the seminal plasma has also been shown to positively correlate with the degree of varicocele (Barbieri et al. 1999), resulting in

increased sperm DNA fragmentation. Sperm DNA fragmentation has been shown to be reduced after varicocelectomy treatment, concomitantly with increased in pregnancy rates (Baker et al. 2013).

Lastly, an increase in the level of DNA fragmentation in infertile men can be attributed to abnormal histone to protamine exchange (Simon et al. 2011a; Zhang et al. 2006), sometimes observed as an abnormal protamine content or ratio (Castillo et al. 2011; Aoki et al. 2006). The mechanism by which diminished or altered protamination results in DNA fragmentation may be associated with a loss of the "protective" nature that protamination confers on sperm DNA.

Systematic Analysis of the Consequences of Sperm DNA Fragmentation on Assisted Reproduction Technologies

The existing literature regarding the effects of sperm DNA fragmentation on ART outcomes are controversial. A recent study showed a strong influence of sperm DNA fragmentation on male reproductive health and suggested that sperm DNA testing should be incorporated into routine clinical use (Simon et al. 2017b). In contrast, earlier meta-analyses and reviews did not support the clinical use of sperm DNA fragmentation (Collins et al. 2008; Zini and Sigman 2009). Therefore, for this chapter, we have performed an updated literature search and analysis of the association of sperm DNA fragmentation with ART outcomes, as measured using the four most commonly used assays (TUNEL, SCSA, SCD, and Comet). Our literature search identified 70 articles that included 94 study methodologies, TUNEL assay (35 studies), SCSA (30 studies), Comet assay (10 studies), and SCD assay (19 studies). Based on the treatment type, these studies involve standard-type insemination of IVF (30 studies), ICSI (41 studies), and IVF + ICSI mixed (23 studies) studies (Table 6.1).

Studies with overlapping data, inappropriate sampling method, assays that are less commonly used (neutral Comet assay, in situ nick translation assay, and acridine orange slide-based staining method), and studies with insufficient data were excluded from this systematic analysis. A drawback of this systematic analysis is that, in some studies, there were differences in the definition of threshold values for DNA fragmentation assays, study design, lack of control for female factors, diverse patient group, nonconsecutive recruitment of patients, and variations in the protocols used to measure DNA fragmentation assays, while in some studies, the inclusion and exclusion criteria of subject selection were not clearly stated.

Effect of Sperm DNA Damage on In Vitro Fertilization Rates

Of the 94 studies that analyzed sperm DNA fragmentation with ART outcomes, 18 did not evaluate and study the relationship between fertilization rates and sperm DNA fragmentation. The remaining 76 studies (26 IVF, 32 ICSI, and 18 mixed IVF + ICSI studies) involved 8711 treatment cycles (3149 IVF, 2558 ICSI, and 3004 mixed IVF + ICSI cycles). Forty percent (30/76 studies; including 14 TUNEL, 5 SCSA, 7 SCD, and 4 Comet studies) reported a significant inverse relationship between sperm DNA fragmentation and fertilization rate, whereas the other 46 studies (16 TUNEL, 19 SCSA, 6 SCD, and 5 Comet) showed no significant relationship between these parameters (Table 6.1).

There appears to be a stronger effect in standard IVF compared to ICSI. Fifty eight percent of the studies (15/26) reported a significant inverse relationship between sperm DNA fragmentation and fertilization rate compared to ICSI (25% or 8/32) and mixed IVF + ICSI studies (39% or 7/18). One possible explanation of this effect is that during IVF fertilization, the sperm fertilizing the oocyte is randomly selected based on the sperm-oocyte interaction, in contract to the ICSI process where the most morphologically normal and motile sperm are injected into the oocytes (Ola et al. 2001). Such selection of sperm for ICSI fertilization may result in selection of sperm with low DNA fragmentation, as sperm

Table 6.1 Description of studies associating sperm DNA fragmentation with ART outcomes

Study	ART	DD test	(n)	Fertilization rate	Embryo quality	Clinical pregnancy	Miscarriage	Live birth
Anifandis et al. (2015)	IVF + ICSI	SCD	139	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant	NA
Avendano et al. (2010)	ICSI	TUNEL	36'	NA	Significant	Significant	NA	NA
Bakos et al. (2007)	IVF	TUNEL	45	Significant	Nonsignificant	Nonsignificant	NA	NA
	IVF + ICSI	TUNEL	113	Nonsignificant	Nonsignificant	Significant	NA	NA
	ICSI	TUNEL	68	Nonsignificant	Nonsignificant	Significant	NA	NA
Benchaib et al. (2003)	IVF	TUNEL	50	Significant	Nonsignificant	Nonsignificant	NA	NA
	ICSI	TUNEL	54	Significant	Nonsignificant	Nonsignificant	NA	NA
Benchaib et al. (2007)	IVF	TUNEL	84	Nonsignificant	Nonsignificant	Nonsignificant	Significant	NA
	ICSI	TUNEL	218	Significant	Significant	Nonsignificant	Significant	NA
Boe-Hansen et al. (2005b)	IVF	SCSA	139	NA	NA	Nonsignificant	NA	NA
	ICSI	SCSA	47	NA	NA	Nonsignificant	NA	NA
Borini et al. (2006)	IVF	TUNEL	82	Significant	NA	Nonsignificant	Nonsignificant	NA
	ICSI	TUNEL	50	Nonsignificant	NA	Significant	Significant	NA
Bungum et al. (2007)	IVF	SCSA	388	Nonsignificant	Nonsignificant	NA	Nonsignificant	Nonsignificant
	ICSI	SCSA	223	Nonsignificant	Nonsignificant	NA	Nonsignificant	Nonsignificant
Caglar et al. (2007)	ICSI	TUNEL	56	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
Check et al. (2005)	ICSI	SCSA	106	NA	Significant	Nonsignificant	NA	NA
Dar et al. (2013)	ICSI	SCSA	153	Nonsignificant	NA	Nonsignificant	Nonsignificant	NA
Daris et al. (2010)	ICSI	TUNEL	20	Nonsignificant	NA	NA	NA	NA
Esbert et al. (2011)	IVF + ICSI	TUNEL	178	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant	NA
Fang et al. (2011)	IVF	SCSA	111	Significant	Nonsignificant	Nonsignificant	NA	NA
Frydman et al. (2008)	IVF	TUNEL	117	Nonsignificant	Nonsignificant	Significant	Significant	Significant
Gandini et al. (2004)	IVF	SCSA	12	Nonsignificant	NA	Nonsignificant	NA	NA
	ICSI	SCSA	22	Nonsignificant	NA	Nonsignificant	NA	NA
Gosalvez et al. (2013)	ICSI	SCD	81	NA	NA	Significant	NA	NA
Gu et al. (2009)	IVF	SCD	136	Significant	NA	Significant	NA	NA
Gu et al. (2011)	IVF	SCD	67	Significant	Nonsignificant	Significant	NA	NA
Guerin et al. (2005)	IVF + ICSI	SCSA	100	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
Hammadeh et al. (2006)	IVF	TUNEL	26	Nonsignificant	NA	NA	NA	NA
	ICSI	TUNEL	22	Nonsignificant	NA	NA	NA	NA
Hammadeh et al. (2008)	IVF	TUNEL	26	Nonsignificant	NA	Nonsignificant	NA	NA
	ICSI	TUNEL	22	Nonsignificant	NA	Nonsignificant	NA	NA

Study	ART	DD test	(n)	Fertilization rate	Embryo quality	Clinical pregnancy	Miscarriage	Live birth
Henkel et al. (2003)	IVF	TUNEL	208	Nonsignificant	Nonsignificant	NA	NA	NA
	ICSI	TUNEL	54	Significant	Nonsignificant	NA	NA	NA
Host et al. (1999)	IVF	TUNEL	175	Significant	Significant	Nonsignificant	NA	NA
	ICSI	TUNEL	60	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
Huang et al. (2005)	IVF	TUNEL	204	Significant	Nonsignificant	Nonsignificant	NA	NA
	ICSI	TUNEL	86	Significant	Nonsignificant	Nonsignificant	NA	NA
Jiang et al. (2011)	IVF	SCSA	137	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
	ICSI	SCSA	50	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
Kennedy et al. (2011)	ICSI	SCSA	233	NA	NA	Nonsignificant	Significant	Significant
Larson et al. (2000)	IVF + ICSI	SCSA	24	Nonsignificant	Nonsignificant	Significant	NA	NA
Larson-Cook et al. (2003)	IVF + ICSI	SCSA	82	Nonsignificant	Nonsignificant	Significant	NA	NA
Lazaros et al. (2013)	ICSI	SCSA	36	Significant	Significant	Significant	NA	NA
Lewis et al. (2004)	ICSI	Comet	77	Nonsignificant	NA	Significant	NA	NA
Li et al. (2011)	IVF	SCD	51	Significant	Nonsignificant	NA	NA	NA
Lin et al. (2008)	IVF	SCSA	137	Nonsignificant	Nonsignificant	Nonsignificant	Significant	NA
	ICSI	SCSA	86	Nonsignificant	Nonsignificant	Nonsignificant	Significant	NA
Lopes et al. (1998)	ICSI	TUNEL	131	Significant	Nonsignificant	NA	NA	NA
Lopez et al. (2013)	IVF + ICSI	SCD	152	NA	NA	Nonsignificant	NA	NA
Marchetti et al. (2002)	IVF	TUNEL	111	Significant	NA	NA	NA	NA
Meseguer et al. (2011)	IVF + ICSI	SCD	210	NA	NA	Significant	NA	NA
Micinski et al. (2009)	ICSI	SCSA	60	Significant	Nonsignificant	Significant	NA	NA
Morris et al. (2002)	IVF + ICSI	Comet	60	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant	NA
Muriel et al. (2006)	IVF + ICSI	SCD	85	Significant	Significant	Nonsignificant	NA	NA
Nasr-Esfahan et al. (2005)	ICSI	Comet	28	Nonsignificant	Significant	NA	NA	NA
Ni et al. (2014)	IVF	SCD	1380	NA	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant
	ICSI	SCD	355	NA	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant
Nicopoullos et al. (2008)	ICSI	SCSA	56	Nonsignificant	NA	Nonsignificant	NA	NA
Nijs et al. (2009)	IVF + ICSI	SCSA	205	Significant	NA	Nonsignificant	NA	NA
Nijs et al. (2011)	IVF + ICSI	SCSA	278	Nonsignificant	NA	Nonsignificant	NA	NA
Nunez-Calonge et al. (2012)	ICSI	SCD	70	Nonsignificant	NA	Significant	NA	NA
Ozmen et al. (2007)	ICSI	TUNEL	42	Nonsignificant	NA	Significant	Significant	Significant
Payne et al. (2005)	IVF + ICSI	SCSA	95	Significant	Nonsignificant	Nonsignificant	NA	NA

(continued)

Table 6.1 (continued)

Study	ART	DD test	(n)	Fertilization rate	Embryo quality	Clinical pregnancy	Miscarriage	Live birth
Pregl Breznik et al. (2013)*	IVF	SCD	133	Significant	Significant	NA	NA	NA
	ICSI	SCD	133	Significant	Significant	NA	NA	NA
Rama Raju et al. (2012)	IVF + ICSI	SCD	247	Nonsignificant	Significant	Significant	NA	NA
Sanchez-Martin et al. (2013)	ICSI	SCD	40	NA	NA	NA	NA	NA
Seli et al. (2004)	IVF + ICSI	TUNEL	49	NA	Significant	Nonsignificant	NA	NA
Sharbatoghli et al. (2012)	ICSI	SCD	120	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
	ICSI	TUNEL	120	Nonsignificant	Nonsignificant	Significant	NA	NA
Simon et al. (2010)	IVF	Comet	219	Significant	Significant	Significant	Nonsignificant	Nonsignificant
	ICSI	Comet	116	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant	Significant
Simon et al. (2011a)	IVF	Comet	70	Significant	Significant	Significant	NA	NA
Simon and Lewis (2011)	IVF	Comet	73	Significant	Significant	Significant	NA	NA
	ICSI	Comet	22	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
Simon et al. (2014a)	IVF + ICSI	Comet	238	Significant	Significant	Significant	NA	NA
	IVF + ICSI	TUNEL	238	Significant	Nonsignificant	Significant	NA	NA
	IVF + ICSI	SCSA	102	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
Smit et al. (2010)	IVF + ICSI	SCSA	27	NA	NA	Significant	NA	NA
Speyer et al. (2010)	IVF	SCSA	192	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant	NA
	ICSI	SCSA	155	Nonsignificant	Nonsignificant	Significant	Nonsignificant	NA
Stevanato et al. (2008)	IVF + ICSI	TUNEL	35	NA	NA	NA	NA	NA
Sun et al. (1997)	IVF	TUNEL	143	Significant	Significant	NA	NA	NA
Tarozzi et al. (2009)	IVF	TUNEL	82	NA	NA	Nonsignificant	NA	NA
	ICSI	TUNEL	50	NA	NA	Significant	NA	NA
Tavalaee et al. (2009)	IVF + ICSI	SCD	92	Nonsignificant	NA	Nonsignificant	NA	NA
Tomsu et al. (2002)	IVF	Comet	40	NA	Significant	Nonsignificant	NA	NA
Velez de la Calle et al. (2008)	IVF + ICSI	SCD	622	Significant	Significant	Nonsignificant	NA	NA
Virro et al. (2004)	IVF + ICSI	SCSA	249	Nonsignificant	Significant	Significant	NA	NA
Yang et al. (2013)	ICSI	SCSA	62	NA	NA	Significant	Nonsignificant	NA
Zeyad et al. (2018)	ICSI	TUNEL	84	Nonsignificant	NA	Nonsignificant	NA	NA
Zheng et al. (2018)	IVF	SCD	161	Nonsignificant	Significant	Significant	NA	NA
Zini et al. (2005)	ICSI	SCSA	60	Nonsignificant	Significant	Nonsignificant	Nonsignificant	NA

TUNEL terminal deoxyuridine nick-end labeling assay, *SCD* Sperm Chromatin Dispersion assay, *SCSA* Sperm Chromatin Structure assay, *IVF* in vitro fertilization, *ICSI* intracytoplasmic sperm injection, *NA* data not available

*Pregl Breznik et al. (2013) –IVF and ICSI cycles of treatment were not specified

motility and sperm morphology are negatively associated with sperm DNA fragmentation (Borini et al. 2006; Huang et al. 2005; Caglar et al. 2007; Lin et al. 2008; Simon and Lewis 2011).

Effect of Sperm DNA Damage on Embryo Development

From the systematic review (Table 6.1), we identified 62 eligible studies (22 IVF, 24 ICSI, and 16 mixed IVF + ICSI studies) that analyzed sperm DNA fragmentation with embryo quality, of which 21 studies (34%) showed a significant association between embryo quality and sperm DNA fragmentation. The 62 studies involved 9116 treatment cycles (4193 IVF, 2445 ICSI, and 2478 mixed IVF + ICSI cycles). In 34% (21/62) of the studies (5 TUNEL, 4 SCSA, 6 SCD, and 6 Comet), a significant inverse relationship between sperm DNA fragmentation and embryo quality was reported, whereas the remaining 41 studies (17 TUNEL, 15 SCSA, 6 SCD, and 3 Comet) showed no significant relationship between these parameters.

Studies using the Comet assay more commonly reported an adverse effect (67%), whereas 22% of TUNEL, 21% of SCSA, and 50% of SCD studies reported adverse effects of sperm DNA fragmentation on embryo quality. In terms of the type of assisted treatment, 36% of IVF, 29% of ICSI, and 38% of mixed IVF + ICSI studies reported adverse effect of sperm DNA fragmentation on embryo quality. Our analysis showed a differential association between sperm DNA fragmentation and embryo quality; when the studies were segregated into groups based on assay types, sperm DNA fragmentation detected by the alkaline Comet assay was strongly associated with poor embryo quality when compared to other assays. This association may be due to the sensitivity of the Comet assay, which measures both single- and double-stranded DNA fragmentation following complete chromatin decondensation, or may be due to the small number of studies (Simon et al. 2014b).

Effect of Sperm DNA Damage on ART Success

An extensive review of the existing literature and meta-analysis of studies testing the effect of DNA fragmentation on ART treatment were recently published by our group (Simon et al. 2017b). In this meta-analysis (56 studies), clinical pregnancy was analyzed in 3734 IVF treatment cycles from 16 studies, 2282 ICSI treatment cycles from 24 studies, and 2052 mixed IVF + ICSI treatment cycles from 16 studies. An overall relationship between sperm DNA fragmentation and clinical pregnancy outcome from 56 studies (including 8068 ART cycles) supported a strong and significant association between the two parameters [Odds Ratio (OR): = 1.68; 95% CI: 1.49–1.89, P <0.0001] (Simon et al. 2017b). The meta-analysis showed a strong relationship between sperm DNA fragmentation and clinical pregnancy outcome based on the type of treatment. A significant association between sperm DNA fragmentation and clinical pregnancy was observed for IVF treatment (OR = 1.65; 95% CI: 1.34–2.04; P <0.0001), ICSI treatment (OR = 1.31; 95% CI, 1.08–1.59; P = 0.0068), and combined IVF + ICSI treatment (OR = 2.37; 95% CI: 1.89–2.97; P <0.0001) (Simon et al. 2017b).

The meta-analysis suggested that DNA fragmentation measured by TUNEL (n = 2098 cycles from 18 studies; OR = 2.22; 95% CI: 1.61–3.05; P <0.0001), SCD (n = 2359 cycles from 8 studies; OR = 1.98; 95% CI: 1.19–3.3; P = 0.0086), and Comet (n = 798 cycles from 7 studies; OR = 3.56; 95% CI: 1.78–7.09; P = 0.0003) assays reported a significant relationship with clinical pregnancy outcome. However, the association between the two parameters using SCSA studies was not statistically significant (n = 2813 cycles from 23 studies; OR = 1.22; 95% CI: 0.93–1.61; P = 0.1522) (Simon et al. 2017b).

Our results are in contrast with previously published meta-analysis (Collins et al. 2008; Practice Committee of the American Society for Reproductive Medicine 2013; Li et al. 2006; Zhao et al. 2014) as these studies were unable to

show any relationship between sperm DNA fragmentation and clinical pregnancy outcome. Our recent meta-analysis (Simon et al. 2017b) concludes that a modest but significant association between sperm DNA fragmentation and clinical pregnancy rates is present in all three ART treatment groups (IVF, ICSI, and mixed IVF + ICSI studies) with a variable effect according to the type of sperm DNA assay. A moderate relationship between the two parameters may be due to the failure of prior studies to control for strict patient inclusion criterion, such as the failure of most studies to not exclude couples with female factors infertility. Studies in which more than half of the couples had been diagnosed with female infertility resulted in lower odds of predicting a success via DNA damage analysis (Payne et al. 2005; Frydman et al. 2008; Meseguer et al. 2011), whereas studies in which patients with female infertile factor were controlled, the odds to predicting a successful pregnancy have significantly increased irrespective of the type of DNA fragmentation testing method (Simon et al. 2011b; Giwercman et al. 2010).

Association of Sperm DNA Damage with Pregnancy Loss

Robinson et al. performed a meta-analysis evaluating the relationship between sperm DNA damage and pregnancy loss. The results of the meta-analysis suggested a significant increase in miscarriage in patients with high DNA fragmentation compared with those with low DNA fragmentation (Risk risk (RR): 2.16; 95% CI: 1.54–3.03; P <0.0001) (Robinson et al. 2012). The meta-analysis also reported a strong association of DNA fragmentation on miscarriages, observed when DNA fragmentation was measured in the raw semen (RR: 1.65; 95% CI: 1.66–2.33; P <0.0001) as well as the density gradient prepared subpopulation (RR: 3.47; 95% CI: 2.13–5.63; P <0.0001). These results are in support of previous meta-analysis (Zini et al. 2008) where a positive impact of sperm DNA fragmentation on spontaneous pregnancy loss was observed.

Although the specific mechanism(s) by which sperm DNA damage leads to increased pregnancy loss is not understood, it is well known that many factors contribute to this problem (Ford and Schust 2009). Interestingly, the negative impact of sperm DNA fragmentation is more pronounced in animal models where induced sperm DNA damage leads to abnormal embryo development, reduced implantation rate, and frequent pregnancy loss (Ahmadi and Ng 1999; Fatehi et al. 2006). Such prolonged effects of sperm DNA fragmentation, also known as the late paternal effect (Tesarik et al. 2004), may be in part due to the inability of the oocyte to repair the damaged sperm chromatin when it exceeds the threshold value (Simon et al. 2014a).

Effect of Sperm DNA Damage with Intrauterine Insemination Success

Our literature search identified ten studies that analyzed the association between sperm DNA fragmentation and IUI outcome. A total of 1673 IUI cycles were analyzed using SCSA (7 studies), TUNEL (2 studies), and SCD (1 study) assays. The results from five of the seven studies by SCSA (Saleh et al. 2003b; Bungum et al. 2004, 2007, 2008; Yang et al. 2011) and one study using the TUNEL assay (Duran et al. 2002) suggested a significant statistical difference in the level of sperm DNA fragmentation between the clinically pregnant and non-pregnant groups. Conclusive results were not published in two studies using SCSA (Boe-Hansen et al. 2006; Alkhayal et al. 2013), while no correlations were reported in two studies: using TUNEL assay (Thomson et al. 2011) and using SCD assay (Muriel et al. 2006).

Data were available to construct a two-by-two table from six of the seven studies performed using SCSA. The remaining five studies were used to construct a meta-analysis consisting of 1135 IUI cycles and with an overall pregnancy rate of 18.23%, resulting in an odds ratio of 5.61 (CI: 2.59–12.16; Z statistics: 4.37; p <0.0001) and relative risk of 1.17 (CI: 1.12–1.22; p

<0.0001) indicating a strong association between sperm DNA fragmentation and IUI outcome (unpublished data). The positive and negative predictive values were 18.96% and 96.00%, respectively. This model provided a high sensitivity (96.30%) but low specificity (17.76%) values. Our recent meta-analysis suggests a slight but significant ability of DNA fragmentation to predict IUI success, which is in contrast to the previous meta-analysis that included four of the ten studies presented above for the analysis (Practice Committee of the American Society for Reproductive Medicine 2013).

Sperm DNA Fragmentation as a Biomarker

Approximately, 30% of couples having fertility issues are diagnosed with unexplained infertility. In couples with unexplained infertility, sperm DNA fragmentation is elevated (Simon et al. 2013; Feijó and Esteves 2014). Simon et al. analyzed 147 unexplained infertile men for sperm DNA fragmentation using the Comet assay and reported that 84% of these unexplained infertile men had DNA fragmentation above the 25% cutoff value used to determine fertile from infertile men (Simon et al. 2013). In addition, the study reported that 41% of men categorized with unexplained infertility issues have sperm DNA fragmentation above the threshold of 52% fragmentation, a level previously shown to categorize the probability of a clinical pregnancy following IVF treatment (Simon et al. 2013). In another study using the SCSA assay, Oleszcuk et al. reported that 26% of men diagnosed with unexplained infertility had high DNA fragmentation index (Oleszczuk et al. 2013). Similarly, studies using TUNEL and SCD assays have reported that men with unexplained infertility have high levels of sperm DNA fragmentation (Feijó and Esteves 2014). These studies suggest that, to some extent, sperm DNA fragmentation assays may be helpful as a biomarker to identify men with fertility problems even when they are presented with normal semen analysis, as reported in unexplained infertility.

It has been shown that sperm are vulnerable to xenobiotic agents, resulting in DNA fragmentation (Aitken and De Iuliis 2007). The exposure to xenobiotics can be classified into three major types such as occupational exposure, environmental exposure, and pharmacological exposure. Workers in contact with polycyclic aromatic hydrocarbon exposure have higher sperm DNA fragmentation (Hsu et al. 2006). Elevated levels of DNA fragmentation were also observed in workers associated with waste incineration (Oh et al. 2005). Men working in the factories in contact with organic molecules such as styrene (Migliore et al. 2002), men working in the insecticide and pesticide industries (Xia et al. 2005), and men exposed to organic chemicals (Migliore et al. 2002) also have increased in sperm DNA fragmentation.

Pharmacological intervention for the treatment of diseases can result in genotoxic to sperm and male germ cells. A well-known example for such intervention is cyclophosphamide, which is used as a chemotherapeutic agent to treat cancer (Hales et al. 2005). In addition, environmental estrogens and similar compounds can have profound effects on male fertility, including affecting sperm DNA fragmentation (Anderson et al. 2003). Other environmental pollutions that have the ability to induce DNA fragmentation include organo-chlorides (Spano et al. 2005) and smog (Evenson and Wixon 2005). Therefore, sperm DNA fragmentation may not only be useful to identify male reproductive health status but also can serve as a biomarker to diagnose men exposed to xenobiotics.

Conclusion

General semen quality parameters (sperm concentration, sperm motility, sperm morphology, and total sperm count) have shown little or no correlation with fertility outcome in populations of first pregnancy planners (Bonde et al. 1998; Andersen et al. 2002; Cooper et al. 2010; Buck Louis et al. 2014). In contrast, studies correlating sperm DNA fragmentation with time to pregnancy (Spano et al. 2000; Evenson et al. 1999)

show a strong association between the two parameters. In addition, men with infertility issues are showed to have higher levels of sperm DNA fragmentation when compared with fertile men, suggesting a strong association between sperm DNA fragmentation and male infertility (Simon et al. 2011b).

The meta-analyses and systematic review presented here demonstrate that sperm DNA fragmentation is a good predictor of IUI failure and is associated with IVF pregnancy but less so with ICSI outcomes. Sperm DNA fragmentation is also negatively associated with embryo development and implantation and positively associated with miscarriage rates. Based on the evidence presented here, we suggest that sperm DNA fragmentation is closely associated with male infertility and it is independent of semen parameters. In addition, the level of sperm DNA fragmentation could influence various parameters of ART outcomes.

Controversy still exists regarding the clinical implementation of DNA fragmentation assays. Future studies should carefully consider the cost effectiveness and clinical utility of routine screening, versus targeted analysis. Furthermore, while preliminary data are intriguing, more data are still needed regarding the clinical utility of clinical interventions, such as antioxidant therapy and testicular sperm aspiration (TESE). Lastly, until testing procedures can become standardized, it will be nearly impossible to solve issues of variability and ultimate utility of the assay.

References

Abd-Elmoaty MA et al (2010) Increased levels of oxidants and reduced antioxidants in semen of infertile men with varicocele. Fertil Steril 94(4):1531–1534

Agarwal A, Allamaneni SS (2005) Sperm DNA damage assessment: a test whose time has come. Fertil Steril 84(4):850–853

Agarwal A et al (2014) Reactive oxygen species and sperm DNA damage in infertile men presenting with low level leukocytospermia. Reprod Biol Endocrinol 12:1–8

Ahmadi A, Ng SC (1999) Fertilizing ability of DNA-damaged spermatozoa. J Exp Zool 284(6):696–704

Aitken RJ (2012) Aetiology of defective sperm function and DNA damage in the male germ line. J Reprod Immunol 94(1):7–8

Aitken RJ, De Iuliis GN (2007) Origins and consequences of DNA damage in male germ cells. Reprod Biomed Online 14(6):727–733

Aitken RJ, De Iuliis GN (2010) On the possible origins of DNA damage in human spermatozoa. Mol Hum Reprod 16(1):3–13

Aitken RJ, Koppers AJ (2011) Apoptosis and DNA damage in human spermatozoa. Asian J Androl 13(1):36–42

Aitken RJ et al (2005) Impact of radio frequency electromagnetic radiation on DNA integrity in the male germline. Int J Androl 28(3):171–179

Alkan I et al (1997) Reactive oxygen species production by the spermatozoa of patients with idiopathic infertility: relationship to seminal plasma antioxidants. J Urol 157(1):140–143

Alkhayal A et al (2013) Sperm DNA and chromatin integrity in semen samples used for intrauterine insemination. J Assist Reprod Genet 30(11):1519–1524

Andersen AG et al (2002) Time to pregnancy in relation to semen quality assessed by CASA before and after sperm separation. Hum Reprod 17(1):173–177

Anderson D et al (2003) Oestrogenic compounds and oxidative stress (in human sperm and lymphocytes in the Comet assay). Mutat Res 544(2–3):173–178

Anifandis G et al (2015) Sperm DNA fragmentation measured by Halosperm does not impact on embryo quality and ongoing pregnancy rates in IVF/ICSI treatments. Andrologia 47(3):295–302

Aoki VW et al (2005) DNA integrity is compromised in protamine-deficient human sperm. J Androl 26(6):741–748

Aoki VW et al (2006) Sperm protamine 1/protamine 2 ratios are related to in vitro fertilization pregnancy rates and predictive of fertilization ability. Fertil Steril 86(5):1408–1415

Aravindan GR et al (1997) Susceptibility of human sperm to in situ DNA denaturation is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. Exp Cell Res 236(1):231–237

Avendano C et al (2010) DNA fragmentation of normal spermatozoa negatively impacts embryo quality and intracytoplasmic sperm injection outcome. Fertil Steril 94(2):549–557

Baker K et al (2013) Pregnancy after varicocelectomy: impact of postoperative motility and DFI. Urology 81(4):760–766

Bakos HW et al (2007) Elevated glucose levels induce lipid peroxidation and DNA damage in human spermatozoa. Aust N Z J Obstet Gynaecol 47:A1–A1

Barbieri ER et al (1999) Varicocele-associated decrease in antioxidant defenses. J Androl 20(6):713–717

Barroso G, Morshedi M, Oehninger S (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. Hum Reprod 15(6):1338–1344

Benchaib M et al (2003) Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. Hum Reprod 18(5):1023–1028

Benchaib M et al (2007) Sperm deoxyribonucleic acid fragmentation as a prognostic indicator of assisted reproductive technology outcome. Fertil Steril 87(1):93–100

Bianchi PG et al (1993) Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. Biol Reprod 49(5):1083–1088

Boe-Hansen GB, Ersboll AK, Christensen P (2005a) Variability and laboratory factors affecting the sperm chromatin structure assay in human semen. J Androl 26(3):360–368

Boe-Hansen GB, Ersbøll AK, Christensen P (2005b) Variability and laboratory factors affecting the sperm chromatin structure assay in human semen. J Androl 26(3):360–368

Boe-Hansen GB et al (2006) The sperm chromatin structure assay as a diagnostic tool in the human fertility clinic. Hum Reprod 21(6):1576–1582

Bonde JPE et al (1998) Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. Lancet 352(9135):1172–1177

Borini A et al (2006) Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Hum Reprod 21(11):2876–2881

Buck Louis GM et al (2014) Semen quality and time to pregnancy: the Longitudinal Investigation of Fertility and the Environment Study. Fertil Steril 101(2):453–462

Bungum M et al (2004) The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. Hum Reprod 19(6):1401–1408

Bungum M et al (2007) Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Hum Reprod 22(1):174–179

Bungum M et al (2008) Sperm chromatin structure assay parameters measured after density gradient centrifugation are not predictive for the outcome of ART. Hum Reprod 23(1):4–10

Caglar GS et al (2007) Semen DNA fragmentation index, evaluated with both TUNEL and Comet assay, and the ICSI outcome. In Vivo 21(6):1075–1080

Castillo J et al (2011) Protamine/DNA ratios and DNA damage in native and density gradient centrifuged sperm from infertile patients. J Androl 32(3):324–332

Check JH et al (2005) Effect of an abnormal sperm chromatin structural assay (SCSA) on pregnancy outcome following (IVF) with ICSI in previous IVF failures. Arch Androl 51(2):121–124

Collins JA, Barnhart KT, Schlegel PN (2008) Do sperm DNA integrity tests predict pregnancy with in vitro fertilization? Fertil Steril 89(4):823–831

Cooper TG et al (2010) World Health Organization reference values for human semen characteristics. Hum Reprod Update 16(3):231–245

Dar S et al (2013) In vitro fertilization-intracytoplasmic sperm injection outcome in patients with a markedly high DNA fragmentation index (>50%). Fertil Steril 100(1):75–80

Daris B et al (2010) Sperm morphological abnormalities as indicators of DNA fragmentation and fertilization in ICSI. Arch Gynecol Obstet 281(2):363–367

Donnelly ET, McClure N, Lewis SE (2001) Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. Fertil Steril 76(5):892–900

Duran EH et al (2002) Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. Hum Reprod 17(12):3122–3128

Erenpreiss J et al (2002) Effect of leukocytospermia on sperm DNA integrity: a negative effect in abnormal semen samples. J Androl 23(5):717–723

Erenpreiss J et al (2006) Sperm chromatin structure and male fertility: biological and clinical aspects. Asian J Androl 8(1):11–29

Esbert M et al (2011) Impact of sperm DNA fragmentation on the outcome of IVF with own or donated oocytes. Reprod Biomed Online 23(6):704–710

Evenson D, Jost L (2000) Sperm chromatin structure assay is useful for fertility assessment. Methods Cell Sci 22(2–3):169–189

Evenson DP, Wixon R (2005) Environmental toxicants cause sperm DNA fragmentation as detected by the Sperm Chromatin Structure Assay (SCSA). Toxicol Appl Pharmacol 207(2. Suppl):532–537

Evenson DP, Darzynkiewicz Z, Melamed MR (1980) Relation of mammalian sperm chromatin heterogeneity to fertility. Science 210(4474):1131–1133

Evenson DP et al (1999) Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 14(4):1039–1049

Evenson DP, Larson KL, Jost LK (2002) Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl 23(1):25–43

Fang L, et al. (2011) [A study on correlation between sperm DNA fragmentation index and age of male, various parameters of sperm and in vitro fertilization outcome]. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 28(4):432–435

Fatehi AN et al (2006) DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after the first cleavages. J Androl 27(2):176–188

Feijó CM, Esteves SC (2014) Diagnostic accuracy of sperm chromatin dispersion test to evaluate sperm deoxyribonucleic acid damage in men with unexplained infertility. Fertil Steril 101(1):58–63.e3

Fernández JL, Gosálvez J (2002) Application of FISH to detect DNA damage. DNA breakage detection-FISH (DBD-FISH). Methods Mol Biol 203:203–216

Fernandez JL et al (2003) The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl 24(1):59–66

Ford HB, Schust DJ (2009) Recurrent pregnancy loss: etiology, diagnosis, and therapy. Rev Obstet Gynecol 2(2):76–83

Fraser L (2004) Structural damage to nuclear DNA in mammalian spermatozoa: its evaluation techniques and relationship with male infertility. Pol J Vet Sci 7(4):311–321

Frydman N et al (2008) Adequate ovarian follicular status does not prevent the decrease in pregnancy rates associated with high sperm DNA fragmentation. Fertil Steril 89(1):92–97

Gandini L et al (2004) Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. Hum Reprod 19(6):1409–1417

Giwercman A et al (2010) Sperm chromatin structure assay as an independent predictor of fertility in vivo: a case-control study. Int J Androl 33(1):e221–e227

Gorczyca W, Gong J, Darzynkiewicz Z (1993) Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. Cancer Res 53(8):1945–1951

Gosalvez J et al (2013) Can DNA fragmentation of neat or swim-up spermatozoa be used to predict pregnancy following ICSI of fertile oocyte donors? Asian J Androl 15(6):812–818

Gu LJ et al (2009) Sperm chromatin anomalies have an adverse effect on the outcome of conventional in vitro fertilization: a study with strictly controlled external factors. Fertil Steril 92(4):1344–1346

Gu LJ, et al. (2011) [Effects of abnormal structure of sperm chromatin on the outcome of in vitro fertilization and embryo transfer]. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 28(2):156–159

Guerin P, et al. (2005) [Impact of sperm DNA fragmentation on ART outcome]. Gynecol Obstet Fertil 33(9):665–668

Hales BF, Barton TS, Robaire B (2005) Impact of paternal exposure to chemotherapy on offspring in the rat. J Natl Cancer Inst Monogr (34):28–31

Hammadeh ME et al (2006) Comparison of reactive oxygen species concentration in seminal plasma and semen parameters in partners of pregnant and non-pregnant patients after IVF/ICSI. Reprod Biomed Online 13(5):696–706

Hammadeh ME et al (2008) Reactive oxygen species, total antioxidant concentration of seminal plasma and their effect on sperm parameters and outcome of IVF/ICSI patients. Arch Gynecol Obstet 277(6):515–526

Henkel R et al (2003) DNA fragmentation of spermatozoa and assisted reproduction technology. Reprod Biomed Online 7(4):477–484

Host E et al (1999) DNA strand breaks in human sperm cells: a comparison between men with normal and oligozoospermic sperm samples. Acta Obstet Gynecol Scand 78(4):336–339

Høst E et al (1999) DNA strand breaks in human sperm cells: a comparison between men with normal and oligozoospermic sperm samples. Acta Obstet Gynecol Scand 78(4):336–339

Hsu PC et al (2006) Sperm DNA damage correlates with polycyclic aromatic hydrocarbons biomarker in coke-oven workers. Int Arch Occup Environ Health 79(5):349–356

Huang CC et al (2005) Sperm DNA fragmentation negatively correlates with velocity and fertilization rates but might not affect pregnancy rates. Fertil Steril 84(1):130–140

Hughes CM et al (1996) A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. Mol Hum Reprod 2(8):613–619

Hughes CM, McKelvey-Martin VJ, Lewis SEM (1999) Human sperm DNA integrity assessed by the Comet and ELISA assays. Mutagenesis 14(1):71–75

Irvine DS et al (2000) DNA integrity in human spermatozoa: relationships with semen quality. J Androl 21(1):33–44

Jiang HH, et al. (2011) [Sperm chromatin integrity test for predicting the outcomes of IVF and ICSI]. Zhonghua Nan Ke Xue 17(12):1083–1086

Kennedy C et al (2011) Sperm chromatin structure correlates with spontaneous abortion and multiple pregnancy rates in assisted reproduction. Reprod Biomed Online 22(3):272–276

Klaude M et al (1996) The comet assay: mechanisms and technical considerations. Mutat Res 363(2):89–96

Koca Y et al (2009) Antioxidant activity of seminal plasma in fertile and infertile men. Arch Androl 49(5):355–359

Lackner JE et al (2008) Effect of leukocytospermia on fertilization and pregnancy rates of artificial reproductive technologies. Fertil Steril 90(3):869–871

Larson KL et al (2000) Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. Hum Reprod 15(8):1717–1722

Larson-Cook KL et al (2003) Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. Fertil Steril 80(4):895–902

Lazaros L et al (2013) Sperm flow cytometric parameters are associated with ICSI outcome. Reprod Biomed Online 26(6):611–618

Lewis SE, Agbaje IM (2008) Using the alkaline comet assay in prognostic tests for male infertility and assisted reproductive technology outcomes. Mutagenesis 23(3):163–170

Lewis SE et al (2004) An algorithm to predict pregnancy in assisted reproduction. Hum Reprod 19(6):1385–1394

Li N, Jiang L (2011) Effect of sperm DNA on the outcome of in vitro fertilization-embryo transfer. Guangxi Med J 33(3):257–260

Li Z et al (2006) Correlation of sperm DNA damage with IVF and ICSI outcomes: a systematic review and meta-analysis. J Assist Reprod Genet 23(9–10):367–376

Lin MH et al (2008) Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in in vitro fertilization and intracytoplasmic sperm injection, but

might be related to spontaneous abortion rates. Fertil Steril 90(2):352–359
Lopes S et al (1998) Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil Steril 69(3):528–532
Lopez G et al (2013) Diagnostic value of sperm DNA fragmentation and sperm high-magnification for predicting outcome of assisted reproduction treatment. Asian J Androl 15(6):790–794
Manicardi GC et al (1995) Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. Biol Reprod 52(4):864–867
Marchetti C et al (2002) Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. Hum Reprod 17(5):1257–1265
McKelvey-Martin VJ et al (1997) Two potential clinical applications of the alkaline single-cell gel electrophoresis assay: (1). Human bladder washings and transitional cell carcinoma of the bladder; and (2). Human sperm and male infertility. Mutat Res 375(2):93–104
Meseguer M et al (2011) Effect of sperm DNA fragmentation on pregnancy outcome depends on oocyte quality. Fertil Steril 95(1):124–128
Micinski P et al (2009) The sperm chromatin structure assay (SCSA) as prognostic factor in IVF/ICSI program. Reprod Biol 9(1):65–70
Migliore L et al (2002) Assessment of sperm DNA integrity in workers exposed to styrene. Hum Reprod 17(11):2912–2918
Morris ID (2002) Sperm DNA damage and cancer treatment. Int J Androl 25(5):255–261
Morris ID et al (2002) The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. Hum Reprod 17(4):990–998
Muriel L et al (2006) Value of the sperm chromatin dispersion test in predicting pregnancy outcome in intrauterine insemination: a blind prospective study. Hum Reprod 21(3):738–744
Nasr-Esfahani MH et al (2005) Effect of sperm DNA damage and sperm protamine deficiency on fertilization and embryo development post-ICSI. Reprod Biomed Online 11(2):198–205
Ni W et al (2014) Effect of sperm DNA fragmentation on clinical outcome of frozen-thawed embryo transfer and on blastocyst formation. PLoS One 9(4):e94956
Nicopoullos JD et al (2008) Sperm DNA fragmentation in subfertile men: the effect on the outcome of intracytoplasmic sperm injection and correlation with sperm variables. BJU Int 101(12):1553–1560
Nijs M et al (2009) Chromomycin A3 staining, sperm chromatin structure assay and hyaluronic acid binding assay as predictors for assisted reproductive outcome. Reprod Biomed Online 19(5):671–684
Nijs M et al (2011) Correlation between male age, WHO sperm parameters, DNA fragmentation, chromatin packaging and outcome in assisted reproduction technology. Andrologia 43(3):174–179
Nunez-Calonge R et al (2012) An improved experimental model for understanding the impact of sperm DNA fragmentation on human pregnancy following ICSI. Reprod Sci 19(11):1163–1168
Oh E et al (2005) Evaluation of immuno- and reproductive toxicities and association between immunotoxicological and genotoxicological parameters in waste incineration workers. Toxicology 210(1):65–80
Ola B et al (2001) Should ICSI be the treatment of choice for all cases of in-vitro conception? Considerations of fertilization and embryo development, cost effectiveness and safety. Hum Reprod 16(12):2485–2490
Oleszczuk K et al (2013) Prevalence of high DNA fragmentation index in male partners of unexplained infertile couples. Andrology 1(3):357–360
Oliva R (2006) Protamines and male infertility. Hum Reprod Update 12(4):417–435
Olive PL et al (2001) Analysis of DNA damage in individual cells. Methods Cell Biol 64:235–249
Ostling O, Johanson KJ (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 123(1):291–298
Ozmen B et al (2007) Relationship between sperm DNA damage, induced acrosome reaction and viability in ICSI patients. Reprod Biomed Online 15(2):208–214
Pasqualotto FF et al (2001) Oxidative stress in normospermic men undergoing infertility evaluation. J Androl 22(2):316–322
Payne JF et al (2005) Redefining the relationship between sperm deoxyribonucleic acid fragmentation as measured by the sperm chromatin structure assay and outcomes of assisted reproductive techniques. Fertil Steril 84(2):356–364
Practice Committee of the American Society for Reproductive Medicine (2013) The clinical utility of sperm DNA integrity testing: a guideline. Fertil Steril 99(3):673–677
Pregl Breznik B, Kovacic B, Vlaisavljevic V (2013) Are sperm DNA fragmentation, hyperactivation, and hyaluronan-binding ability predictive for fertilization and embryo development in in vitro fertilization and intracytoplasmic sperm injection? Fertil Steril 99(5):1233–1241
Rama Raju GA et al (2012) Noninsulin-dependent diabetes mellitus: effects on sperm morphological and functional characteristics, nuclear DNA integrity and outcome of assisted reproductive technique. Andrologia 44 Suppl 1:490–498
Robinson L et al (2012) The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod 27(10):2908–2917
Sailer BL, Jost LK, Evenson DP (1995) Mammalian sperm DNA susceptibility to in situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. J Androl 16(1):80–87

Saleh RA et al (2002) Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa. Fertil Steril 78(6):1215–1224

Saleh RA et al (2003a) Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. Fertil Steril 79:1597–1605

Saleh RA et al (2003b) Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. Fertil Steril 80(6):1431–1436

Sanchez-Martin P et al (2013) Increased pregnancy after reduced male abstinence. Syst Biol Reprod Med 59(5):256–260

Seli E et al (2004) Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. Fertil Steril 82(2):378–383

Sergerie M et al (2005a) Longitudinal study of sperm DNA fragmentation as measured by terminal uridine nick end-labelling assay. Hum Reprod 20(7):1921–1927

Sergerie M et al (2005b) Sperm DNA fragmentation: threshold value in male fertility. Hum Reprod 20(12):3446–3451

Shamsi MB, Kumar R, Dada R (2008) Evaluation of nuclear DNA damage in human spermatozoa in men opting for assisted reproduction. Indian J Med Res 127(2):115–123

Sharbatoghli M et al (2012) Relationship of sperm DNA fragmentation, apoptosis and dysfunction of mitochondrial membrane potential with semen parameters and ART outcome after intracytoplasmic sperm injection. Arch Gynecol Obstet 286(5):1315–1322

Sikka SC, Rajasekaran M, Hellstrom WJ (1995) Role of oxidative stress and antioxidants in male infertility. J Androl 16(6):464–468

Simon L, Lewis SE (2011) Sperm DNA damage or progressive motility: which one is the better predictor of fertilization in vitro? Syst Biol Reprod Med 57(3):133–138

Simon L et al (2010) Clinical significance of sperm DNA damage in assisted reproduction outcome. Hum Reprod 25(7):1594–1608

Simon L et al (2011a) Relationships between human sperm protamines, DNA damage and assisted reproduction outcomes. Reprod Biomed Online 23(6):724–734

Simon L et al (2011b) Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. Fertil Steril 95(2):652–657

Simon L et al (2013) Sperm DNA damage has a negative association with live-birth rates after IVF. Reprod Biomed Online 26(1):68–78

Simon L et al (2014a) Paternal influence of sperm DNA integrity on early embryonic development. Hum Reprod 29(11):2402–2412

Simon L et al (2014b) Comparative analysis of three sperm DNA damage assays and sperm nuclear protein content in couples undergoing assisted reproduction treatment. Hum Reprod 29(5):904–917

Simon L et al (2017a) Sperm DNA damage output parameters measured by the alkaline Comet assay and their importance. Andrologia 49(2)

Simon L et al (2017b) A systematic review and meta-analysis to determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome. Asian J Androl 19(1):80–90

Singh NP, Stephens RE (1998) X-ray-induced DNA double-strand breaks in human sperm. Mutagenesis 13(1):75–79

Singh NP et al (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175(1):184–191

Smit M et al (2010) Decreased sperm DNA fragmentation after surgical varicocelectomy is associated with increased pregnancy rate. J Urol 183(1):270–274

Spanò M et al (1998) The applicability of the flow cytometric sperm chromatin structure assay in epidemiological studies. Asclepios. Hum Reprod 13(9):2495–2505

Spano M et al (2000) Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. Fertil Steril 73(1):43–50

Spano M et al (2005) Exposure to PCB and p, p'-DDE in European and Inuit populations: impact on human sperm chromatin integrity. Hum Reprod 20(12):3488–3499

Speyer BE et al (2010) Fall in implantation rates following ICSI with sperm with high DNA fragmentation. Hum Reprod 25(7):1609–1618

Stevanato J et al (2008) Semen processing by density gradient centrifugation does not improve sperm apoptotic deoxyribonucleic acid fragmentation rates. Fertil Steril 90(3):889–890

Sun JG, Jurisicova A, Casper RF (1997) Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod 56(3):602–607

Tarozzi N et al (2007) Clinical relevance of sperm DNA damage in assisted reproduction. Reprod Biomed Online 14(6):746–757

Tarozzi N et al (2009) Anomalies in sperm chromatin packaging: implications for assisted reproduction techniques. Reprod Biomed Online 18(4):486–495

Tavalaee M, Razavi S, Nasr-Esfahani MH (2009) Influence of sperm chromatin anomalies on assisted reproductive technology outcome. Fertil Steril 91(4):1119–1126

Tesarik J, Greco E, Mendoza C (2004) Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. Hum Reprod 19(3):611–615

Thomson LK, Zieschang JA, Clark AM (2011) Oxidative deoxyribonucleic acid damage in sperm has a negative impact on clinical pregnancy rate in intrauterine insemination but not intracytoplasmic sperm injection cycles. Fertil Steril 96(4):843–847

Tomsu M, Sharma V, Miller D (2002) Embryo quality and IVF treatment outcomes may correlate with different sperm comet assay parameters. Hum Reprod 17(7):1856–1862

Twigg JP, Irvine DS, Aitken RJ (1998) Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. Hum Reprod 13(7):1864–1871

Velez de la Calle JF et al (2008) Sperm deoxyribonucleic acid fragmentation as assessed by the sperm chromatin dispersion test in assisted reproductive technology programs: results of a large prospective multicenter study. Fertil Steril 90(5):1792–1799

Virro MR, Larson-Cook KL, Evenson DP (2004) Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. Fertil Steril 81(5):1289–1295

Xia Y et al (2005) Genotoxic effects on spermatozoa of carbaryl-exposed workers. Toxicol Sci 85(1):615–623

Yang XY, et al. (2011) [Sperm chromatin structure assay predicts the outcome of intrauterine insemination]. Zhonghua Nan Ke Xue 17(11):977–83

Yang XY, et al. (2013) [Impact of sperm DNA fragmentation index and sperm malformation rate on the clinical outcome of ICSI]. Zhonghua Nan Ke Xue 19(12):1082–1086

Zeyad A et al (2018) Relationships between bacteriospermia, DNA integrity, nuclear protamine alteration, sperm quality and ICSI outcome. Reprod Biol 18(1):115–121

Zhang X, Gabriel MS, Zini A (2006) Sperm nuclear histone to protamine ratio in fertile and infertile men: evidence of heterogeneous subpopulations of spermatozoa in the ejaculate. J Androl 27(3):414–420

Zhao J et al (2014) Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Fertil Steril 102(4):998–1005 e8

Zheng WW et al (2018) Sperm DNA damage has a negative effect on early embryonic development following in vitro fertilization. Asian J Androl 20(1):75–79

Zini A, Sigman M (2009) Are tests of sperm DNA damage clinically useful? Pros and cons. J Androl 30(3):219–229

Zini A et al (2001) Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. Fertil Steril 75(4):674–677

Zini A et al (2005) Potential adverse effect of sperm DNA damage on embryo quality after ICSI. Hum Reprod 20(12):3476–3480

Zini A et al (2008) Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. Hum Reprod 23(12):2663–2668

Oxidative Damage to Sperm DNA: Attack and Defense

7

Joel R. Drevet and R. J. Aitken

Abstract

Due to its particular "silent" metabolic state, without transcription or translation, and a low level of cytosolic protective activities, mature sperm is a cellular type of aerobic organisms particularly at risk of oxidative damage. Despite the efforts of the male genital tract to treat this problem, a subcellular compartment of the sperm, the nucleus, and consequently, the paternal DNA cannot be effectively protected. There is an accumulation of evidence that oxidative damage to sperm DNA is quite common in male infertilities/subfertilities with potential harmful impacts on reproductive success, including the transgenerational inheritance of a paternal chromosomal lot carrying mutations.

Keywords

Reactive oxygen species · Male infertility · Lipid peroxidation · Oxidative DNA damage · DNA repair

Observations that the sperm cells of a large proportion of infertile men have high levels of reactive oxygen species (ROS) and that their antioxidant content is low compared to fertile men have been reported for several years (Aitken et al. 2012; Henkel 2011; Lewis et al. 1995). At present, it is fairly commonly accepted that oxidative stress interferes with the fertilization potential of sperm, damages DNA, and even affects the epigenetic profile of sperm cells (Aitken et al. 2014; Aitken 2016). Despite this recognized situation, oxidative DNA lesions in semen are still neglected, even though they could have significant clinical consequences for male fertilizing potential, optimal embryonic development, and the health and well-being of the offspring. In this chapter, we first summarize why the nucleus and DNA of sperm cells are so sensitive to oxidative damage, when and where sperm cells are at risk of oxidative damage to DNA, where this damage is concentrated in the sperm cell, whether oxidative damage can also affect epigenetic information transmitted by sperm cells, and what are the possible consequences when a sperm with an oxidatively damaged nucleus fertilizes an oocyte.

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In a second section, we present how sperm cells are protected from oxidative DNA damage and discuss the relevance of a therapeutic strategy focused on oral antioxidant supplementation.

Why Spermatozoa DNA Is Susceptible to Oxidative Damage?

Due to its particular architecture and biology, the sperm cell is at greater risk of oxidative damage to DNA than any other cell. The main reason for this vulnerability is the spermatozoon's inability to fight oxidative insults because the cell is "silent" by nature (it does not transcribe and, despite some reports to the contrary, is generally assumed to be translationally inactive or to translate poorly). In this respect, the spermatozoa cannot mount a stress response as any other cell would. In addition, the sperm cell is generally lacking in cytosolic antioxidant protective capacity in the form of enzymes (such as catalase, superoxide dismutase, and glutathione peroxidase) and nonenzymatic small molecular mass scavengers (such as polyamines, taurine, lipoic acid, and vitamins C and E) that counteract ROS because uniquely, this cell type divests itself of most of its cytoplasm prior to its release from the germinal epithelium. We add to this picture additional facts such as the following: 1) having lost most of its cytosol, the nuclear compartment of the sperm is readily exposed to environmental ROS; 2) the plasma membrane of the sperm cell has a particular lipid composition rich in polyunsaturated fatty acids (PUFAs) that are very susceptible to a peroxidative process that generates even more aggressive ROS and other toxic metabolites (Jones et al. 1978; Jones et al. 1979); and 3) the sperm cells themselves produce ROS. It is therefore not surprising to note that the sperm nucleus and its DNA should be at risk of oxidative damage.

As a consequence of such factors, spermatozoa under conditions of oxidative stress easily generate cytotoxic lipid aldehydes such as malondialdehyde (MDA) and, above all, 4-hydroxynonenal (4-HNE) as a result of membranous lipid peroxidation (Lenzi et al. 1996; Aitken et al. 2012; Ayala et al. 2014; Moazamian et al. 2015). 4-HNE is a very toxic lipid aldehyde that has been shown to efficiently alkylate proteins, induce DNA damage, stimulate the production of inflammatory markers and, also, interfere with mitochondria function leading to more ROS generation entertaining and amplifying the situation of oxidative stress in spermatozoa as in any other cells (Aitken et al. 2012; Shoeb et al. 2014). A very recent review stresses the importance of 4-HNE production in male infertility (Walters et al. 2018) and how it could eventually be circumvented via either antioxidant supplementation or a direct action on lipoxygenase enzymes that contribute to 4-HNE such as the arachidonate 15-lipoxygenase (ALOX15). It turns out that ALOX15 could well be a pertinent target to manipulate in order to decrease sperm oxidative damage as it was shown elsewhere to mediate oxidative stress in mouse spermatozoa (Brütsch et al. 2015).

Where Are Sperm Cells at Risk of DNA Oxidative Damage?

In a physiological context, from its genesis in the germinal epithelium to the moment of fertilization in the female genital tract, spermatozoa may experience oxidative attacks. Spermatozoa themselves are good producers of ROS, especially hyperactivated ones at the onset of capacitation since ROS are known to facilitate this process (Aitken et al. 1995. Aitken et al. 1998b; Aitken and Curry 2011). Briefly, because ROS can inhibit tyrosine phosphatase activity, promote cAMP generation, and facilitate sperm plasma membrane cholesterol efflux via the oxidation in oxysterols, they are key actors in this sperm maturation process which is an essential requirement for fertilization to occur (Aitken 1997; Leclerc et al. 1997; Aitken et al. 1998a; Lewis and Aitken 2001; O'Flaherty et al. 2006; Awda and Buhr 2010; Aitken and Curry 2011; Aitken 2011; Brouwers et al. 2011; Donà et al. 2011). The dark side of this process is the self- damaging effect of ROS produced by capacitated spermatozoa affecting the plasma membrane, mitochondria

function, and ultimately the sperm DNA. Thus, even in a physiological context, mature spermatozoa may endure DNA oxidative damage especially when the condensation of the sperm nucleus is not optimized (De Iuliis et al. 2009).

Outside this physiological situation, in both men and women, oxidative stress within the genital tract can have several origins, being either grossly systemic or local. Inflammatory and infectious situations are classically associated with increasing level of ROS in the vicinity of sperm cells since hydrogen peroxide (H2O2), a harmful ROS, is a key player in the resolution of such situations. It was therefore not surprising to see in clinical practice that leukocytospermia is strongly associated with sperm DNA oxidative damage (Vorilhon et al. 2018). To a lesser extent, asthenozoospermia was also found associated with increased sperm DNA oxidative damage in line with what could be expected in situations of defective mitochondrial functions and superoxide anion leakage. Exposures to environmental toxicants, as well as environmental physical and mechanical stressors such as radiation and heat, are classical sources of oxidative stress that spermatozoa could face (Houston et al. 2016; Houston et al. 2018). A non-balanced diet and many medical treatments are also sources of oxidative stress that could easily affect spermatozoa during their life span whether it is during their generation in the testis or during their post-testicular life. The multiplicity of situations leading to systemic or local oxidative stress around sperm cells whether there are physiological, pathological, or even artificial as in the case of assisted reproductive technologies (ART) lets us suspect that oxidative DNA damage should be a rather common feature of this particular cell type (Aitken 2016). This is exactly what was observed in a clinical context at the Clermont-Ferrand public infertility clinic where a cohort of males entering the andrology lab for fertility assessment or/and an ART program was monitored for the presence of 8-OHdG**,** a precocious marker of sperm DNA oxidative damage. The result of such a survey was that 2/3 of men showed moderate to high levels of 8-OHdG whatever the origin of the couple infertility and whatever their individual fertility status as measured by the standard WHO parameters (Cooper et al. 2010) addressing sperm count, morphology, and motility (Vorilhon et al. 2018). If this local observation reflects on the worldwide situation, sperm DNA oxidative damage would appear to be a very common form of insult to the paternal DNA; even more frequent than sperm DNA fragmentation which is estimated to involve about 1 out of 7 males from couple having difficulties to conceive (Giwercman et al. 2010).

Sperm Nuclear Oxidative Damage and Its Developmental Impacts (Putative Mutational Risks and Transgenerational Effects)

Sperm Nuclear and DNA Damage

Oxidative damage to the sperm nucleus can take multiple forms. Firstly, oxidative alterations can promote nuclear decondensation and DNA fragmentation. Nuclear DNA is, to a large extent, protected from oxidative damage because it has been condensed to the point of crystallization as a result of the remodeling of sperm chromatin that occurs during spermiogenesis. During this process, about 85% of the human sperm histones are eliminated and replaced by small basic proteins, rich in arginine, called protamines. These molecules, due to their net positive charge, are able to neutralize the negative charges carried by phosphate groups in the DNA skeleton and, by overcoming electrostatic repulsion between adjacent DNA strands, allow the high level of compaction of the DNA typical of the spermatic nucleus. The DNA of the mitochondria of the semen is not conditioned in this way and is therefore more vulnerable to oxidative attacks (Kocer et al. 2015). This may not be relevant in terms of embryonic developmental potential because paternal mitochondria are destroyed after fertilization to make way for the maternal mitochondrial line. However, it should be noted that the increased vulnerability of mitochondrial DNA to oxidative attack provides an opportunity to monitor such oxidative DNA damage in spermatozoa for a diagnostic perspective (Sawyer et al. 2003).

It is not entirely clear at this stage whether the oxidation of sperm DNA and its fragmentation are closely correlated. Obviously, the fragmentation of sperm DNA, to some extent, has an oxidative origin since the high concentration of ROS (especially hydrogen peroxide) has the ability to cause single and double strand breaks (SSB, DSB) in most cell types. Furthermore, human and mouse spermatozoa have been shown to lack a fully functional DNA repair machinery, as they only possess the first enzyme in the base-excision repair pathway, OGG1, which removes the oxidized base leaving a vulnerable abasic site. The second step in this base excision repair pathway, which utilizes APE1 to create a nick in the phosphodiester backbone of the AP site in readiness for the insertion of a new base, is missing from these cells. However, APE1 and other downstream constituents of the base-excision repair pathway are present in abundance within the oocyte. It has therefore been concluded that the effective repair of oxidative DNA damage in spermatozoa involves a collaboration between the male and female germ lines; the spermatozoon removing the oxidized base, while the oocyte completes the repair process, through the insertion of a new base into the damaged site, following fertilization (Smith et al. 2013a, b). Therefore, the oxidation of sperm DNA could lead to paternal DNA fragmentation and other forms of defect, as a result of inadequate or aberrant oocyte repair after fertilization, a situation that cannot be measured by solely evaluating the fragmentation of the sperm nucleus in a semen sample.

When H2O2 levels around sperm cells are very high, it could directly lead to DNA breaks, and, in that sense when sperm DNA fragmentation is recorded, it may be partly due to excessive DNA oxidation. However, it is important to keep in mind that absence of DNA fragmentation in the semen should not be interpreted as absence of oxidative DNA alterations, a shortcut frequently made in the clinic, because DNA oxidation, if not monitored, may be moderate to high without leading to dramatic sperm DNA fragmentation. This situation, in which sperm DNA oxidation is seen to be disconnected from sperm DNA fragmentation, has been clearly demonstrated in several mouse models of post-testicular oxidative stress in which high levels of DNA oxidation were recorded in cauda epididymis sperm without any evidence of increased DNA fragmentation (Chabory et al. 2009; Noblanc et al. 2013; Kocer et al. 2015). In these models where spermatozoa were challenged by mild reducing condition, low nuclear condensation and/or increased susceptibility to nuclear decondensation was recorded (Noblanc et al. 2013). Thus, nuclear oxidation and DNA fragmentation of semen are two conditions that should be considered separately to avoid misleading diagnoses.

On the contrary, sperm nuclear condensation and oxidation are quite well-associated parameters. This is due to the fact that sperm nuclear compaction is completed in the epididymis via an oxidative process involving the creation of inter- and intramolecular cross bridges between, and within, nuclear protamines. These events result in the further condensation of the sperm nucleus ultimately locking it into a compacted state. Disulfide bridging of sperm protamines is enabled by the presence of a well-controlled luminal concentration of hydrogen peroxide in the epididymis (Drevet 2006) and enzymes (protein disulfide isomerase and glutathione peroxidase) in the sperm nucleus (Chabory et al. 2009). This finely controlled process can be challenged by systemic or local factors, which can lead to the production of ROS, as mentioned above. There is, therefore, a delicate balance between physiological oxidation which will allow optimal nuclear compaction of the sperm and harmful nuclear oxidation. Excessive production of ROS by mature sperm cells, in addition to affecting sperm membranes and amplifying ROS production, may transiently increase nuclear condensation of sperm cells but will quickly promote spontaneous DNA fragmentation resulting in nuclear decondensation and increased oxidative damage to DNA and proteins associated with chromatin.

Despite the compaction of nuclear DNA into dense doughnut-shaped structures called toroids (which comprise 50 to 100 kb of DNA), there are still areas of the mammalian nuclear genome that are vulnerable to oxidative attacks. These corre-

spond to the less dense genomic regions still organized into nucleosomes (where only 146 bp of DNA is associated *per* histone octamer). Depending on the species looked at, the share of paternal DNA still loosely compacted in nucleosomes is very different. In the mouse, only 1 to 2% of histones are left after spermiogenesis, while in the human this figure goes up to 12 to 15% (although there is some controversy about it with some authors claiming that it could be less and closer to 5 to 7%). Whatever the precise level of histone retention, these findings reveal that the human sperm nucleus is rather less compacted than the mouse counterpart since there are a lot more chromosomal regions maintained in nucleosomal arrangements making them more prone to suffer from oxidative DNA damage (De Iuliis et al. 2009; Noblanc et al. 2013). This concept was supported by the finding that human sperm DNA oxidation is a very common phenomenon affecting 66% of the men tested, whether they were classified fertile or infertile following the WHO criteria (Vorilhon et al. 2018). A fundamental difference between human and mouse spermatozoa was also suggested by the fact that 8-OHdG oxidative marks were found to affect the entire nuclear compartment in human sperm, while the regions affected by oxidative damage in mouse spermatozoa were a lot more discrete (Vorilhon et al. 2018; Noblanc et al. 2013; Kocer et al. 2015; Champroux et al. 2018a). Experimentally, a quantitative PCR technique has been used to demonstrate conclusively that the nuclear genes of human spermatozoa are more vulnerable to oxidative attack than the murine equivalent (Bennetts and Aitken 2005). In the mouse nucleus, it has recently been shown that persistent regions of histone-bound sperm DNA belong to two categories (Johnson et al. 2011; Noblanc et al. 2013). In the first category, nucleosomes were found at irregular intervals in large regions of DNA associated with protamines within nuclear toroids. In the second category, histone-rich DNA regions have also been found in the small DNA strands connecting protamine toroids to one another – the so- called interlinker regions (Kocer et al. 2015). It is interesting to note that these short DNA strands were also attached to the nuclear matrix of the sperm, anchoring the chromosomes at the periphery and at the base of the sperm head (Noblanc et al. 2013). The peripheral location of these nucleosomal nuclear domains and their less condensed nature make them particularly vulnerable to oxidative DNA damage (Noblanc et al. 2013). In addition, because of the way sperm chromosomes are organized in the sperm head, some chromosomes have interconnected regions that are more vulnerable to damage than others (Noblanc et al. 2013; Kocer et al. 2015). From a quantitative perspective, the oxidation of DNA bases is not a minor problem. As an illustration, in the mouse models of mild post-testicular oxidative damage we analyzed, even though the level of luminal epididymal oxidative stress is rather low (Chabory et al. 2009; Noblanc et al. 2013), the numbers of oxidized regions on mouse chromosomes were considerable. In one model, more than 15,000 DNA regions (with an average length of 300 bp each) were found significantly oxidized, including a set of 1000 highly oxidized regions (Kocer et al. 2015). This situation has to be compared with the observation that in a wild-type mouse, less than 60 sperm chromosomal regions were found significantly oxidized on average (Kocer et al. 2015). A theoretical calculation estimates that, in the mildly oxidized transgenic context, about one million guanine residues could be oxidized and will have to be replaced by the BER pathway of the oocyte. Moreover, this is only the visible tip of the iceberg since while guanine is the most sensitive base to oxidation, it is not the only base affected since all other bases can suffer oxidative damage. The replacement of all these oxidized bases puts great pressure on the oocyte repair systems, increasing the risk of errors or/and the impossibility of repair. In the absence of 8-OHdG repair, an increased risk of de novo mutations by transversion (following Hoogsteen base pairing between 8-oxoG with adenine) in embryonic cells and their transmission in offspring will occur (Ohno et al. 2014). These issues can have enormous implications for the optimal completion of the embryonic developmental program and, potentially, the health of the offspring and beyond.

Oxidative Alteration of the Sperm Nucleus and its Possible Consequences on Epigenetic Information

In addition to DNA, there are reasons to suspect that the proteins and RNA components of the sperm nucleus may also be affected by oxidation, thus altering the epigenetic information carried by the paternal nucleus (Champroux et al. 2018b).

Oxidation of Nuclear Proteins

Oxidation of sperm protamines does not seem to be a major problem because these proteins will be quickly removed from the paternal nucleus after fertilization. On the other hand, oxidative alteration of the sperm DNA regions still bound to nucleosomes will activate the oocyte BER pathway that may not faithfully replace the appropriate histone/histone variants in these sites of oxidative attack. It is therefore not difficult to imagine that changes in the composition of paternal nucleosomes (the so-called "histone" code) may lead to subtle changes in the expression of the concerned genes in the developing embryo. This aspect has not yet been studied.

Oxidation of Methylated Cytosine Residues

Another obvious example where alterations in the nuclear oxidation of sperm can influence epigenetic information concerns the different chemical modifications of the conventional methylcytosine (meC) mark. Immediately after fertilization, there is a particular cycle of demethylation that mainly concerns the paternal nucleus (McLay and Clarke 2003). Demethylation begins with the oxidation of meC to a hydroxy methylcytosine residue (hmeC) by the action of the TET enzyme machinery. HmeC will then be transformed into formylmethyl-cytosine (fmC), carboxymethyl-cytosine (camC), and finally cytosine (Wu and Zhang, 2017). The activity of TET is nothing less than an oxidation of meC. Spontaneous post-testicular oxidative alterations of mature sperm cells can therefore generate excessive transformations of meC into hmeC (M. Gentil, unpublished data), possibly modifying the kinetics and landscape of paternal DNA demethylation. Considering the fact that some regions of the male pronucleus are meant to escape the post-fertilization demethylation process, it is likely that if the meC pattern of these regions has been transformed into hmeC, a significant change in the pattern of imprinting within the male genome will result. The involvement of oxidative stress in CpG mutagenesis in the male germ line could therefore have a profound effect on the expression of paternally imprinted genes later in development. In addition, it has also been reported that the presence of 8-OHdG in a CpG doublet may impair the methylation process of the adjacent cytosine by interfering with methyl transferase activity, again leading to changes in the pattern of paternally imprinted regions (Wachsman 1997; Wu and Ni, 2015).

Oxidation of Sperm-Associated RNAs

Recently, it has been reported that the contribution of sperm to the embryo is not limited to paternal DNA. Spermatozoa also provides a complex set of noncoding short and long RNAs (ncRNAs) that have been assigned regulatory functions. These ncRNAs constitute another form of paternal epigenetic inheritance. In 2016, two research groups reported that male sperm cells under specific environmental conditions such as unbalanced nutrition or behavioral stress have different RNA loads that are, in turn, responsible for transmission of the father's phenotype to the offspring (Sharma et al. 2016, Chen et al. 2016). Current unpublished studies from our team suggest that post-testicular (i.e., epididymal) exposure to moderate oxidizing conditions alters the profile of sperm ncRNAs, which may potentially affect embryonic development.

The consequences of such oxidative damage to sperm DNA and RNA for the health and well-being of the offspring, are extremely important issues that still needs to be assessed. One impor-

tant feature to keep in mind is that sperm DNA oxidation (eventually leading to sperm DNA fragmentation) does not, of itself, impair the fertilizing ability of spermatozoa, particularly when ICSI (intra-cytoplasmic sperm injection) is used as the insemination procedure (Aitken et al. 1998a, b). Therefore, it is quite possible that a sperm cell with high levels of oxidative DNA damage could fertilize an oocyte and disrupt normal embryonic development. We may see the consequences of this mechanism in action in the high rates of cancer observed in the offspring of heavy male smokers (Lee et al. 2009). Unfortunately, we may see other examples of this association with the unrestricted use of ICSI. Already, there is an increase in the rate of spontaneous abortions associated with ICSI, but not with IVF, which may be partly due to oxidative damage to the sperm nucleus (Zhao et al. 2014). The risk of seeing further impacts on fetuses that develop into adults should be considered.

Endogenous and Exogenous Defense of Spermatozoa Against Oxidative DNA Damage

As mentioned above, mature sperm cells are not very well equipped to counteract the harmful effects of ROS. The low cytosol content, the particular lipid composition of plasma membranes that promote the production of ROS in the event of oxidation (Moazamian et al. 2015), and their inability to mount a genetically mediated antioxidant stress response make them easily vulnerable to oxidative damage. They therefore depend essentially on their environment to protect themselves from oxidative stress. As the focus of this chapter is on oxidative damage to the DNA of sperm cell, the following section will focus on the intrinsic and extrinsic arsenal of enzymes and scavengers protecting sperm from nuclear oxidative alterations. The first factors that come to mind when we think of the nuclear protection of sperm against oxidative damage are protamines. Protamines with their high content of cysteine residues and their associated thiol groups are involved in protecting sperm DNA from oxidative damage (Liang et al. 1999).

Although, during epididymal maturation, many thiol groups of protamines are oxidized to disulfide bonds, which stiffens the organization of the toroids and encloses the nucleus of the sperm in a condensed state, there are still free thiol groups that have the ability to mitigate oxidative attacks by blocking the action of ROS. Zinc also contributes directly to the nuclear protection of sperm. Zinc is incorporated into the nucleus of the sperm cell during spermiogenesis and the zinc content of the sperm chromatin is estimated to be one to one with protamine (Bjorndhal and Kvist 2010). In its conventional tertiary zinc finger structure, zinc mobilizes cysteine and histidine residues to form inter- and intramolecular zinc bridges (as is the case between proteins and DNA). By blocking thiol groups on protamines, zinc can therefore, to some extent, help protect them from oxidation to disulfide bridges, as a salt bridge involving zinc, thiol groups of histidine cysteine, and imidazole groups are rather stable and are not very sensitive to oxidation or reduction (Bjorndhal and Kvist 2010).

Apart from the anti-ROS action of protamine and zinc, sperm cells are poorly equipped to fight insults caused by ROS. It is the role of the epididymal environment and, later, of the seminal plasma, to ensure this protection. To this end, it has been demonstrated that the mouse caput epididymis synthesizes and secretes a significant amount of a H2O2 recycling enzyme belonging to the glutathione peroxidase family. The importance of this activity in protecting sperm (and in particular the paternal nucleus) from oxidative damage has been confirmed by observations that, in transgenic mice without such activity, sperm stored in the cauda epididymis have a high level of DNA oxidation. In addition, when transgenic males were mated with WT female mice, there was an increase in miscarriages, embryonic, and congenital malformations that were attributed to the oxidized state of paternal DNA and probably to the oocyte's inability to successfully repair such damage (Chabory et al. 2009; Aitken et al. 2009). In addition to the secreted caput GPx, the mouse cauda epididymis also secretes a plasma-

type GPX that contributes to ROS recycling events in the sperm storage compartment. Although this epididymal protection of sperm in transit provided by GPx has been found in several mammalian species, it does not fully apply to the human model which appears to rely more on peroxiredoxins (PRDX) than GPx (Fernandez and O'Flaherty 2018). Regardless of the type of ROS recycling enzyme used, it should be noted that maintaining an optimal ROS balance in the testis and particularly in the epididymis is of great importance. This protection is all the more important in the epididymis because at this stage in the life of sperm cells, they are transcriptionally and translationally silent and have no way of countering the harmful actions of ROS. One of the few responses that a sperm cell can mount in response to oxidative damage is apoptosis. Sperm cells retain the possibility of committing suicide through an almost classic apoptotic process that will also generate ROS (Koppers et al. 2011). In addition to the enzymatic recycling activities of ROS, which also include catalase and SOD2, epididymal and seminal fluids provide many nonenzymatic antioxidant molecules against toxic oxygen-derived molecules. Conventional hydrophilic and lipophilic scavengers, including vitamins, polyamines, carnitine, taurine, and trace elements such as selenium, are present in epididymal and seminal fluids and contribute to the protection of sperm cells.

Whether or not it is relevant to enhance the antioxidant capacity of these fluids through antioxidant supplementation has been the subject of a debate that has stirred (and continues to stir) the scientific and clinical community (for a recent review see: Aitken et al. 2019). The rationale is logical, as it is clear that infertile men often have high levels of ROS and/or reduced antioxidant capacity in their seminal plasma and spermatozoa. Several oral antioxidant supplementation trials have been conducted over the years, but we are still waiting for a rigorous, carefully designed, large-scale, multicenter, double-blind, placebo-controlled, and randomized trial using rigorous selection criteria for both male and female patients. In addition, the dose of antioxidants, the choice of molecules to be used alone or in combination, and the duration of treatment are all aspects to be taken into serious consideration. A meta-analysis conducted in 2011 by Gharagozloo and Aitken, in which 20 clinical studies were rigorously evaluated, showed with little doubt that antioxidant supplementation reduces oxidative stress in semen and increases mobility in asthenozoosperm patients (Gharagozloo and Aitken 2011).

It remains to be seen how this translates into an improvement in the pregnancy rate. Indeed, because the latter is influenced by so many different factors, such confirmation may be a long time coming since it would require a very large and expensive trial to determine whether oral antioxidant supplements can significantly influence fertility. It may be more realistic to focus on whether such supplements are able to reverse biochemical markers of oxidative stress (lipid aldehyde formation, oxidative DNA damage, etc.…) in a sustained and reliable manner. We might be optimistic about the potential success of such trials because a carefully designed oral antioxidant supplementation has recently been reported to protect rodent sperm DNA from the harmful effects of acutely induced or chronic ROS and to result in improved reproductive success (Gharagozloo et al. 2016).

Despite the appeal of oral antioxidant supplementation to reduce oxidative damage to sperm DNA, caution should be exercised in developing these therapeutic approaches. If oxidative stress to the DNA of the sperm cell is a problem, so too is reductive stress (which may be the result of excess antioxidant) an undesirable situation (Cohen-Bacrie et al. 2009, Menezo et al. 2010; Gharagozloo and Aitken 2011). This is easily understandable considering that epididymal sperm maturation partly uses oxidative events (disulfide bridging of protamines) to stabilize the sperm nucleus and lock it into an optimally condensed state. If sperm cells are exposed to an excess of antioxidant, the risk is to promote decondensation of the nucleus which potentially has an impact on its sensitivity to other damage and also on the motility of the cell (if the sperm head is larger). Therefore, it is of paramount importance before any attempt at exogenous anti-

oxidant treatment to assess the level of oxidation of the DNA in the patient's sperm cell. We recently reported the optimization of a test using flow cytometry and an antibody against the oxidized guanine residue 8-OHdG that allowed us to determine a pathological threshold for the oxidation of human spermatozoa DNA in the clinic (Vorilhon et al. 2018). The systematic use of such a test in combination or not with a test designed to assess sperm DNA fragmentation would allow the selection of patients who would be best able to benefit from antioxidant supplementation.

Conclusion

Evidence from a wide variety of species over several decades has clearly demonstrated that the male reproductive system is very vulnerable to oxidative stress. When the ROS attack is severe, we may see a disruption of spermatogenesis or a suppression of sperm fertilizing potential, depending on where in the process of sperm production and maturation the oxidative stress occurs. Because sperm DNA is more vulnerable to oxidative stress than the cellular machinery controlling fertilization (Aitken et al. 1998a, b), it is also possible to arrive at a situation with lower levels of oxidative stress, wherein sperm production and fertilizing potential are not affected but the integrity of sperm DNA and RNA is seriously compromised. Under these circumstances, fertilization may be achieved with DNA/RNA damaged spermatozoa, with possible consequences for the developmental normality of the embryo and long-term health trajectory of the offspring. This is a particularly important concern in relation to the safety of assisted conception procedures, especially when ICSI is used as the insemination strategy (Aitken et al. 2018). The data from animal models are incontrovertible in demonstrating that oxidative damage to the male germ line can have detrimental impacts on embryonic development and offspring health as a consequence of such mechanisms; there is now an urgent need to address this question in our own species.

Authors' Roles J.R.D. drafted the article, which was then edited and revised by R.J.A. Both authors approved the final version of this article.

Conflict of Interest J.R.D. and R.J.A. are scientific advisors of a US-based biotech company (Celloxess LLC, New Jersey, USA) which has a commercial interest in the detection and treatment of oxidative stress.

References

Aitken RJ (1997) Molecular mechanisms regulating human sperm function. Mol Hum Reprod 3(3):169–173

Aitken RJ (2011) The capacitation-apoptosis highway: oxysterols and mammalian sperm function. Biol Reprod 85(1):9–12. https://doi.org/10.1095/biolreprod.111.092528

Aitken RJ (2016) Oxidative stress and the ethiology of male infertility. J Assist Reprod Genet 33(12):1691–1692

Aitken RJ (2018) Not every sperm is sacred; a perspective on male infertility. Mol Hum Reprod 24(6):287–298

Aitken RJ, Curry BJ (2011) Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. Antioxid Redox Signal 14(3):367–381. https://doi.org/10.1089/ars.2010.3186

Aitken RJ, Paterson M, Fisher H et al (1995) Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. J Cell Sci 108:2017–2025

Aitken RJ, Gordon E, Harkiss D et al (1998a) Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol Reprod 59(5):1037–1046

Aitken RJ, Harkiss D, Knox W et al (1998b) A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. J Cell Sci 111:645–656

Aitken RJ, De Iuliis GN, McLachlan RI (2009) Biological and clinical significance of DNA damage in the male germ line. Int J Androl 32(1):46–56. https://doi.org/10.1111/j.1365-2605.2008.00943.x

Aitken RJ, Jones KT, Robertson SA (2012) Reactive oxygen species and sperm function in sickness and in health. J Androl 33(6):1096–1106. https://doi.org/10.2164/jandrol.112.016535

Aitken RJ, Smith TB, Jobling MS et al (2014) Oxidative stress and male reproductive health. Asian J Androl 16(1):31–38. https://doi.org/10.4103/1008-682X

Aitken RJ, De Iuliis G, Drevet JR (2019) Role of oxidative stress in the etiology of male infertility and the potential therapeutic value of antioxidants. In: Henkel R, Samanta L, Agarwal A (eds) Oxidants, antioxidants, and impact of the oxidative stress in male reproduction. Academic Press-Elsevier, Oxford, UK, pp 90–100

Awda BJ, Buhr MM (2010) Extracellular signal-regulated kinases (ERKs) pathway and reactive oxygen species regulate tyrosine phosphorylation in capacitating boar spermatozoa. Biol Reprod 83(5):750–758. https://doi.org/10.1095/biolreprod.109.082008

Ayala A, Muñoz MF, Argüelles S (2014) Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxidative Med Cell Longev 2014:1. https://doi.org/10.1155/2014/360438

Bennetts LE, Aitken RJ (2005) A comparative study of oxidative DNA damage in mammalian spermatozoa. Mol Reprod Dev 71(1):77–87

Björndahl L, Kvist U (2010) Human sperm chromatin stabilization: a proposed model including zinc bridges. Mol Hum Reprod 16(1):23–29. https://doi.org/10.1093/molehr/gap099

Brouwers JF, Boerke A, Silva PF et al (2011) Mass spectrometric detection of cholesterol oxidation in bovine sperm. Biol Repord 85(1):128–136. https://doi.org/10.1095/biolreprod.111.091207

Brütsch SH, Wang CC, Li L, Stender H et al (2015) Expression of inactive glutathione peroxidase 4 leads to embryonic lethality, and inactivation of the Alox15 gene does not rescue such knock-in mice. Antioxid Redox Signal 22(4):281–293. https://doi.org/10.1089/ars.2014.5967

Chabory E, Damon C, Lenoir A et al (2009) Epididylis seleno-independent glutathione peroxidase 5 maintains sperm DNA integrity in mice. J Clin Invest 119(7):2074–2085. https://doi.org/10.1172/JCI38940

Champroux A, Damon-Soubeyrand C, Goubely C et al (2018a) Nuclear integrity but not Topology of mouse sperm chromosome is affected by oxidative DNA damage. Genes (Basel);9(10). pii: E501. https://doi.org/10.3390/genes9100501

Champroux A, Cocquet J, Henry-Berger J et al (2018b) A decade of exploring the mammalian sperm Epigenome: paternal epigenetic and transgenerational inheritance. Front Cell Dev Biol 6:50. https://doi.org/10.3389/fcell.2018.00050

Chen Q, Yan M, Cao Z et al (2016) Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. Science 351(6271):397–400. https://doi.org/10.1126/science.aad7977

Cohen-Bacrie P, Belloc S, Ménézo YJ et al (2009) Correlation between DNA damage and sperm parameters: a prospective study of 1,633 patients. Fertil Steril 91(5):1801–1805. https://doi.org/10.1016/j.fertnstert.2008.01.086

Cooper TG, Noonan E, von Eckardstein S et al (2010) World Health Organization reference values for human semen characteristics. Hum Reprod Update 16(3):231–245. https://doi.org/10.1093/humupd/dmp048

De Iuliis GN, Thomson LK, Mitchell LA et al (2009) DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2′-deoxyguanosine, a marker of oxidative stress. Biol Reprod 81(3):517–524. https://doi.org/10.1095/biolreprod.109.076836

Donà G, Fiore C, Andrisani A et al (2011) Evaluation of correct endogenous reactive oxygen species content for human sperm capacitation and involvement of the NADPH oxidase system. Hum Reprod 26(12):3264–3273. https://doi.org/10.1093/humrep/der321

Drevet JR (2006) The antioxidant glutathione peroxidase family and spermatozoa: a complex story. Mol Cell Endocrinol 250(1–2):70–79

Fernandez MC, O'Flaherty C (2018) Peroxiredoxin 6 is the primary antioxidant enzyme for the maintenance of viability and DNA integrity in human spermatozoa. Hum Reprod 33:1394. https://doi.org/10.1093/humrep/dey221

Gharagozloo P, Aitken RJ (2011) The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. Hum Reprod 26:1628–1640

Gharagozloo P, Gutiérrez-Adán A, Champroux A et al (2016) A novel antioxidant formulation designed to treat male infertility associated with oxidative stress: promising preclinical evidence from animal models. Hum Reprod 31(2):252–262. https://doi.org/10.1093/humrep/dev302

Giwercman A, Lindstedt L, Larsson M et al (2010) Sperm chromatin structure assay as an independent predictor of fertility in vivo: a case-control study. Int J Androl 33(1):e221–e227. https://doi.org/10.1111/j.1365-2605.2009.00995

Henkel RR (2011) Leukocytes and oxidative stress: dilemma for sperm function and male fertility. Asian J Androl 13(1):43–52. https://doi.org/10.1038/aja.2010.76

Houston BJ, Nixon B, King BV et al (2016) The effects of radiofrequency electromagnetic radiation on sperm function. Reproduction 152(6):R263–R276

Houston BJ, Nixon B, Martin JH et al (2018) Heat exposure induces oxidative stress and DNA damage in the male germ line. Biol Reprod 98(4):593–606. https://doi.org/10.1093/biolre/ioy009

Johnson GD, Lalancette C, Linnemann AK et al (2011) The sperm nucleus: chromatin, RNA, and the nuclear matrix. Reproduction 141(1):21–36. https://doi.org/10.1530/REP-10-0322

Jones R, Mann T, Sherins RJ (1978) Adverse effects of peroxidized lipid on human spermatozoa. Proc R Soc Lond B Biol Sci 201(1145):413–417

Jones R, Mann T, Sherins R (1979) Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. Fertil Steril 31(5):531–537

Kocer A, Henry-Berger J, Noblanc A et al (2015) Oxidative DNA damage in mouse sperm chromo-

somes: Size matters. Free Radic Biol Med 89:993–1002. https://doi.org/10.1016/j.freeradbiomed

Koppers AJ, Mitchell LA, Wang P et al (2011) Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxidative DNA damage in human spermatozoa. Biochem J 436(3):687–698. https://doi.org/10.1042/BJ20110114

Leclerc P, de Lamirande E, Gagnon C (1997) Regulation of protein-tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. Free Radic Biol Med 22(4):643–656

Lee KM, Ward MH, Han S et al (2009) Paternal smoking, genetic polymorphisms in CYP1A1 and childhood leukemia risk. Leuk Res 33:250–258

Lenzi A, Picardo M, Gandini L et al (1996) Lipids of the sperm plasma membrane: from polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. Hum Reprod Update 2(3):246–256

Lewis B, Aitken RJ (2001) A redox-regulated tyrosine phosphorylation cascade in rat spermatozoa. J Androl 22(4):611–622

Lewis SE, Boyle PM, McKinney KA et al (1995) Total antioxidant capacity of seminal plasma is different in fertile and infertile men. Fertil Steril 64(4):868–870

Liang R, Senturker S, Shi X et al (1999) Effects of Ni(II) and Cu(II) on DNA interaction with the N-terminal sequence of human protamine P2: enhancement of binding and mediation of oxidative DNA strand scission and base damage. Carcinogenesis 20(5):893–898

McLay DW, Clarke HJ (2003) Remodelling the paternal chromatin at fertilization in mammals. Reproduction 125(5):625–633

Ménézo Y, Dale B, Cohen M (2010) DNA damage and repair in human oocytes and embryos: a review. Zygote 18(4):357–365. https://doi.org/10.1017/S0967199410000286

Moazamian R, Polhemus A, Connaughton H et al (2015) Oxidative stress and human spermatozoa: diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. Mol Hum Reprod 21(6):502–515. https://doi.org/10.1093/molehr/gav014

Noblanc A, Damon-Soubeyrand C, Karrich B et al (2013) DNA oxidative damage in mammalian spermatozoa: where and why is the male nucleus affected? Free Radic Biol Med 65:719–723. https://doi.org/10.1016/j.freeradbiomed.2013.07.044

O'Flaherty C, de Lamirande E, Gagnon C (2006) Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. Free Radic Biol Med 41(4):528–540

Ohno M, Sakumi K, Fukumura R et al (2014) 8-oxoguanine causes spontaneous de novo germline mutations in mice. Sci Rep 4:4689. https://doi.org/10.1038/srep04689

Sawyer DE, Mercer BG, Wiklendt AM et al (2003) Quantitative analysis of gene-specific DNA damage in human spermatozoa. Mutat Res 529(1–2):21–34

Sharma U, Conine CC, Shea JM et al (2016) Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals. Science 351(6271):391–396. https://doi.org/10.1126/science.aad6780

Shoeb M, Ansari NH, Srivastava SK et al (2014) 4-Hydroxynonenal in the pathogenesis and progression of human diseases. Curr Med Chem 21(2):230–237

Smith TB, De Iuliis GN, Lord T et al (2013a) The senescence-accelerated mouse prone 8 as a model for oxidative stress and impaired DNA repair in the male germ line. Reproduction 146(3):253–262. https://doi.org/10.1530/REP-13-0186

Smith TB, Dun MD, Smith ND et al (2013b) The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. J Cell Sci 126(Pt 6):1488–1497. https://doi.org/10.1242/jcs.121657

Vorilhon S, Brugnon F, Kocer A et al (2018) Accuracy of human sperm DNA oxidation quantification and threshold determination using an 8-OHdG immunodetection assay. Hum Reprod 33(4):553–562. https://doi.org/10.1093/humrep/dey038

Wachsman JT (1997) DNA methylation and the association between genetic and epigenetic changes: relation to carcinogenesis. Mutat Res 375(1):1–8

Walters JLH, De Iuliis GN, Dun MD (2018) Pharmacological inhibition of arachidonate 15-lipoxygenase protects human spermatozoa against oxidative stress. Biol Reprod 98(6):784–794. https://doi.org/10.1093/biolre/ioy058

Wu Q, Ni X (2015) ROS-mediated DNA methylation pattern alterations in carcinogenesis. Curr Drug Targets 16(1):13–19

Wu X, Zhang Y (2017) TET-mediated active DNA demethylation: mechanism, function and beyond. Nat Rev Genet 18(9):517–534. https://doi.org/10.1038/nrg.2017.33

Zhao J, Zhang Q, Wang Y et al (2014) Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Fertil Steril 102:998–1005

Interventions to Prevent Sperm DNA Damage Effects on Reproduction

8

Sandro C. Esteves

Abstract

Excessive oxidation and antioxidant imbalance resulting from several conditions may cause sperm DNA damage, which, in turn, affect male fertility, both natural and assisted. Sperm DNA damage transferred to the embryo might also affect the health of offspring. Several conditions associated with excessive oxidative stress are modifiable by the use of specific treatments, lifestyle changes, and averting exposure to environmental/occupational toxicants. Here, we discuss the strategies to reduce sperm DNA damage with a focus on clinical and surgical interventions.

Keywords

Sperm DNA fragmentation · Male infertility · Semen analysis · Assisted reproductive technology · Varicocele · Unexplained infertility · Intrauterine insemination · In vitro fertilization · Intracytoplasmic sperm injection · Recurrent pregnancy loss · Testicular sperm · Lifestyle modifications

Introduction

The semen of men with difficulties to conceive often contains abnormal levels of sperm with damaged DNA (Saleh et al. 2002; Sergerie et al. 2005; Hamada et al. 2012; Esteves et al. 2015a). Apoptosis triggered by testicular conditions and oxidative stress (OS) during sperm transit through the male reproductive tract seem to be the primary causes of sperm DNA damage (Muratori et al. 2015). The source of OS can range from a specific clinical condition such as a varicocele and a subclinical genital infection to age, obesity, smoking, and environmental exposure to toxicants (Agarwal et al. 2016a; Esteves et al. 2014; Majzoub et al. 2016; Hamada et al. 2013).

Sperm DNA damage is associated with male infertility and decreased chances of conception, both natural and assisted (Esteves et al. 2017a; Simon et al. 2017; Robinson et al. 2012; Zhao et al. 2014a). Among pregnancies achieved by IVF and ICSI, the risk of miscarriage is increased if the male partners have elevated levels of sperm DNA damage in semen (Robinson et al. 2012; Zhao et al. 2014a). Also, there is a growing concern that an underlying DNA damage could be transferred to the embryo by defective sperm and

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thus affect the health of resulting offspring (Aitken 2017).

The sperm vulnerability to oxidative DNA damage relates to many non-mutually factors including (i) plasma membrane rich in polyunsaturated fatty acid (PUFA), (ii) limited cytosolic content, and (iii) truncated DNA damage detection and repair mechanisms (Dada 2017). PUFA is highly susceptible to reactive oxygen species (ROS), and in situations of ROS excess or poor antioxidant activity, PUFA amplifies the generation of ROS in a vicious oxidative stress circle (Champroux et al. 2016). Upon reaching the sperm nucleus, ROS can promote harm by modifying bases, creating abasic sites, chromatin protein cross-linking, and DNA strand breaks (both single and double) depending on the intensity of the oxidative attack. For instance, high levels of ROS lead to the formation of oxidized base adducts such as 8-oxo-7,8-dihydro2′-deoxyguanosine (8OHdG), the primary oxidative product of sperm DNA. The spermatozoon has a single enzyme, namely, 8-oxoguanine DNA glycosylase 1 (OGG1), to cleave oxidized base adducts such as 8OHdG out of the DNA. OGG1 removes the base adducts and creates a relatively unstable abasic site that is more prone to fragmentation (Aitken 2017; Lopes et al. 1998; Feng et al. 2003).

The oxidatively induced sperm DNA damage may dysregulate expression of many genes critical for fertilization, embryo development, and implantation (Dada 2017). Moreover, 8-OHdG residues can cause transversion mutations (G-C to T-A) which might alter gene expression if not repaired by the oocyte enzymes before the zygote's S-phase (Aitken 2017; Feng et al. 2003). Indeed, the spermatozoon is dependent on the oocyte for completing the removal of damaged DNA and oxidized adducts. However, little is known about the effectiveness and fidelity of DNA repair at the oocyte level once fertilization has occurred. In particular, aged oocytes are more likely to have inefficient repair mechanisms, and as a result persistence of DNA lesions and mutagenic bases might increase the risk of embryo genetic and epigenetic abnormalities (Aitken 2017; Dada 2017; Champroux et al. 2016).

Many conditions associated with OS and sperm DNA damage, as mentioned above, are potentially modifiable. It is therefore essential that clinicians carry out a thorough evaluation including detailed history taking and clinical examination to identify such factors. Interventions to prevent or decrease sperm DNA damage are desirable as a means to increase reproductive outcomes and minimize the risk of transmitting sperm genetic defects to the resulting offspring. In this chapter, we discuss the strategies that might be used alone or combined to reduce sperm DNA damage. Specifically, our focus is on clinical or surgical interventions that can be applied to the affected male.

Clinical Recommendations for Sperm DNA Damage Testing

Given the essential role of sperm DNA integrity for normal embryo development and pregnancy outcome (Esteves et al. 2017a; Simon et al. 2017; Robinson et al. 2012; Zhao et al. 2014a; Esteves 2016), assessments of sperm DNA damage have been used to obtain information about sperm DNA quality, particularly for the evaluation of a possible male factor contributing to infertility (Agarwal et al. 2016a, b; Esteves et al. 2014, 2017b; Majzoub et al. 2017a).

A 2017 clinical practice guideline (CPG) issued by the Society for Translational Medicine provides evidence-based guidance for recommending SDF testing (Box 8.1) (Agarwal et al. 2017a). According to the guideline, male patients with risk factors for OS, including but not limited to lifestyle conditions (e.g., smoking, obesity, metabolic syndrome), varicocele, genital infections, advanced age, and exposure to toxicants (e.g., environmental, licit or illicit drugs, radiation, chemotherapy), should be tested for sperm DNA damage. Testing has also been recommended after failed intrauterine insemination (IUI), IVF, or ICSI cycle provided no other apparent reasons exist to explain that failure. Couples with unexplained infertility and those suffering from recurrent pregnancy loss—defined as two or more pregnancy losses from the time of conception until 24 weeks of gestation—could also benefit from sperm DNA damage testing (Agarwal et al. 2017a).

Box 8.1: Clinical Practice Guidelines for Sperm DNA Fragmentation Testing in Male Infertility by the Society for Translational Medicine

A. Sperm DNA fragmentation testing[a]:
Neat semen sample should be used for SDF testing
A fixed ejaculatory abstinence before collection of semen sample should be applied
A standardized protocol with stringent quality control is essential for a reliable SDF testing result
SDF threshold reflects the probability on reproductive outcome[b]
B. Recommendations:
Clinical varicocele:
SDF testing is recommended in patients with grade 2/3 varicocele with normal conventional semen parameters (grade C recommendation)
SDF testing is recommended in patients with grade 1 varicocele with borderline/abnormal conventional semen parameter results (grade C recommendation)
Unexplained infertility/IUI failure/RPL:
SDF testing should be offered to infertile couples with RPL or prior to initiating IUI (grade C recommendation)
Early IVF or ICSI may be an alternative to infertile couple with RPL or failed IUI (grade C recommendation)
IVF and/or ICSI failure:
SDF testing is indicated in patients with recurrent failure of assisted reproduction (grade C recommendation)
The use of testicular sperm rather than ejaculated sperm may be beneficial in men with oligozoospermia, high SDF, and recurrent IVF failure (grade B–C recommendation)
Borderline abnormal (or normal) semen parameters with risk factor:
SDF testing should be offered to patients who have a modifiable lifestyle risk factor of male infertility (grade C recommendation)

Adapted from Agarwal et al. (2017a).
SDF sperm DNA fragmentation, *RPL* recurrent pregnancy loss, *IUI* intrauterine insemination, *IVF* conventional in vitro fertilization, *ICSI* intracytoplasmic sperm injection
[a]Grade B–C recommendation
[b]SDF levels represent one of the many variables that can affect a couple's reproductive outcome

Interventions to Decrease Sperm DNA Fragmentation

Varicocele Repair

Varicocele is considered the primary correctable cause of male infertility. It can impair sperm quality and fertility via various mechanisms, in particular, oxidative stress (Hamada et al. 2013; Agarwal et al. 2012). The most accepted theory is that ROS generation is related to scrotal hyperthermia, testicular hypoxia, reflux of adrenal/renal metabolites, cadmium accumulation, and epididymal response (Cho et al. 2016).

The seminal levels of OS markers, including ROS, nitric oxide, and lipid peroxidation products, are higher in both fertile and infertile men with varicocele than that in counterparts without varicocele (Hamada et al. 2013; Agarwal et al. 2012, 2017b; Blumer et al. 2012; Mostafa et al. 2009; Zylbersztejn et al. 2013; Mehraban et al. 2005; Sakamoto et al. 2008). Likewise, infertile men with varicocele have diminished seminal antioxidant capacity when compared to their fertile counterparts (Sakamoto et al. 2008; Pasqualotto et al. 2008; Mostafa et al. 2001). Under the presence of a varicocele, in particular, large ones (Köksal et al. 2000; Allamaneni et al. 2004; Ishikawa et al. 2007; Abd-Elmoaty et al. 2010; Pasqualotto et al. 2000; Sharma et al. 1999; Hendin et al. 1999), ROS and nitrogen species are released in the endothelial cells of the dilated pampiniform plexus, testicular cells (germ cells, Leydig cells, macrophages, and peritubular cells), and principal cells of the epididymis (Agarwal et al. 2012; Hurtado de Catalfo et al. 2007). The excessive ROS negatively affect the sperm membrane and chromatin by causing sperm membrane lipid peroxidation and induce nuclear and mitochondrial DNA breaks (Cho et al. 2016; Blumer et al. 2012; Chen et al. 2008).

Thus, the imbalance between excessive ROS production and antioxidant protection causes alterations in nuclear and mitochondrial sperm DNA, including base modifications, strand breaks, and chromatin cross-links, consequently affecting the overall sperm DNA quality and ultimately rendering a subset of varicocele men

less fertile (Esteves et al. 2012, 2015a; Robinson et al. 2012; Cho et al. 2016; Blumer et al. 2012; Tremellen 2008; Esteves and Agarwal 2017). However, it is still unclear how infertility is prevented in fertile varicocele men. Individual factors, including antioxidant enzymes such as catalase, superoxide dismutase, vitamin C, and glutathione peroxidase, could protect fertile men from the deleterious effect of varicocele (Agarwal et al. 2012), and other protective mechanisms, including a slower rate of germ cell apoptosis, enhanced turnover machinery for the oxidized proteins preventing their aggregation, and reduced cellular signal-transducing effects of ROS, might act synergistically (Hamada et al. 2013). By contrast, the disruption of these protective mechanisms could exacerbate the harmful effects of oxidation in infertile varicocele men.

In men with varicocele, sperm DNA damage is probably a critical endpoint of OS via ROS (Fig. 8.1). This fact is supported by (i) the frequent observation of a concomitant impairment in sperm DNA integrity and altered oxidative stress markers in such men and (ii) the amelioration of OS markers and sperm DNA damage after varicocele repair (reviewed by Roque and Esteves) (Roque and Esteves 2018).

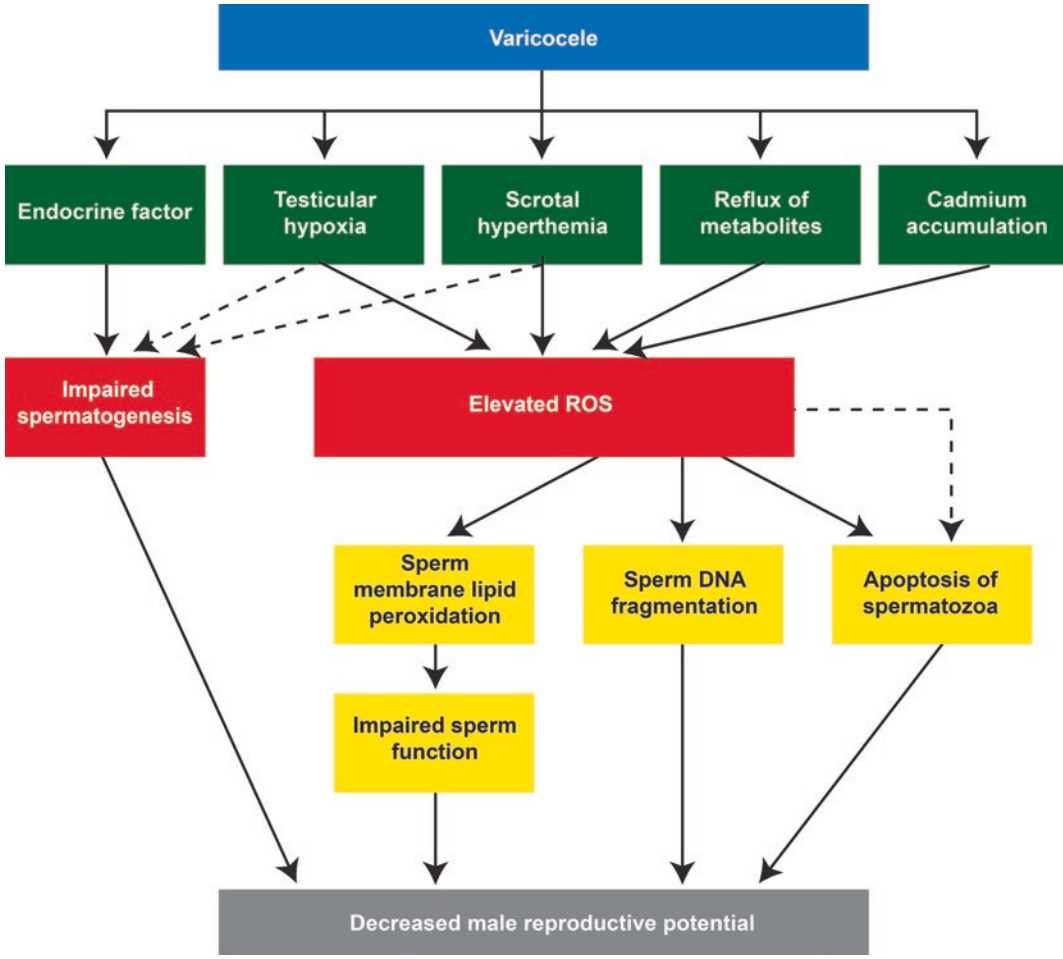

Fig. 8.1 Pathophysiology of varicocele and its association with reactive oxygen species and sperm DNA fragmentation (solid lines and dotted lines indicate direct and indirect effects, respectively). (Reprint from Cho et al. (2016), under the terms of the Creative Commons Attribution-Non-Commercial- ShareAlike License, which permits non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited)

Approximately half of men with clinical varicoceles exhibit high seminal sperm DNA damage, and varicocele repair has been proved to effectively reduce the oxidatively induced sperm DNA damage (Hamada et al. 2013; Sakamoto et al. 2008; Zini and Dohle 2011; Zini et al. 2005; Werthman et al. 2008; Moskovtsev et al. 2009; Smit et al. 2010; Kadioglu et al. 2014; Telli et al. 2015; Sun et al. 2018; Zaazaa et al. 2018; Lacerda et al. 2011; Li et al. 2012; Baker et al. 2013; Pourmand et al. 2014; Tavalaee et al. 2015; La Vignera et al. 2012; Ni et al. 2014; Mohammed et al. 2015; Alhathal et al. 2016; Ni et al. 2016; Abdelbaki et al. 2017) (Table 8.1). Despite the use of different assays to measure sperm DNA damage, different design, and variable sample size, these studies unequivocally report a significant decrease in sperm DNA damage rates after varicocele repair. The vast majority of men benefit from the intervention, with 78% to 90% of

Table 8.1 Summary of studies evaluating the effect of varicocele repair on sperm DNA damage

Study (year)	*N*	Main results
Zini et al. (2005)	37	SDF rates by SCSA before and after surgery (6 months interval): 27.7 ± 2.9% vs. 24.6 ± 2.7% ($p = 0.04$)
Sakamoto et al. (2008)	30	TUNEL-positive sperm before and after surgery (6 months interval): 79.6 ± 13.6% vs. 27.5 ± 19.4% ($p < 0.001$). TUNEL results of controls not provided
Werthman et al. (2008)	11	90% of the patients showed a significant decrease in the rates of SDF by SCSA 3–6 months after varicocelectomy
Moskovtsev et al. (2009)	37	Improvements in SDF by SCSA were observed in 78% of the patients subjected to the combination of varicocelectomy and antioxidants (pre, 44.7 ± 12.8%; post, 28.4 ± 9.5%; $p < 0.03$)
Smit et al. (2010)	49	Improvements in SDF assessed by SCSA were observed in the treated subjects (pre, 35.2%; post, 30.2%; $p = 0.019$)
Lacerda et al. (2011)	21	Comet class I cells (undamaged DNA) increased after varicocelectomy (49.6 ± 23.1% to 64.5 ± 13.6%; $p = 0.011$)
Zini and Dohle (2011)	25	Improvements in SDF by SCSA were observed in the treated subjects (pre, 18 ± 11%; post (4 months), 10 ± 5% ($p = 0.0009$); post (6 months), 7 ± 3%; $N = 19$ subjects; $p = 0.0021$)
La Vignera et al. (2012)	30	SDF rates by TUNEL before and after surgery (4 months): 5.0 ± 3.0% vs. 2.1 ± 0.4% ($p < 0.05$)
Li et al. (2012)	19	DFI rates by SCSA before and after surgery (3 months interval): 28.4 ± 15.6% vs. 22.4 ± 12.9% ($p = 0.018$)
Baker et al. (2013)	22	DFI by TUNEL decreased from 40.8% (pre-op mean) to 24.5% (post-op mean) (mean % change, −16.2; 95% CI, −7.3 to −25.2; $p = 0.001$)
Kadioglu et al. (2014)	92	DFI by TUNEL decreased from 42.6% to 20.5% 6 months after surgery ($p < 0.001$); higher preoperative DFI was associated with a larger decrease in postoperative DFI
Ni et al. (2014)	42	In grade 3 group, P1/P2 mRNA ($p < 0.05$) and DFI by SCSA ($p < 0.01$) were significantly improved while in grade 2 group only DFI was improved ($p < 0.05$). In grade 1 patients, no differences were noted in P1/P2 mRNA ratio and DFI
Pourmand et al. (2014)	100	DFI by TUNEL improved from before to 6 months after surgery in both groups (group 1, 14.0% vs. 9.5%, $p = 0.02$; group 2, 13.9% vs. 8.5%, $p < 0.001$)
Mohammed et al. (2015)	75	DFI by acridine orange reduced after varicocelectomy (pre vs. post, 32.4 ± 7.4% and 20.0 ± 4.1%, $p = 0.05$), but no significant changes were detected regarding DNA chromatin decondensation (25.4 ± 8.8% vs. 22.0 ± 4.1)
Tavalaee et al. (2015)	23	%DFI by TUNEL (15.9 ± 1.2% pre-op vs. 10.8 ± 1.1% post-op, $p < 0.001$), %sperm with protamine deficiency (46.7 ± 2.6% pre-op vs. 39.4 ± 2.6% post-op, $p = 0.02$), and %sperm with OS (47.6 ± 6.6% pre-op vs. 36.6 ± 3.8% post-op, $p = 0.03$) improved 3 months after surgery
Telli et al. (2015)	72	Mean DFI by acridine orange using flow cytometry was 34.5 ± 3.3% and 28.2 ± 3.5% before and after varicocelectomy ($p = 0.024$) with a follow-up of 6.2 ± 2.4 months

(continued)

Table 8.1 (continued)

Study (year)	*N*	Main results
Alhatalet al. (2016)	29	%DFI by SCSA decreased after surgery (from 20.0 ± 10.6% to 12.0 ± 5.7%; $p = 0.001$). AB% staining (from 13.5 ± 7.0% to 5.4 ± 2.7%; $p = 0.0004$) and %5-IAF (16.3 ± 6.0% to 5.4 ± 2.7%; $p = 0.0004$) also decreased after surgery
Ni et al. (2016)	51	Varicocelectomy reduced SDF (by SCSA) in patients with grades 1–3 clinical varicocele: grade 1 ($n = 19$): pre 23.5 ± 7.5%, post (3 months) 20.8 ± 5.6%, post (6 months) 19.5 ± 5.5%, $p < 0.01$; grade 2 ($n = 18$): pre 27.7 ± 9.0%, post (3 months) 22.9 ± 5.2%, post (6 months) 22.4 ± 4.5%, $p < 0.01$;grade 3, pre 30.0 ± 8.2%, post (3 months) 23.3 ± 5.4%, post (6 months) 21.8 ± 5.9%, $p < 0.01$
Abdelbaki et al. (2017)	60	%DFI by SCSA (18.8 ± 7.2%, $p < 0.001$) and ROS levels (3.3 ± 1.3 Log(ROS + 1) photons/min, $p < 0.001$) decreased after varicocelectomy whereas TAC levels increased (2.0 ± 0.5 mM) at a 3-month follow-up.
Sun et al. (2018)	358	DFI by SCSA was reduced after varicocelectomy at 1-year follow-up (unilateral, 21.6 ± 7.1% pre-op vs. 11.8 ± 6.0% post-op; bilateral, 23.0 ± 8.1% pre-op vs. 12.1 ± 6.8% post-op (*p* value not specified)
Zaazaa et al. (2018)	80	DFI (by SCD) improvement percentages showed the highest improvement in men subjected to varicocelectomy followed by MC stabilizer (26.8%) compared with varicocelectomy alone (18.2%; $p = 0.04$) and MC stabilizer alone (16.8%; $p = 0.02$)

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N number of participating subjects, *SDF* sperm DNA fragmentation, *DFI* DNA fragmentation index, *SCSA* sperm chromatin structure assay, *SCD* sperm chromatin dispersion assay, *AB staining* Alcian blue staining, *5-IAF* 5-Iodoacetamidofluorescein, *TAC* total antioxidant capacity, *MC* mast cell

the treated patients exhibiting lower sperm DNA damage rates 3–6 months after varicocele repair (Werthman et al. 2008; Moskovtsev et al. 2009).

Several of the studies mentioned above also evaluated the impact of varicocele repair on oxidative stress markers, sperm chromatin compaction, or other advanced sperm function characteristics (Tables 8.2 and 8.3). Decreases in such markers were noticeable in most studies, thus underscoring the association between varicocele, OS, and SDF (Lacerda et al. 2011; Li et al. 2012; Baker et al. 2013; Pourmand et al. 2014; Tavalaee et al. 2015; La Vignera et al. 2012; Ni et al. 2014; Mohammed et al. 2015; Alhathal et al. 2016; Ni et al. 2016; Abdelbaki et al. 2017). Yet, some authors were unable to confirm the reduction of OS after varicocele repair (Lacerda et al. 2011; Baker et al. 2013), rendering it unclear as to why not all men with signs of OS improve after the intervention.

Improvements in sperm DNA integrity after varicocele repair seem to translate into increased pregnancy outcomes. In one study, Smit et al. prospectively evaluated 49 men with clinical varicocele, oligozoospermia, and at least 1-year infertility duration subjected to varicocelectomy (Smit et al. 2010). Couples that conceived naturally or with ART had lower postoperative sperm DNA damage levels (26.6 ± 13.7%) assessed by sperm chromatin structure assay (SCSA) than those who did not (37.3 ± 13.9%, $p = 0.013$). In another study, Ni et al. evaluated 42 subfertile patients with varicocele grades 2 and 3 and abnormal seminal parameters subjected to microsurgical varicocelectomy (Ni et al. 2014). The seminal levels of sperm DNA damage by SCSA were significantly higher preoperatively in the patient group than in semen donor controls. After 3–6 months postoperatively, sperm DNA damage decreased overall (preoperative, 28.4%; postoperative, 22.4%; $p = 0.018$), despite remaining higher than controls. However, sperm DNA damage levels among patients who achieved pregnancy naturally after varicocele repair (20.6 ± 3.5%) were not significantly different than controls (11.5 ± 3.9%) and were lower than both preoperative values (27.4 ± 6.3%; $p < 0.01$) and nonpregnant patients (24.7 ± 6.5%; $p < 0.010$). Lastly, Mohammed et al. prospectively evaluated 75 infertile men with clinical varicocele and abnormal semen parameters and found that couples with positive pregnancy outcome at 1-year

Table 8.2 Studies evaluating the effect of varicocele repair on sperm DNA damage including controls or concomitant assessment of oxidative stress markers

Study (year)	Design	Patients	Controls	Sperm DNA damage assay	Oxidative stress and/or other sperm function markers	Varicocele repair method	Main results
Lacerda et al. (2011)	Prospective cohort	21 adolescents between 15 and 19 years old with grades 2 or 3 varicocele subjected to varicocelectomy	NA	Comet	Mitochondrial activity and thiobarbituric acid-reactive substances (TBARS) levels	Microsurgical subinguinal varicocele repair	Comet class I cells (undamaged DNA) increased after varicocelectomy (49.6 ± 23.1% to 64.5 ± 13.6%; $p = 0.011$) Percentage of sperm with mostly inactive mitochondria (diaminobenzidine [DAB] class III) decreased after varicocelectomy (20.2 ± 4.9 to 17.1 ± 3.2; $p = 0.013$). The TBARS levels remained unaltered
Li et al. (2012)	Not specified	19 infertile men (mean age, 33.1 years) with clinical varicocele (left, 19 patients; bilateral, 2 patients) subjected to varicocelectomy	19 normozoospermic controls	SCSA	Not assessed	Microsurgical subinguinal varicocele repair	SDF was higher in men with varicocele (28.4 ± 15.6%) than controls (DFI, 17.4 ± 5.3%; $p = 0.007$) DFI decreased from 28.4 ± 15.6% before surgery to 22.4 ± 12.9% 3 months postoperatively ($p = 0.018$) and postoperative DFI in varicocele patients was similar to controls
Baker et al. (2013)	Retrospective cohort	22 men with clinical varicocele subjected to varicocelectomy	NA	TUNEL	ROS and TAC levels	Microsurgical subinguinal varicocele repair	DFI decreased from a preoperative mean of 40.8% to a postoperative mean of 24.5% (mean % change, −16.2; 95% CI, −7.3 to −25.2; $p = 0.001$). A higher preoperative DFI was associated with a larger decrease in postoperative DFI (r2 = 0.53; $p = 0.01$) DFI results in pregnant and nonpregnant couples did not differ (22.2 ± 14.4 vs. 25.7 ± 14.5%, respectively) Mean TAC decreased from 2292 uM preoperatively to 1885 uM postoperatively ($p = 0.03$) and the percentage of patients with a TAC above the normal value (1420 uM) decreased from 86% preoperatively to 71% postoperatively; however, postoperative TAC remained above the normal reference value for the majority of subjects. There was no statistically significant change in ROS levels after surgery

(continued)

Table 8.2 (continued)

Study (year)	Design	Patients	Controls	Sperm DNA damage assay	Oxidative stress and/or other sperm function markers	Varicocele repair method	Main results
Pourmand et al. (2014)	Randomized controlled trial	100 infertile men with clinical left varicocele ($N = 78$) or subclinical ($N = 22$)) varicocele subjected to varicocelectomy alone (group 1) or varicocelectomy plus 750 mg L-carnitine orally daily for 6 months (group 2)	NA	TUNEL	Protamine damage	Not specified	DFI decreased from before to 6 months after surgery in both groups (group 1, 14.0% vs. 9.5%, $p = 0.02$; group 2, 13.9% vs. 8.5%, $p < 0.001$), but results were not different between groups Improvement in protamine damage from before to 6 months after surgery in group 2 only (44.9% vs. 33.7%, $p < 0.001$)
Tavalaee et al. (2015)	Not specified	23 infertile men (mean age, 31.3 years) with grades 2 or 3 left varicocele subjected to varicocelectomy	NA	TUNEL	Protamine deficiency (chromomycin A3), oxidative stress (DCFH-DA staining), and global DNA methylation (immunostaining)	Not specified	%DFI (15.9 ± 1.2% pre-op vs. 10.8 ± 1.1% post-op, $p < 0.001$), %sperm with protamine deficiency (46.7 ± 2.6% pre-op vs. 39.4 ± 2.6% post-op, $p = 0.02$), and %sperm with OS (47.6 ± 6.6% pre-op vs. 36.6 ± 3.8% post-op, $p = 0.03$) improved 3 months after surgery Percentage of sperm exhibiting global DNA methylation and intensity of DNA methylation also improved after surgery, although the differences were not significant—except in the group of oligozoospermic patients ($p = 0.03$) when compared with preoperative results

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DCFH-DA 2′,7′-dichlorodihydrofluorescein diacetate, *DFI* DNA fragmentation index, *MDA* malondialdehyde, *NA* not applicable, *NR* not reported, *OS* oxidative stress, *ROS* reactive oxygen species, *SCSA* sperm chromatin structure assay, *SCD* sperm chromatin dispersion assay, *SDF* sperm DNA fragmentation, *TAC* total antioxidant capacity

Table 8.3 Studies evaluating the effect of varicocelectomy on sperm DNA fragmentation, including both controls and concomitant assessment of oxidative stress/sperm function markers

Study (year)	Design	Patients	Controls	Sperm DNA damage assay	Oxidative stress and/or other sperm function markers	Varicocele repair method	Main results
Sakamoto et al. (2008)	Retrospective cohort	30 infertile men with grades 2 or 3 varicocele (15 oligozoospermic and 15 normozoospermic) subjected to varicocele repair	15 age-matched healthy controls without varicocele and with normal semen characteristics 15 oligozoospermic infertile men without varicocele	TUNEL	Nitric oxide (NO), 8-hydroxy-2′-deoxyguanosine (8-OHdG), hexanoyl-lysine (HEL), superoxide dismutase (SOD) activity, interleukin (IL)-6, IL-8, and tumor necrosis factor-alpha in seminal plasma	Microsurgical subinguinal varicocele repair	The percentage of TUNEL-positive sperm 6 months after surgery was significant lower than before (post-op, 27.5 ± 19.4%; pre-op, 79.6 ± 13.6%; $p < 0.001$). TUNEL results of controls not provided Seminal plasma NO concentration and SOD activity of normozoospermic patients with varicocele were significantly higher than that of controls ($p < 0.05$). In oligozoospermic patients, the NO, IL-6, and HEL levels in seminal plasma in men with varicocele were significantly higher than in those without There was a significant reduction in the level of NO, HEL, 8-OHdG, and SOD activity after surgery
La Vignera et al. (2012)	Not specified	30 men (mean age, 26.5 years) with oligoasthenoteratozoospermia and grade 3 left varicocele subjected to varicocelectomy	30 normozoospermic controls without varicocele	TUNEL	Mitochondrial membrane potential (MMP), phosphatidylserine externalization (Annexin V/PI assay), and chromatin compactness	Microsurgical subinguinal varicocele repair	SDF rates significantly decreased after surgery (4 months) from 5.0 ± 3.0% to 2.1 ± 0.4% ($p < 0.05$), and these postoperative results were similar to that of healthy controls (2.0 ± 1.0%) After surgery, a lower percentage of spermatozoa with low MMP was observed compared with baseline (2.0 ± 0.6% vs. 28.0 ± 4.0%, respectively; $p < 0.05$), and results were not different than controls (2.0 ± 0.6%). The percentages of spermatozoa with PS externalization (3.0 ± 3.0% vs. 9.0 ± 4.0%; $p < 0.05$) and decondensed chromatin (6.0 ± 0.5% vs. 22.0 ± 4.0%; $p < 0.05$) were lower than baseline, and results were not different than controls (4.0 ± 2.0% and 6.0 ± 2.0%, respectively)
Ni et al. (2014)	Prospective cohort	42 infertile men with clinical left varicocele (grade 1, 15 patients; grade 2, 16 patients; grade 3, 11 patients) and abnormal semen analysis (sperm count <15 M/mL and/or %motility<32%) subjected to varicocelectomy	10 normozoospermic fertile controls	SCSA	Sperm protamine-1/2 mRNA ratio	Microsurgical varicocele repair	Mean DFI and protamine-1/2 mRNA ratio were significantly higher in the preoperative group than in the control group (27.4 ± 6.3% vs. 11.5% ± 3.9% and 2.1 ± 1.1 vs. 1.1 ± 0.1, respectively; $p < 0.01$) DFI results in patients who achieved pregnancy after varicocele repair (20.6 ± 3.5%) were not significantly different than controls (11.5 ± 3.9%) but were both lower than preoperative values (27.4 ± 6.3%; $p < 0.01$) and the results of nonpregnant patients (24.7 ± 6.5%; $p < 0.01$) In grade 3 group P1/P2 mRNA ($p < 0.05$) and DFI ($p < 0.01$) were significantly improved while in grade 2 group only DFI was improved ($p < 0.05$). In grade 1 patients, no differences were noted in P1/P2 mRNA ratio and DFI

(continued)

Table 8.3 (continued)

Study (year)	Design	Patients	Controls	Sperm DNA damage assay	Oxidative stress and/or other sperm function markers	Varicocele repair method	Main results
Mohammed et al. (2015)	Prospective cohort	75 infertile men (mean age, 31 years) with clinical varicocele (any grade) and altered semen parameters subjected to varicocelectomy	40 healthy fertile volunteers (mean age, 30.2 years) without varicocele	Acridine orange	Sperm chromatin decondensation by flow cytometry	Subinguinal varicocele repair with loop magnification	Baseline DFI and sperm chromatin decondensation were lower in controls than patients (18.2 ± 4.8% vs. 32.4 ± 7.4, $p = 0.003$; 12.8 ± 2.2% vs. 25.4 ± 8.8%, $p = 0.005$) DFI reduced after varicocelectomy (pre vs. post, 32.4 ± 7.4% and 20.0 ± 4.1%, $p = 0.05$), but no significant changes were detected regarding DNA chromatin decondensation (25.4 ± 8.8% vs. 22.0 ± 4.1) Positive pregnancy outcome at 1-year follow-up ($n = 15$) had significantly lower DFI (16.4 ± 6.4%) than those who did not (24.2 ± 4.1%, $p = 0.04$), but there was no significant difference observed in sperm DNA decondensation among couples who conceived or did not (20.3 ± 6.8 vs. 23.5 ± 5.4)
Alhathal et al. (2016)	Prospective cohort	29 infertile men with clinical varicocele and abnormal semen parameters subjected to varicocelectomy	6 healthy sperm donors with normal sperm parameters	SCSA	Sperm DNA decondensation (aniline blue and iodoacetamide fluorescein)	Microsurgical subinguinal varicocele repair	Preoperative sperm %DFI (20 ± 10.6 vs. 7.4 ± 5%; $p = 0.01$), %positive AB staining (13.5 ± 7.0 vs. 2.5 ± 1%; $p = 0.0009$), and % positive 5-IAF (16.3 ± 6.0 vs. 1.7 ± 1.0%; $p = 0.0001$) of infertile men with varicocele were significantly higher than that of healthy donors The %DFI decreased significantly after surgery (from 20.0 ± 10.6% to 12.0 ± 5.7%; $p = 0.001$). Similarly, the %AB staining (from 13.5 ± 7.0% to 5.4 ± 2.7%; $p = 0.0004$) and %5-IAF (16.3 ± 6.0% to 5.4 ± 2.7%; P0.0004) also decreased after surgery

Ni et al. (2016)	Not specified	51 men with clinical varicocele and abnormal semen analysis subjected to varicocelectomy	15 men with subclinical varicocele, 22 normozoospermic men with clinical varicocele, and 25 healthy fertile donors	SCSA	Assessment of lipid peroxidation by measurement of seminal MDA concentration	Microsurgical retroperitoneal high ligation of varicose veins	SDF levels were elevated in men with clinical varicocele (all grades: range, 20.6 ± 4.1% to 30.03 ± 8.3%) compared to controls (12.0 ± 7.9%) and subclinical varicocele (14.9 ± 5.1%) ($P < 0.05$) Varicocelectomy reduced SDF in patients with grades 1–3 clinical varicocele and altered semen parameters: Grade 1 ($n = 19$): pre 23.5 ± 7.5%, post (3 months) 20.8 ± 5.6%, post (6 months) 19.5 ± 5.5%; $P < 0.01$ Grade 2 ($n = 18$): pre 27.7 ± 9.0%, post (3 months) 22.9 ± 5.2%, post (6 months) 22.4 ± 4.5%; $P < 0.01$ Grade 3: pre 30.0 ± 8.2%, post (3 months) 23.3 ± 5.4%, post (6 months) 21.8 ± 5.9%; $P < 0.01$ Among men with clinical varicocele, DFI and MDA levels in couples who achieved pregnancy were lower than nonpregnant couples ($P < 0.05$) Seminal MDA levels were higher in men with clinical varicocele (all grades) than subclinical varicocele and fertile controls ($P < 0.01$). After surgery, a significant reduction in seminal MDA was observed at 3 (grade 2, $P < 0.01$; grade 3, $P < 0.05$) and 6 (grade 1, $P < 0.05$; grade 2 and 3, $P < 0.01$) months, which was almost equal to the level of control group A positive correlation was observed between sperm DFI and seminal MDA ($r = 0.504$, $P < 0.01$)
Abdelbaki et al. (2017)	Prospective controlled cohort	60 infertile men (median age: 31 years) with clinical varicocele (left: 35 patients; bilateral: 25 patients) and abnormal semen parameters subjected to varicocelectomy	20 normozoospermic healthy fertile men with normal standard semen variables according to WHO criteria	SCSA	Measurement of ROS and TAC levels	Inguinal varicocele repair with loop magnification	A higher %DFI (29.9 ± 8.3%) and ROS level (4.49 ± 0.9 Log(ROS + 1) photons/min) and a lower TAC (0.97 ± 0.4 mM) were found in varicocele patients than controls (7.56 ± 2.84%, 2.62 ± 0.8 Log(ROS + 1) photons/min, and 1.5 ± 0.5 mM, respectively) The DFI% had a positive correlation ($r = 0.654$; $p < 0.001$) with ROS levels, grade of varicocele and duration of infertility, and a significant negative correlation with TAC ($r = –0.79$; $p < 0.001$) %DFI (18.8 ± 7.2%; $p < 0.001$) and ROS levels (3.3 ± 1.3 Log(ROS + 1) photons/min; $p < 0.001$) decreased after varicocelectomy whereas TAC levels increased (2.0 ± 0.5 mM) at a 3-month follow-up

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DFI DNA fragmentation index, *MDA* malondialdehyde, *MMP* mitochondrial membrane potential, *OS* oxidative stress, *ROS* reactive oxygen species, *SCSA* sperm chromatin structure assay, *SCD* sperm chromatin dispersion assay, *SDF* sperm DNA fragmentation, *TAC* total antioxidant capacity

follow-up had had significantly lower DNA fragmentation index (DFI, 16.4 ± 6.4%) than those who did not (24.2 ± 4.1%, $p = 0.04$) (Mohammed et al. 2015).

By contrast, Baker et al. evaluating a cohort of 24 infertile men with clinical varicocele who underwent microsurgical varicocele repair showed that although DFI was reduced after varicocele repair, results were not different in pregnant and nonpregnant couples (DFI, 22.2 ± 14.4 vs. 25.7 ± 14.5%, respectively) (Baker et al. 2013). In another report, Nasr-Esfahani et al. observed that an increase in chromatin compaction after varicocelectomy might not translate in higher pregnancy rates (Nasr-Esfahani et al. 2009). Criticism of this study is that chromomycin A3—a test of sperm nuclear decondensation (SND)—was utilized for measuring sperm DNA damage. SND refers to defects in chromatin compaction (e.g., protamine mispackage via defective DNA–protein cross-linking), which is intrinsically associated with the later stage of spermatogenesis (Aitken 2017; Gosálvez et al. 2015). Although defective chromatin compaction makes the DNA more vulnerable to ROS attack—and as a consequence sperm DNA damage could occur—this effect depends on the seminal redox properties and OS levels (Esteves et al. 2017c). Excessive ROS affect the membranes by lipid peroxidation and chromatin by inducing DNA breaks (Cho et al. 2016; Blumer et al. 2012; Chen et al. 2008). Given the ubiquity of OS in varicocele, we believe that tests assessing sperm DNA fragmentation should be preferable over those that assess chromatin compaction (Esteves et al. 2017c, d).

Collectively, the current evidence supports OS as a central factor in the pathophysiology of varicocele-related infertility. The testis and epididymis react to OS via several mechanisms—including the generation of antioxidants that may maintain fertility potential in men with varicocele. Failure of these mechanisms might explain testicular/epididymal dysfunction and infertility in a subset of men with varicocele. Increased sperm DNA damage—as often seen in men with clinical varicocele—is likely the endpoint of the oxidative-induced damage. The existing evidence is reassuring as to the effectiveness of varicocele repair to alleviating oxidatively induced sperm DNA damage and increasing the likelihood of pregnancy, both natural and assisted. Therefore, practitioners providing care to infertile couples should advise those men with palpable varicoceles of the connection between sperm DNA damage and OS and discuss varicocele repair as a way of both decreasing sperm DNA damage and potentially improving fertility.

Changes in Lifestyle

Environment and Smoking

Several studies have consistently reported the negative impact of smoking and exposure to environmental/occupational chemicals on sperm DNA integrity. Workers exposed to polycyclic aromatic hydrocarbon, ionizing radiation, and organophosphate and carbamate pesticides exhibited elevated levels of sperm DNA damage in semen (Jeng et al. 2016; Sánchez-Peña et al. 2004; Miranda-Contreras et al. 2015; Jamal et al. 2016; Zhou et al. 2016). Increased levels of bisphenol A—an environmental endocrine disruptor used for the production of plastics and resins—are associated with increased sperm DNA damage (Gandhi et al. 2017). The reproductive toxicity of lead poisoning on sperm DNA damage has also been documented (Gandhi et al. 2017).

Furthermore, an association between exposure to air pollutants, such as PM2.5, PM10, NOx, SO2, and O3, and sperm DNA damage has been documented (Lafuente et al. 2016; Radwan et al. 2016). Tobacco users tend to have increased levels of ROS, 8-hydroxy-2′-deoxyguanosine (8-OHdG), and sperm DNA damage SDF in the semen (Kumar et al. 2015). It is, therefore, sound to advice infertile men to avoid exposure to such toxicants as much as possible. However, data on the effects of averting exposure to environmental/occupational chemicals and smoking cessation on sperm DNA damage is lacking.

Diet

Recent data indicate that dietary patterns could influence sperm DNA damage. In a 2018 study

from Poland, 336 men with sperm count within the WHO 2010 normal ranges seeking fertility were interviewed as regards their dietary patterns (Jurewicz et al. 2018). Patients were classified into three groups according to scores of each dietary pattern, namely, Western, Mixed, or Prudent. The Prudent dietary pattern was characterized by high intakes of fish, chicken, fruit, cruciferous vegetables, tomatoes, leafy green vegetables, legumes, and whole grains, whereas the Western pattern included high intakes of red and processed meat, butter, high-fat dairy, refined grains, pizza, snacks, high energy drinks, mayonnaise, and sweets. After controlling for ejaculatory abstinence, age, smoking, past diseases, and alcohol consumption, the authors observed that a Prudent dietary pattern was associated with increased sperm counts, higher testosterone serum levels, and decreased percentages of sperm with DNA damage than the Western dietary pattern (DFI by SCSA, 15.2% ± 10.4 vs. 17.9% ± 8.1; $p < 0.05$).

In a 2016 study from India, a decline in OS markers and an improvement in sperm DNA integrity were noted following adoption of meditation and yoga-based lifestyle modification (Rima et al. 2016). The authors assessed the levels of ROS, DFI, 8-hydroxy-2′-deoxyguanosine (8-OHdG), and telomere length in 56 fathers of children with childhood cancer (retinoblastoma) and 50 controls (fathers of healthy children) according to yoga, meditation practice and smoking status at day 0, and after 3 and 6 months of intervention. The intervention program lasted for 2 hours each day and lasted for 6 months, comprising of theory and practice sessions. The seminal mean ROS levels (RLU/s/million, 36.1 ± 1.8 vs. 20.5 ± 2.7; $p < 0.01$), sperm DFI by SCSA (31.5% ± 6.7 vs. 21.9% ± 9.4; $p < 0.01$), and 8-OHdG (66.0 pg/mL ± 2.9 vs. 23.1 pg/mL ± 2.7; $p < 0.01$) levels were higher in fathers of children with retinoblastoma than in controls, whereas the relative mean telomere length in the sperm was shorter in the former (telomere to single copy gene ratio, 0.35 ± 0.02 vs. 0.38 ± 0.02; $p < 0.05$). Levels of ROS were reduced in tobacco users ($p < 0.05$) as well as in alcohol users ($p < 0.05$) after the adoption of meditation and yoga-based lifestyle modification. DFI reduced ($p < 0.05$) after 6 months of yoga and meditation practice in both groups, whereas the levels of 8-OHdG were reduced after 3 months ($p < 0.05$) and 6 months ($p < 0.05$) of interventions.

Obesity

Recently, some studies have suggested a link between increased male BMI and reduced live birth outcomes (Campbell et al. 2015; Houfflyn et al. 2017; Craig et al. 2017). With the prevalence of obesity rising it becomes important to examine whether these effects are associated with sperm DNA quality. In a 2017 systematic review and meta-analysis involving men from IVF/male infertility clinics and the general population, the authors examined the effect of BMI on sperm DNA fragmentation index (DFI) (Sharma et al. 2017). Patients were stratified and compared according to BMI categories based on WHO classification, namely, normal, overweight (OW), and obese (OB). The meta-analysis included a total of 7 studies, involving 3250 subjects. Overall, DFI was slightly increased in both overweight men MD = 0.62 (95% CI [−2.20, 3.44], $I^2 = 93\%$) and obese men MD = 0.64 (95% CI [−3.79, 5.07], $I^2 = 94\%$), when compared with normal BMI men, but these results were not statistically significant. The pooled effect estimates were not materially affected by the test method (SCSA or TUNEL) or study population (general population vs. infertile men). Heterogeneity was high across all comparisons, suggesting a marked variation across the included studies. Based on the high heterogeneity across the included studies, the authors concluded that the clinical implications of the association between BMI and SDF deserve further investigation (Sharma et al. 2017).

By contrast, very few studies have examined the effects of weight loss on improvement in sperm quality, in particular, SDF (Best et al. 2017). To our knowledge, only a small cohort study involving eight obese men looked at this issue (Faure et al. 2014). In this report, a nutritionist-led personalized dietary program coupled with exercise was used to reduce intra-abdominal fat over a 3- to 8-month period. All

men had unexplained infertility and at least 25% sperm with DNA fragmentation. The DNA quality of six who had their semen samples analyzed both before and after the intervention improved, and all partners achieved pregnancy, either naturally or assisted. Given the paucity of data, it remains difficult to draw any conclusions regarding the benefits of weight loss on sperm DNA integrity until further high-quality studies are conducted.

Collectively, objective evidence indicates that lifestyle factors, including smoking and exposure to environmental/occupational chemicals, obesity, and advanced paternal age, have a negative effect on sperm DNA quality. Dietary and lifestyle changes could reduce SDF. Although further research is warranted to determine how these changes may translate into better reproductive outcomes, information provided by SDF testing gives substantial grounds for implementing lifestyle changes as well as monitoring patient compliance in health prevention programs. Knowledge of the SDF status can be used to strengthen patient counseling and allow clinicians to provide a more realistic prognosis of every treatment strategy the couple wishes to pursue.

Antioxidants

Antioxidants (AOX) can be consumed through diet or as an oral supplement. Some studies have examined the clinical utility of oral antioxidant intake as a means to decrease the oxidatively induced sperm DNA damage (reviewed by Majzoub et al. 2017b). While most studies included small patient cohorts and used short treatment protocols, in general, they conveyed a beneficial effect for antioxidants on measures of SDF (Table 8.4) (Tunc et al. 2009; Ménézo et al. 2007; Kodama et al. 1997; Omu et al. 2008; Greco et al. 2005a, b; Martínez-Soto et al. 2016; Fraga et al. 1991; Abad et al. 2013; Vani et al. 2012; Gual-Frau et al. 2015; Piomboni et al. 2008).

On the other hand, the impact of AOX on the likelihood of establishing a pregnancy, either natural or assisted, has been poorly studied. In a Cochrane review of 48 randomized controlled clinical trials (RCT) pooling data from 4179 subfertile men taking AOX, only four trials included live birth data. The authors reported significant improvement in the live birth rate (OR, 4.21; 95% CI, 2.08 to 8.51; $p < 0.0001$) and clinical pregnancy rate (OR, 3.43; 95% CI, 1.92 to 6.11; $p < 0.0001$) among couples whose male partners were taking AOX, but the results were derived from small RCTs, thus making the quality of evidence equivocal (Showell et al. 2014).

Notably, some data indicate that the indiscriminate long-term use of high AOX dose might induce a state of "reductive stress," which enhances ROS generation by mitochondria (Bauersachs and Widder 2010). In one study in which male partners of couples with failed IVF/ICSI cycles were prescribed a daily oral antioxidant treatment consisting of vitamins C and E (400 mg each), β-carotene (18 mg), zinc (500 μmol) and selenium (1 μmol) for 90 days, the authors observed that while SDF was significantly decreased, sperm decondensation was increased by 25% overall (Ménézo et al. 2007). The authors speculated that due to its high redox potential, vitamin C could reduce cystine to two cysteine moieties and open the interchain disulfide bridges in protamines. High rates of decondensed sperm might offset the positive effect of AOX on SDF as it can result in asynchronous chromosome condensation (Aitken et al. 2014).

The author's personal prescription of oral antioxidants (AOX) to infertile men is presented in Box 8.2. In the author's practice, AOX are given for approximately 3 months, and the effect of the intervention is monitored using sperm DNA damage testing. Notably, the benefit of oral AOX is not universal. In a 2009 study, a 3-month course of a vitamin-based AOX treatment reduced SDF in about 50% of treated subjects (Moskovtsev et al. 2009). In this study, the authors found that the effect of AOX therapy, as used, could be age-dependent because patients older than 40 years had no decrease in SDF after treatment. Equally important is to acknowledge the fact that the magnitude of reduction in SDF rates could be

Table 8.4 Characteristics and main outcome measures of studies reporting ICSI outcomes with testicular versus ejaculated sperm in non-azoospermic men with high sperm DNA fragmentation in the semen

Author and year	Design	Subjects and cohort size (*N*)	SDF testing method	SDF cutoff values	SDF results (%)	Sperm retrieval method	Fertilization rate (%)	Clinical pregnancy rate (%)	Live birth rate (%)
Greco et al. (2005c)	Case series; intervention applied in consecutive patients	Predominantly normozoospermic infertile men (18) Couples with history of ICSI failure	TUNEL	15%	23.6 ± 5.1% (E) and 4.8 ± 3.6% (T)[b]	TESE and TESA	74.9[c]	44.4 (T)[d]	NR
Esteves et al. (2015b)	Prospective cohort	Oligozoospermic infertile men (172) Couples with no history of ICSI failure	SCD	30%	40.9% ± 10.2% (E) and 8.3% ± 5.3% (T)[b]	TESE and TESA	69.4 (E) vs. 56.1 (T)	40.2 (E) vs. 51.9 (T)	26.4 (E) vs. 46.7 (T)
Bradley et al. (2016)	Retrospective cohort	Predominantly oligozoospermic men infertile men (228)[a]	SCIT	29%	NR	TESE and TESA	66.0 (E) vs. 57.0 (T)	27.5 (E) vs. 49.5 (T)	24.2 (E) vs. 49.8 (T)
Pabuccu et al. (2017)	Retrospective cohort	Normozoospermic infertile men (71) Couples with history of ICSI failure	TUNEL	30%	41.7 ± 8.2 (E)	TESA	74.1 ± 20.7 (T) and 71.1 ± 26.9 (E)	41.9 (T) and 20.0 (E)	38.7 (T) vs. 15.0 (E)
Arafa et al. (2018)	Prospective cohort; intervention applied in consecutive patients	Oligozoospermic and normozoospermic infertile men (36) Couples with history of ICSI failure	SCD	30%	56.3 ± 15.3 (E)	TESA	46.4 (T) and 47.8 (E)	38.9 (T) and 13.8 (E)	38.9 (T) vs. 8.0 (E)
Zhang et al. (2019)	Prospective cohort[e]	Oligozoospermic and normozoospermic infertile men (102) Couples with no history of ICSI failure	SCSA	30%	NR	TESA	70.4 (T) vs. 75.0 (E)	36.0 (T) vs. 14.6 (E)	36.0 (T) vs. 9.8 (E)

[a]Number of ICSI cycles; *SDF* sperm DNA fragmentation, *TESE* testicular sperm extraction, *TESA* testicular sperm aspiration, *micro-TESE* microdissection testicular sperm extraction, *NR* not reported, *SCD* sperm chromatin dispersion test, *SCIT* sperm chromatin integrity test, a variation of sperm chromatin structure assay (SCSA), *TUNEL* terminal deoxynucleotidyl transferase dUTP nick end labeling assay, *E* ejaculated sperm, *T* testicular sperm

[b]SDF results from paired ejaculated and testicular specimens of same men

[c]2PN fertilization rate with use of testicular sperm; data from previous cycles with use of ejaculated sperm not provided

[d]The authors reported only one pregnancy with ejaculated sperm which miscarried

[e]Inferred from the study reported data; authors not contacted for providing clarification

small. In the Cochrane meta-analysis mentioned above, DFI rates were reduced by 13.8% overall (95% CI, 10.1–17.5) after AOX therapy (Showell et al. 2014).

Box 8.2: Oral Antioxidant Prescription (Daily Dose)

- Vitamin C 500 mg/d
- Vitamin E 400 mg/d
- Folic acid 2.5 mg/d
- Methyl folate 2.5 mg/d
- Co-enzyme Q10 Ubiquinol 100 mg/d
- Zinc chelate 30 mg/d
- Cooper chelate 1 mg/d
- Selenium 50 mcg/d
- L-carnitine 100 mg/d
- Acetil L-carnitine 500 mg/d
- L-arginine 250 mg/d
- L-tyrosin 100 mg/d
- Lycopene 8 mg/d
- Chrome GTF 100 mcg/d
- Gluthatione setria 250 mg/d
- NAC 400 mg/d

Duration:

- At least 3 months

Source: Dr. Sandro Esteves, ANDROFERT, Campinas, Brazil

The ideal candidates for AOX treatment and the optimal regimen, dosage, and duration are still to be determined. We believe though that the best candidates for AOX treatment are infertile men with confirmed elevated OS markers. In general, abnormal levels of SDF might be used as a surrogate measure of OS, but one should keep in mind that not all SDF results from OS (Gosálvez et al. 2015). Moreover, studies using transgenic animal models indicate that even moderate sperm DNA oxidation not resulting in SDF can cause reproductive failures (Chabory et al. 2009). Therefore, it would be ideal to screen candidates for AOX therapy using techniques that measure ROS.

Despite the complexity of some techniques that are used to measure ROS, which limits their widespread utilization as a routine procedure in the andrology clinic, novel simple and low-cost assays have been developed for rapid assessment of overall OS in the human ejaculate. Examples of these new methods are the semiquantitative nitroblue tetrazolium (NBT) reaction test (Oxisperm®, Halotech® DNA, Spain) and the oxidation–reduction potential (ORP) assay (MiOXSYS, Aytu BioScience) (Gosálvez et al. 2017, 2018; Agarwal et al. 2016c, 2017c). The former is based on NBT that produces a stable colorimetric reaction in a biological sample containing excess superoxide anion whereas the latter provides a quick and accurate direct assessment of OS by using an analyzer and disposable sensors.

Treatment of Male Genital Tract Infection

Sperm DNA fragmentation has been related to male accessory gland infection (MAGI) and urinary tract infection (Esteves et al. 2015a; Ochsendorf 1999; Zeyad et al. 2018; Fraczek et al. 2016), albeit this association is not unequivocal (Dehghan Marvast et al. 2018; Puerta Suarez et al. 2017). In particular, bacteriospermia manifests as acute or chronic inflammation and can lead to an increase in the leukocyte number in the genital tract resulting in elevated ROS production (Ochsendorf 1999), thus negatively affecting semen characteristic, sperm DNA integrity, and pregnancy outcomes (Zeyad et al. 2018). Some specific microbial pathogens have been linked to increased OS and SDF in patients with male accessory gland infection. In a prospective study evaluating 122 asymptomatic, infertile males with a high bacterial count ($>10^5$ colony-forming units (CFU)/ml) and ultrasound evidence of MAGI, aerobes (enterobacteria and Gram-positive bacteria), anaerobes, *Chlamydia trachomatis*, and *Ureaplasma urealyticum* were detected in 58%, 11%, 20%, and 11% of patients, respectively (Vicari 2000). Following antibiotic treatment, seminal white blood cell (WBC) and ROS levels were significantly reduced in all treated patients. The difference between natural

pregnancy rates in treated (28.5%) versus untreated cases (5.4%) was significant ($\chi 2$ test, $p = 0.0097$).

MAGI is usually treated with quinolones (e.g., ofloxacin), tetracyclines (e.g., doxycycline), or macrolides (e.g., azithromycin). These antibiotics have broad antibacterial spectrum against Gram-negative, Gram-positive pathogens as well as *Chlamydia trachomatis* or *Ureaplasma urealyticum*. Also, they are excreted primarily by the kidneys with minimal metabolism and have sufficient penetration into the inflamed sexual glands, low rate of adverse sperm effects following short-term treatment, and specific in vitro susceptibility (Schramm 1986; Cunha and Garabedian-Ruffalo 1980; Naber et al. 1993).

In another study, 14 infertile patients with bacteriospermia and SDF greater than 30% as measured by SCSA, who completed a 2-week course of antibiotics, were evaluated (Moskovtsev et al. 2009). The pathogens identified were *Enterococcus*, enterobacteria (*Enterobacter cloacae* and *Escherichia coli*), and *Ureaplasma urealyticum*. Ciprofloxacin (500 mg twice a day or extended-release (XL) 1000 mg daily) or amoxicillin 500 mg three times a day was given, except for ureaplasma infection, in which azithromycin 250 mg for 5 days was prescribed. The authors found that 93% of the patients had a reduction in SDF after antibiotic treatment. Interestingly, seven patients had a concomitant varicocele and DFI were reduced regardless of its presence (pretreatment, 53.4% ± 24.3, vs. posttreatment, 43.5% ± 20.1; $p < 0.01$) or its absence (pretreatment, DFI 47.4% ± 13.5, vs. posttreatment, DFI 33.6% ± 17.4; $p < 0.02$).

SDF assessed by the sperm chromatin dispersion (SCD) test in patients with MAPI caused by chlamydia or mycoplasma was 3.2 times higher (35.2% ± 13.5%) than in controls without infection (10.8% ± 5.6%) (Gallegos et al. 2008). In this study, seminal leukocytes were 5.2 times higher in patients than in controls. Following specific antibiotic therapy using a macrolide, a tetracycline, or a quinolone, combined with a course of anti-inflammatory agents, SDF decreased significantly in 91% of patients, from 37.7% ± 13.6% to 24.2% ± 11.2% ($p < 0.0001$), thus indicating a 35.7% median DFI relative improvement after treatment. In this study, 85.7% of couples that attempted pregnancy succeeded 3–6 months after therapy. When comparing patients with and without pregnancy success, the only differences found were a lower DFI in the former (32.2% ± 7.6% vs. 43.3% ± 14.1%; $p = 0.047$), as well as sperm morphology.

By contrast, data on the effect of empirical antibiotic treatment to asymptomatic men with increased seminal leukocytes is scanty. Moreover, data concerning the association between seminal leukocytes and OS markers are mixed. In one study evaluating a group of 111 male partners of infertile couples with no symptoms of MAPI, no association was found between the concentration of leukocytes, semen parameters, and the percentage of TUNEL-positive and 8-OHdG-positive spermatozoa (Micillo et al. 2016). Contrary findings were reported by Agarwal et al. studying a group of 472 patients with varying levels of seminal leukocytes. In their study, even the presence of low-level leukocytospermia ($0.1–1.0 \times 10^6$ WBC/mL) was associated with abnormal levels of ROS and sperm DNA fragmentation (Agarwal et al. 2014).

Collectively, MAPI can lead to SDF and DFI rates might be reduced after antibiotic therapy. The decrease in the frequency of spermatozoa with fragmented DNA, as a consequence of therapy, may be relevant to increase the likelihood of pregnancy in infertile men with genitourinary infection.

Treatment of Comorbidities (Thyroid Diseases, Diabetes)

Several studies have indicated a positive association between diabetes/metabolic syndrome and a decline in male fertility potential, including an adverse effect on sperm DNA integrity. Possible causes include an impaired function of the hypothalamic–pituitary–gonadal axis, increased SDF, alterations in the system of advanced glycation end products and their receptor, OS, impaired mitochondrial function, disrupted sympathetic innervation, and increased semen interleukin (IL-

17 and IL-18) levels (Maresch et al. 2018; Lu et al. 2017; Leisegang et al. 2016; Condorelli et al. 2018). The mechanistic effect of an altered sperm function in diabetes mellitus (DM) type 2 seems to relate to an inflammatory condition with increased OS resulting in decreased sperm vitality and increased SDF, whereas DM type 1 alters epididymal voiding causing low ejaculate volume and mitochondrial damage resulting in decreased sperm motility. However, studies evaluating the beneficial effect of insulin and other interventions to restore normoglycemia and counteract the consequences of the metabolic syndrome on male reproduction overall, and SDF in particular, are lacking.

Along the same lines, thyrotoxicosis and hypothyroidism affect testicular function and fertility (La Vignera et al. 2017; La Vignera and Vita 2018). In addition to regulating many functions in the testis, such as proliferation and differentiation of non-germ cells, steroidogenesis, and sperm motility, thyroid hormone plays a role in testicular redox status regulation. Hyperthyroid and hypothyroid patients often have altered serum SHBG as well as free and bioavailable testosterone concentrations. Also, semen characteristics could be disturbed, and such patients have a higher prevalence of sexual disturbances than controls. Nevertheless, it is unknown whether or not thyroid diseases impact sperm DNA quality, and to our knowledge, no data exist regarding the effect of treatment to restore normal thyroid function on sperm DNA integrity.

Empirical Medical Treatment

Sperm DNA fragmentation affects both men with explained as well as those with unexplained or idiopathic infertility. The role of empirical medical therapy to reduce DFI has been explored by some investigators. Specifically, the use of exogenous recombinant FSH has been attempted to overcome SDF in men with unexplained and idiopathic infertility with apparently positive results (Colacurci et al. 2012). The follicle-stimulating hormone (FSH) has an active role on spermatogenesis and spermiogenesis. Moreover, FSH acts synergistically with testosterone to support germ cell survival. In adults, FSH stimulates mitotic and meiotic DNA synthesis in spermatogonia and preleptotene spermatocytes and acts as a survival factor for these premeiotic germ cells by acting on Sertoli cells (Shiraishi and Matsuyama 2017). FSH action on Sertoli cells is mediated by the FSH receptor (FSHR), which possesses several polymorphisms demonstrated to affect receptor sensitivity and expression (Casarini et al. 2014). The beneficial effect of FSH therapy seems to relate to reduced apoptosis and improved qualitative properties of the axoneme, chromatin, and acrosome (Kamischke et al. 1998).

In one cohort study involving 166 patients, a 3-month course of purified human FSH (150 IU 3x/week) resulted in a 10% relative reduction in the frequency of sperm with fragmented DNA (assessed by TUNEL), which was followed by improved pregnancy outcomes in the population who responded to the therapy (Garolla et al. 2017). In another prospective study involving 115 men with unexplained infertility, recombinant FSH administration (150 IU administered subcutaneously (SC) every other day for 3 months) reduced DFI in approximately 70% of patients, with an average relative decrease of 35% compared to baseline (Colacurci et al. 2018). In this study, the authors observed that the improvement on sperm DNA integrity was more pronounced in men with basal DFI lower than 17% (as assessed by TUNEL) and in those with FSH basal levels between 2.16 and 4.27 IU/L. However, no data on pregnancy was provided.

In another prospective study involving 89 men with idiopathic infertility, DFI (by TUNEL) >15%, serum FSH ≤ 8 IU/l, and who were carriers of the FSHR p.N680S homozygous N or S genotype, 150 IU of recombinant FSH administration was given SC every other day (Aitken 2018). Approximately 3 months after the end of the study period, DFI was lower in homozygous carriers of the p.N680S N than p.N680S S allele ($p = 0.008$). The FSHR p.N680S N homozygous genotype is shown to be more sensitive to FSH both in vivo and in vitro. The authors found that the FSHB -211G > T genotype modulated the

observed effect as the patients with this genotype were the most responsive to therapy. The authors suggested that the FSHR genotype could be a pharmacogenetic marker of FSH response. Nevertheless, the number of pregnancies achieved during and after the conclusion of the trial in the groups were not significantly different, six (21.4%) in homozygous carriers of the p.N680S N and six (15.8%) in carriers of the p.N680S S allele.

Collectively, there is still limited data on the role of FSH treatment to improve SDF in idiopathic or unexplained infertility men. Overall, exogenous FSH seems to reduce DFI, an effect that might be modulated by FSH receptor genotype. Further studies are warranted to determine how the effect of this intervention translates into better reproductive outcomes.

Avoid Late Fatherhood

In our society, the number of couples with advanced reproductive age seeking fertility is increasing steadily. Oxidative stress is a common feature in men of advanced age; thus, oxidative base adducts and aldehyde adducts on sperm DNA could lead to mutation and epigenetic changes (Bertoncelli Tanaka et al. 2018). Indeed, some studies indicate a positive relationship between increased paternal age and SDF (Nijs et al. 2011; Varshini et al. 2012; Alshahrani et al. 2014; Kaarouch et al. 2018), which could thus impact the reproductive outcomes (reviewed by Tanaka and Esteves (Bertoncelli Tanaka et al. 2018)) (Table 8.5). Also, the continuous division of male germ cell line during the entire reproductive lifespan and the OS-induced SDF seem to increase the frequency of mutations in the sperm of older men (Aitken 2018). With aging, these mutations can accumulate and increase the risk of genetic- and epigenetic-related diseases in the resulting offspring.

From a practical standpoint, prospective fathers should be counseled to avoid delayed parenthood. One option would be to offer sperm freezing to those considering postponing fatherhood (Bertoncelli Tanaka et al. 2018). Although the freeze-thawing process might adversely impact SDF rates overall (Meamar et al. 2012), there seems to exist a remarkable interindividual variation in chromatin resistance to cryoinjury (Tvrdá et al. 2018). Apparently, both semen of fertile men, in particular young ones, as well as of infertile patients with normal semen characteristics (according to the World Health Organization criteria) resist injury better than that of infertile patients with poor semen quality (Tvrdá et al. 2018; Gómez-Torres et al. 2017). Oral antioxidants can also be utilized as a means to alleviate the OS-induced sperm damage in those men with advanced age seeking fertility. However, likewise in the general male infertility population, it remains to be determined what would be the optimal combination of antioxidants, dosage, and duration. Along the same lines, adoption of a healthy lifestyle and treatment of underlying conditions associated with sperm DNA fragmentation should be discussed with men of advanced age who are willing to conceive. For those undergoing ART at later reproductive age, in particular, if older than 50 years, the use of PGT-A should be considered to avoid the possible negative influence of paternal age on embryo euploidy (Bertoncelli Tanaka et al. 2018) (Fig. 8.2).

Use of Testicular Sperm in Preference over Ejaculated Sperm for ICSI

When SDF remains high after treatment of the underlying factors or no obvious condition is identified to allow treatment, the use of testicular sperm in preference over ejaculated sperm for ICSI seems to be a valid strategy to overcome the oxidative-induced SDF. Current evidence overwhelmingly based on cohort studies suggests the safe utilization of testicular sperm for ICSI in non-azoospermic men with high SDF in semen (Esteves et al. 2017a, b).

The biological plausibility relates to data from both animal and human studies showing lower SDF in testicular sperm than in epididymal sperm as well as in ejaculated sperm (O'Connell et al. 2002; Steele et al. 1999; Suganuma et al. 2005; Hammoud et al. 2017; Moskovtsev et al. 2010;

Table 8.5 Summary of studies evaluating the effect of paternal age on sperm DNA fragmentation and the age cutoff at which the negative effect is observed

Study and year	Country	Number of patients	Population	Main findings	Age cutoff
Nijs et al. (2011)	Belgium	278	Couples seeking ART for male, female, or mixed infertility reasons. Extreme OAT cases and testicular spermatozoa excluded	No statistical correlation between the age of the patient and sperm concentration, sperm progressive motility, and DNA fragmentation. Although no correlation was observed overall between male age and sperm morphology, a weak inverse correlation was found for men aged <35 years old (B = 0.191; $p = 0.027$)	NR
Varshini et al. (2012)	India	504	Couples seeking ART for male infertility reasons	TUNEL-positive DNA damaged sperm was significantly higher in men older than 40 years than in those younger than 40 years ($p < 0.001$)	40
Alshahrani et al. (2014)	USA	472	Non-azoospermic infertile men	No significant differences in conventional semen parameters, TAC, and ROS levels in the four age groups (<30, 31–40, <40, >40). Higher levels of DNA damage were seen in men >40 y when compared with men ≤40 y ($p < 0.05$) as well as in the overall group ($p < 0.01$)	40
Kaarouch et al. (2018)	Morocco	204	Couples seeking ART for male infertility reasons	No significant differences in conventional semen parameters as a function of paternal age. Higher rates of sperm DNA fragmentation (41% vs. 14%; $p = 0.01$), sperm DNA decondensation (43% vs. 25%; $p = 0.01$), and sperm aneuploidy (23% vs. 4%; $p = 0.04$) in men older than 40 years than in those <40 years	40

NR not reported, *CI* confidence interval, *TAC* total antioxidant capacity, *ROS* reactive oxygen species

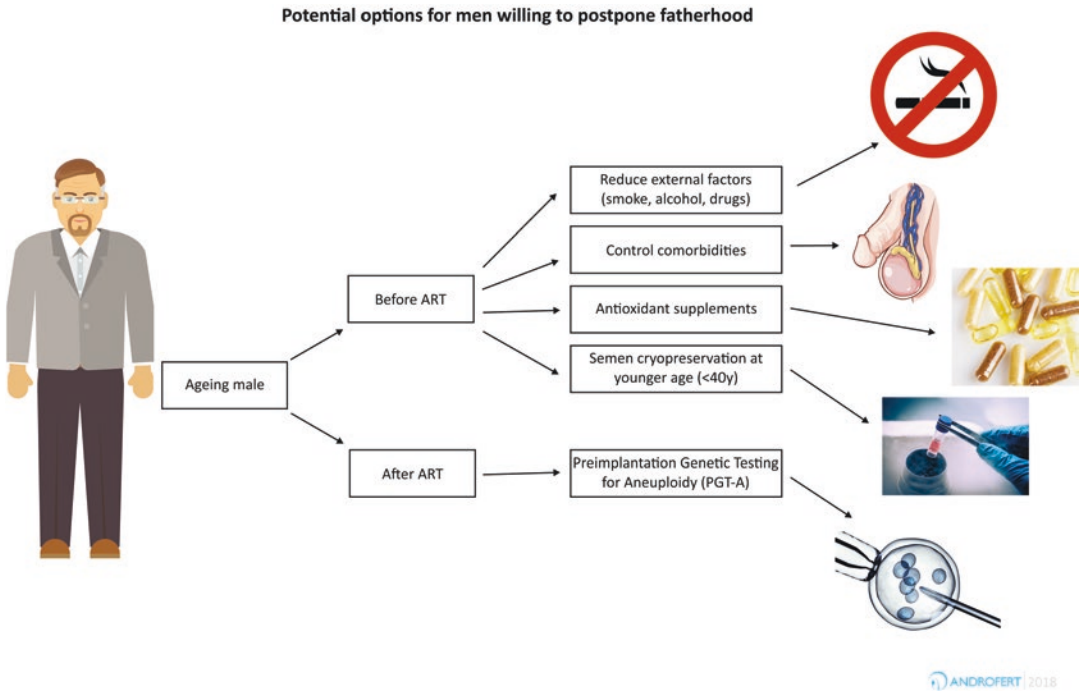

Fig. 8.2 Potential strategies to prevent sperm DNA damage in men willing to postpone fatherhood

Esteves et al. 2015b; Mehta et al. 2015). A 2017 systematic review and meta-analysis examined SDF rates in paired ejaculated and testicular specimens from the same men (Esteves et al. 2017a). The pooled estimates indicated that the mean difference in SDF rates between testicular and ejaculated sperm was 24.6% (95% CI -32.53 to −16.64; $I^2 = 92\%$; $p < 0.001$).

Furthermore, data from a 2019 study using whole exome sequencing molecular karyotype to assess sperm aneuploidy rates in ejaculated and testicular sperm indicated that aneuploidy rates were lower in testicular sperm (Cheung et al. 2019). The authors studied fertile donors and infertile patients (both men with nonobstructive azoospermia and non-azoospermic men with high SDF). Aneuploidy rates in testicular specimens were as low as those of ejaculated samples from fertile donors (1.9% vs. 1.2%), whereas aneuploidy rates were 11.1% for ejaculated specimens of patients ($p < 0.0001$). More importantly, paired assessments in ejaculated and testicular specimens of non-azoospermic men with high SDF showed that both SDF rates and aneuploidy rates were significantly lower in testicular sperm (8% and 1.2%) than in ejaculated sperm (20% and 8.4%).

The integrity of the sperm genome and epigenome is vital for the birth of healthy infants (Krawetz 2005). As the spermatozoon loses most cytosolic antioxidants during spermiogenesis, the male gamete is highly vulnerable to oxidative-induced DNA damage. Low levels of critical DNA repair enzymes might explain the persistence of DNA damage in ejaculated sperm from infertile men (Esteves 2016; Agarwal et al. 2012). The fertilization of oocytes by such sperm through ICSI might result in an increased risk of fertilization failure, embryo arrest, miscarriage, congenital malformations, as well as perinatal and postnatal morbidity (Agarwal et al. 2016b; Lewis and Aitken 2005). Therefore, ICSI using sperm with better chromatin integrity and lower aneuploidy rates might explain, at least in part, the improved reproductive outcome with testicular sperm as seen in various studies.

A pooled analysis of four studies (Esteves et al. 2015b; Bradley et al. 2016; Pabuccu et al. 2017; Greco et al. 2005c) and a total of 507 ICSI cycles and 3840 injected oocytes from couples whose male partners had high SDF in the semen showed that the OR for CPR (3.6; 95% CI 1.94–6.69; $I^2 = 0\%$; $p < 0.0001$) and LBR (OR, 2.6; 95% CI 1.54–4.35; $I^2 = 0\%$; $p = 0.0003$) favored the use of testicular sperm in preference over ejaculated sperm (Esteves et al. 2017a). Likewise, the OR for miscarriage (0.40; 95% CI 0.10–1.65; $I^2 = 34\%$; $p = 0.005$) was also in favor of the testicular sperm group. Further studies published in 2018 corroborate these findings (Arafa et al. 2018; Zhao et al. 2014b) (Table 8.4).

The use of sperm with better genetic quality for ICSI might also result in a positive effect in the offspring health. However, no study has yet investigated on the health of infants born from ICSI using testicular sperm from non-azoospermic men with high SDF. Thus, continuous monitoring and more extensive investigation concerning the offspring health are warranted.

Percutaneous and open sperm retrieval methods are highly effective for harvesting sperm from non-azoospermic men with high SDF in the semen (Esteves et al. 2017b). These procedures are commonly performed on an outpatient basis in association with oocyte retrieval and immediate sperm injection. Since men with high SDF in the semen have various degrees of complete spermatogenesis, unlike men with nonobstructive azoospermia (NOA), sperm retrieval can be carried out using percutaneous or open methods with minimal tissue excision without the aid of microsurgery (Miyaoka et al. 2018). Additionally, the testes of such men are usually adequate in size. Significant adverse effects occasionally seen after SR in men with NOA, such as reduction of testosterone production and potential testis atrophy, are unlikely to occur (Ramasamy et al. 2005). However, given the potential for complications, including pain, swelling, infection, and hematoma, SR should be performed by reproductive urologists who are familiar with testicular anatomy.

Proposed Algorithm for SDF Testing and Use of Testicular Sperm for ICSI in Non-azoospermic Men with High SDF in the Semen

Our current practice at the ANDROFERT Fertility Center is to routinely offer SDF testing for male partners of couples with unexplained infertility, recurrent pregnancy loss, and before intrauterine insemination, conventional IVF, and ICSI, in particular, among couples with a history of failed IUI or ART. In the presence of high SDF, we offer our patients interventions to reduce SDF as previously discussed in this chapter. When SDF levels remain high despite treatment of the underlying conditions—or no apparent factor explaining the abnormal SDF rates is identified—we routinely use testicular sperm for ICSI (Esteves 2018a, b) (Fig. 8.3). Given the still limited clinical evidence supporting the use of Testi-ICSI, the benefits and risks of this option should be discussed with the affected patients.

Conclusions

Given the essential role of sperm DNA integrity for normal embryo development and pregnancy outcome, all efforts should be made to properly counsel infertile men about risks of using sperm with elevated SDF for both natural and assisted insemination. Many conditions associated with SDF can be correctable, including varicocele, lifestyle factors, and genital infections. Correction of underlying factors can alleviate SDF and potentially enable natural conception or increase the likelihood of pregnancy by ART. Moreover, offspring obtained by sperm with low levels of DNA damage have a decreased risk of congenital and epigenetic disorders. A comprehensive male infertility evaluation is essential to identify the causes of infertility and allow treatment to reduce SDF. Although further research is needed to confirm the positive role of interventions on reducing SDF and how they translate in improving fertility, the apparent association between SDF and the risk factors discussed in this chapter makes

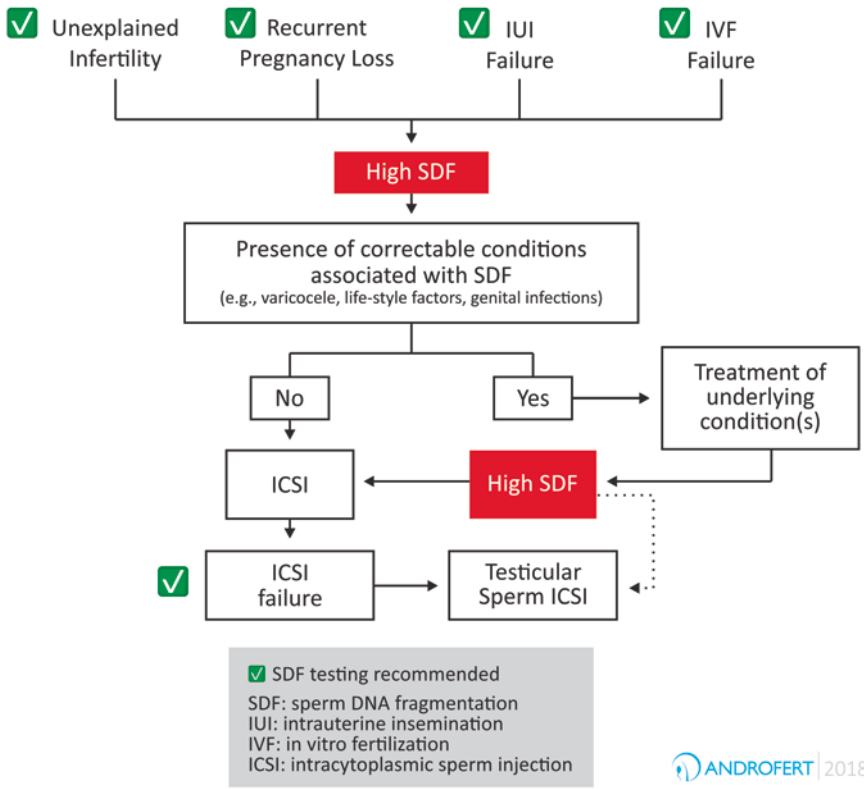

Fig. 8.3 Proposed algorithm for sperm DNA fragmentation testing and use of testicular sperm for ICSI. Solid arrows indicate the preferential decision tree whereas the dotted arrow indicates an optional approach

sperm DNA testing an attractive tool to identify individuals at risk and monitor the response to interventions.

Key Points

- Sperm DNA integrity is essential for healthy human embryo development and successful pregnancy outcome. In addition to the risk of infertility and impaired reproductive outcomes, there is an increased risk of diseases in offspring when natural or artificial inseminations are carried out with specimens containing high frequencies of sperm with fragmented DNA.
- Sperm DNA fragmentation (SDF) tests have been used to obtain information about sperm DNA quality, particularly for the evaluation of a possible male factor contributing to infertility.
- Recently, in 2017, a clinical practice guideline issued by the Society for Translational Medicine has provided recommendations for SDF testing. SDF testing is recommended (i) after failed IUI, IVF, or ICSI cycles provided no other apparent reason exists to explain that failure, (ii) to couples with unexplained infertility and those suffering from recurrent pregnancy loss, and (iii) to patients with risk factors for OS, including but not limited to lifestyle conditions (e.g., smoking, obesity, metabolic syndrome), varicocele, genital infections, advanced age, and exposure to toxicants (e.g., environmental, licit or illicit drugs, radiation, chemotherapy).
- Many conditions associated with SDF can be correctable, including varicocele, lifestyle factors, and genital infections.
- Overwhelming evidence indicates that varicocele causes oxidative-induced SDF. Repair of

palpable varicoceles reduces sperm DNA damage, thus potentially improving fertility.

- Fair evidence indicates that lifestyle factors, including smoking and exposure to environmental/occupational chemicals, obesity, and advanced paternal age, have a negative on sperm DNA quality. Dietary and lifestyle changes could reduce SDF. Further research is warranted to determine how these changes may translate into better reproductive outcomes; information provided by SDF testing gives solid grounds for implementing lifestyle changes as well as monitoring patient compliance in health prevention programs.
- Oral antioxidant intake can be used to decrease the oxidatively induced sperm DNA damage. In general, there is a beneficial effect of antioxidants on measures of SDF. However, the effects of AOX on pregnancy outcomes and the optimal regimen, dosage, duration, and ideal treatment candidates need to be clarified further.
- MAPI increases inflammatory response and reactive oxygen species production, thus causing SDF. Aerobes, anaerobes, *Chlamydia trachomatis*, and *Ureaplasma urealyticum* are the primary pathogens. Antibiotic treatment can reduce seminal white blood cell (WBC) and ROS levels, thus lowering the frequency of sperm with fragmented DNA. Pregnancy success seems to be increased in treated men.
- Empirical therapy with exogenous FSH administration seems to reduce DFI in both men with idiopathic or unexplained infertility, an effect that might be modulated by FSH receptor genotype. Further studies are warranted to determine how the effect of this intervention translates into better reproductive outcomes.
- FSH treatment seems to improve sperm DFI mainly in idiopathic infertile men with the p.N680S homozygous N FSHR.
- Correction of underlying factors can alleviate SDF and potentially enable natural conception or increase the likelihood of pregnancy by ART.
- When SDF remains high after treatment of the underlying factors or no apparent condition is identified to allow potential treatment, the use of testicular sperm (Testi-ICSI) in preference over ejaculated sperm for ICSI might overcome the oxidative-induced SDF.
- Sperm DNA fragmentation is markedly lower in testicular sperm than in the ejaculated sperm. Testi-ICSI may bypass post-testicular chromatin damage caused by OS during sperm transit through the epididymis.
- Data from observational studies suggest that pregnancy outcomes by Testi-ICSI in men with high SDF in the semen are significantly better than that of ejaculated sperm. Additionally, miscarriage rates are lower with the former.
- Current evidence suggests that Testi-ICSI results in higher live birth rates than the current laboratory methods used to select specimens with lower SDF levels.
- Well-designed prospective randomized trials are needed to recommend Testi-ICSI as a means to overcome the oxidative-induced SDF in routine clinical practice. Furthermore, more investigation is required to determine the influence of this approach on offspring health.
- Knowledge of the SDF status can be used to strengthen patient counselling and allow clinicians to provide a more realistic prognosis of every treatment strategy the couple wishes to pursue.

References

Abad C, Amengual MJ, Gosalvez J, Coward K, Hannaoui N, Benet J et al (2013) Effects of oral antioxidant treatment upon the dynamics of human sperm DNA fragmentation and subpopulations of sperm with highly degraded DNA. Andrologia 45:211–216

Abdelbaki SA, Sabry JH, Al-Adl AM, Sabry HH (2017) The impact of coexisting sperm DNA fragmentation and seminal oxidative stress on the outcome of varicocelectomy in infertile patients: a prospective controlled study. Arab J Urol 15:131–139

Abd-Elmoaty MA, Saleh R, Sharma R, Agarwal A (2010) Increased levels of oxidants and reduced antioxidants in semen of infertile men with varicocele. Fertil Steril 94:1531–1534

Agarwal A, Hamada A, Esteves SC (2012) Insight into oxidative stress in varicocele-associated male infertility: part 1. Nat Rev Urol 9:678–690

Agarwal A, Mulgund A, Alshahrani S, Assidi M, Abuzenadah AM, Sharma R et al (2014) Reactive oxygen species and sperm DNA damage in infertile men presenting with low level leukocytospermia. Reprod Biol Endocrinol 12:126

Agarwal A, Cho CL, Esteves SC (2016a) Should we evaluate and treat sperm DNA fragmentation? Curr Opin Obstet Gynecol 28:164–171

Agarwal A, Majzoub A, Esteves SC, Ko E, Ramasamy R, Zini A (2016b) Clinical utility of sperm DNA fragmentation testing: practice recommendations based on clinical scenarios. Transl Androl Urol 5:935–950

Agarwal A, Sharma R, Roychoudhury S, Du Plessis S, Sabanegh E (2016c) MiOXSYS: a novel method of measuring oxidation reduction potential in semen and seminal plasma. Fertil Steril 106:566–573

Agarwal A, Cho CL, Majzoub A, Esteves SC (2017a) The Society for Translational Medicine: clinical practice guidelines for sperm DNA fragmentation testing in male infertility. Transl Androl Urol 6(Suppl 4):720–733

Agarwal A, Cho C-L, Esteves SC, Majzoub A (2017b) Reactive oxygen species and sperm DNA fragmentation. Transl Androl Urol 6(Suppl 4):695–696

Agarwal A, Arafa M, Chandrakumar R, Majzoub A, AlSaid S, Elbardisi H (2017c) A multicenter study to evaluate oxidative stress by oxidation-reduction potential, a reliable and reproducible method. Andrology 5:939–945

Aitken RJ (2017) DNA damage in human spermatozoa; important contributor to mutagenesis in the offspring. Transl Androl Urol 6(Suppl 4):761–764

Aitken RJ (2018) Not every sperm is sacred; a perspective on male infertility. Mol Hum Reprod 24(6):287–298. https://doi.org/10.1093/molehr/gay010

Aitken RJ, Smith TB, Jobling MS, Baker MA, De Iuliis GN (2014) Oxidative stress and male reproductive health. Asian J Androl 16:31–38

Alhathal N, San Gabriel M, Zini A (2016) Beneficial effects of microsurgical varicocoelectomy on sperm maturation, DNA fragmentation, and nuclear sulfhydryl groups: a prospective trial. Andrology 4:1204–1208

Allamaneni SS, Naughton CK, Sharma RK, Thomas AJ Jr, Agarwal A (2004) Increased seminal reactive oxygen species levels in patients with varicoceles correlate with varicocele grade but not with testicular size. Fertil Steril 82:1684–1686

Alshahrani S, Agarwal A, Assidi M, Abuzenadah AM, Durairajanayagam D, Ayaz A et al (2014) Infertile men older than 40 years are at higher risk of sperm DNA damage. Reprod Biol Endocrinol 12:103

Arafa M, AlMalki A, AlBadr M et al (2018) ICSI outcome in patients with high DNA fragmentation: Testicular versus ejaculated spermatozoa. Andrologia 50(1)

Baker K, McGill J, Sharma R, Agarwal A, Sabanegh E Jr (2013) Pregnancy after varicocelectomy: impact of postoperative motility and DFI. Urology 81:760–766

Bauersachs J, Widder JD (2010) Reductive stress: linking heat shock protein 27, glutathione, and cardiomyopathy? Hypertension 55(6):1299–1300

Bertoncelli Tanaka M, Agarwal A, Esteves SC (2018) Paternal age and assisted reproductive technologies: problem solver or trouble maker. Panminerva Med 61:138. https://doi.org/10.23736/S0031-0808.18.03512-7. [Epub ahead of print

Best D, Avenell A, Bhattacharya S (2017) How effective are weight-loss interventions for improving fertility in women and men who are overweight or obese? A systematic review and meta-analysis of the evidence. Hum Reprod Update 23:681–705

Blumer CG, Restelli AE, Giudice PT, Soler TB, Fraietta R, Nichi M et al (2012) Effect of varicocele on sperm function and semen oxidative stress. BJU Int 109:259–265

Bradley CK, McArthur SJ, Gee AJ, Weiss KA, Schmidt U, Toogood L (2016) Intervention improves assisted conception intracytoplasmic sperm injection outcomes for patients with high levels of sperm DNA fragmentation: a retrospective analysis. Andrology 4:903–910

Campbell JM, Lane M, Owens JA, Bakos HW (2015) Paternal obesity negatively affects male fertility and assisted reproduction outcomes: a systematic review and meta-analysis. Reprod Biomed Online 31:593–604

Casarini L, Moriondo V, Marino M, Adversi F, Capodanno F, Grisolia C et al (2014) FSHR polymorphism p.N680S mediates different responses to FSH in vitro. Mol Cell Endocrinol 393:83–91

Chabory E, Damon C, Lenoir A, Kauselmann G, Kern H, Zevnik B et al (2009) Epididymis seleno-independent glutathione peroxidase 5 maintains sperm DNA integrity in mice. J Clin Invest 119:2074–2085

Champroux A, Torres-Carreira J, Gharagozloo P, Drevet JR, Kocer A (2016) Mammalian sperm nuclear organization: resiliencies and vulnerabilities. Basic Clin Androl 26:17

Chen SS, Huang WJ, Chang LS, Wei YH (2008) Attenuation of oxidative stress after varicocelectomy in subfertile patients with varicocele. J Urol 179:639–642

Cheung S, Schlegel PN, Rosenwaks Z, Palermo GD (2019) Revisiting aneuploidy profile of surgically retrieved spermatozoa by whole exome sequencing molecular karyotype. PLoS One 14:e0210079

Cho CL, Esteves SC, Agarwal A (2016) Novel insights into the pathophysiology of varicocele and its association with reactive oxygen species and sperm DNA fragmentation. Asian J Androl 18:186–193

Colacurci N, Monti MG, Fornaro F, Izzo G, Izzo P, Trotta C et al (2012) Recombinant human FSH reduces sperm DNA fragmentation in men with idiopathic oligoasthenoteratozoospermia. J Androl 33:588–593

Colacurci N, De Leo V, Ruvolo G, Piomboni P, Caprio F, Pivonello R et al (2018) Recombinant FSH improves sperm DNA damage in male infertility: a phase II clinical trial. Front Endocrinol (Lausanne) 9:383

Condorelli RA, La Vignera S, Mongioì LM, Alamo A, Calogero AE (2018) Diabetes mellitus and infertility: different pathophysiological effects in type 1 and type 2 on sperm function. Front Endocrinol (Lausanne) 9:268

Craig JR, Jenkins TG, Carrell DT, Hotaling JM (2017) Obesity, male infertility, and the sperm epigenome. Fertil Steril 107:848–859

Cunha BA, Garabedian-Ruffalo SM (1980) Tetracyclines in urology: current concepts. Urology 36:54856

Dada R (2017 Sep) Sperm DNA damage diagnostics: when and why. Transl Androl Urol 6(Suppl 4):691–694

Dehghan Marvast L, Talebi AR, Ghasemzadeh J, Hosseini A, Pacey AA (2018) Effects of Chlamydia trachomatis infection on sperm chromatin condensation and DNA integrity. Andrologia 50(3)

Esteves SC (2016) Novel concepts in male factor infertility: clinical and laboratory perspectives. J Assist Reprod Genet 33:1319–1335

Esteves SC (2018a) Should a Couple with Failed In Vitro Fertilization or Intracytoplasmic Sperm Injection and Elevated Sperm DNA Fragmentation Use Testicular Sperm for the Next Cycle? Eur Urol Focus 4:296–298

Esteves SC (2018b) Testicular versus ejaculated sperm should be used for intracytoplasmic sperm injection (ICSI) in cases of infertility associated with sperm DNA fragmentation | Opinion: Yes. Int Braz J Urol 44:667–675

Esteves SC, Agarwal A (2017) Afterword to varicocele and male infertility: current concepts and future perspectives. Asian J Androl 18:319–322

Esteves SC, Hamada A, Kondray V, Pitchika A, Agarwal A (2012) What every gynecologist should know about male infertility: an update. Arch Gynecol Obstet 286:217–229

Esteves SC, Sharma RK, Gosálvez J, Agarwal A (2014) A translational medicine appraisal of specialized andrology testing in unexplained male infertility. Int Urol Nephrol 46:1037–1052

Esteves SC, Gosálvez J, López-Fernández C, Núñez-Calonge R, Caballero P, Agarwal A et al (2015a) Diagnostic accuracy of sperm DNA degradation index (DDSi) as a potential noninvasive biomarker to identify men with varicocele-associated infertility. Int Urol Nephrol 47:1471–1477

Esteves SC, Sanchez-Martin F, Sanchez-Martin P, Schneider DT, Gosalvez J (2015b) Comparison of reproductive outcome in oligozoospermic men with high sperm DNA fragmentation undergoing intracytoplasmic sperm injection with ejaculated and testicular sperm. Fertil Steril 104:1398–1405

Esteves SC, Roque M, Bradley CK, Garrido N (2017a) Reproductive outcomes of testicular versus ejaculated sperm for intracytoplasmic sperm injection among men with high levels of DNA fragmentation in semen: systematic review and meta-analysis. Fertil Steril 108:456–467

Esteves SC, Agarwal A, Cho CL, Majzoub A (2017b) A Strengths-Weaknesses-Opportunities-Threats (SWOT) analysis on the clinical utility of sperm DNA fragmentation testing in specific male infertility scenarios. Transl Androl Urol 6(Suppl 4):734–760

Esteves SC, Agarwal A, Majzoub A (2017c) The complex nature of the sperm DNA damage process. Transl Androl Urol 6(Suppl 4):S557–S559

Esteves SC, Agarwal A, Majzoub A (2017d) An evidence-based perspective on the role of sperm chromatin integrity and sperm DNA fragmentation testing in male infertility. Transl Androl Urol 6(Suppl 4):S665–S672

Faure C, Dupont C, Baraibar MA, Ladouce R, Cedrin-Durnerin I, Wolf JP et al (2014) In subfertile couple, abdominal fat loss in men is associated with improvement of sperm quality and pregnancy: a case-series. PLoS One 9(2):e86300

Feng Z, Hu W, Amin S, Tang MS (2003) Mutational spectrum and genotoxicity of the major lipid peroxidation product, trans-4-hydroxy-2-nonenal, induced DNA adducts in nucleotide excision repair-proficient and -deficient human cells. Biochemistry 42:7848–7854

Fraczek M, Hryhorowicz M, Gill K, Zarzycka M, Gaczarzewicz D, Jedrzejczak P et al (2016) The effect of bacteriospermia and leukocytospermia on conventional and nonconventional semen parameters in healthy young normozoospermic males. J Reprod Immunol 118:18–27

Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN (1991) Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. Proc Natl Acad Sci U S A 88:11003–11006

Gallegos G, Ramos B, Santiso R, Goyanes V, Gosalvez J, Fernandez JL (2008) Sperm DNA fragmentation in infertile men with genitourinary infection by Chlamydia trachomatis and Mycoplasma. Fertil Steril 90:328–334

Gandhi J, Hernandez RJ, Chen A, Smith NL, Sheynkin YR, Joshi G et al (2017) Impaired hypothalamic-pituitary-testicular axis activity, spermatogenesis, and sperm function promote infertility in males with lead poisoning. Zygote 25:103–110

Garolla A, Ghezzi M, Cosci I, Sartini B, Bottacin A, Engl B et al (2017) FSH treatment in infertile males candidate to assisted reproduction improved sperm DNA fragmentation and pregnancy rate. Endocrine 56:416–425

Gómez-Torres MJ, Medrano L, Romero A, Fernández-Colom PJ, Aizpurúa J (2017) Effectiveness of human spermatozoa biomarkers as indicators of structural damage during cryopreservation. Cryobiology 78:90–94

Gosálvez J, Lopez-Fernandez C, Fernandez JL, Esteves SC, Johnston SD (2015) Unpacking the mysteries of sperm DNA fragmentation: ten frequently asked questions. J Reprod Biotechnol Fertil 4:1–16

Gosálvez J, Coppola L, Fernández JL, López-Fernández C, Góngora A, Faundez R et al (2017) Multi-centre assessment of nitroblue tetrazolium reactivity in human semen as a potential marker of oxidative stress. Reprod Biomed Online 34:513–521

Gosálvez J, Fernández JL, Esteves SC (2018) Response: Nitroblue tetrazolium (NBT) assay. Reprod Biomed Online 36:92–93

Greco E, Romano S, Iacobelli M, Ferrero S, Baroni E, Minasi MG et al (2005a) ICSI in cases of sperm DNA damage: beneficial effect of oral antioxidant treatment. Hum Reprod 20:2590–2594

Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J (2005b) Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. J Androl 26:349–353

Greco E, Scarselli F, Iacobelli M et al (2005c) Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. Hum Reprod 20(1):226–230

Gual-Frau J, Abad C, Amengual MJ, Hannaoui N, Checa MA, Ribas-Maynou J et al (2015) Oral antioxidant treatment partly improves integrity of human sperm DNA in infertile grade I varicocele patients. Hum Fertil (Camb) 18:225–229

Hamada A, Esteves SC, Nizza M, Agarwal A (2012) Unexplained male infertility: diagnosis and management. Int Braz J Urol 38:576–594

Hamada A, Esteves SC, Agarwal A (2013) Insight into oxidative stress in varicoceleassociated male infertility: part 2. Nat Rev Urol 10:26–37

Hammoud I, Bailly M, Bergere M et al (2017) Testicular Spermatozoa Are of Better Quality Than Epididymal Spermatozoa in Patients With Obstructive Azoospermia. Urology 103:106–111

Hendin BN, Kolettis PN, Sharma RK, Thomas AJ Jr, Agarwal A (1999) Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. J Urol 161:1831–1834

Houfflyn S, Matthys C, Soubry A (2017) Male obesity: epigenetic origin and effects in sperm and offspring. Curr Mol Biol Rep 3:288–296

Hurtado de Catalfo GE, Ranieri-Casilla A, Marra FA, de Alaniz MJ, Marra CA (2007) Oxidative stress biomarkers and hormonal profile in human patients undergoing varicocelectomy. Int J Androl 30:519–530

Ishikawa T, Fujioka H, Ishimura T, Takenaka A, Fujisawa M (2007) Increased testicular 8-hydroxy-2′-deoxyguanosine in patients with varicocele. BJU Int 100:863–866

Jamal F, Haque QS, Singh S, Rastogi SK (2016) The influence of organophosphate and carbamate on sperm chromatin and reproductive hormones among pesticide sprayers. Toxicol Ind Health 32:1527–1536

Jeng HA, Pan CH, Chao MR, Chiu CC, Zhou G, Chou CK et al (2016) Sperm quality and DNA integrity of coke oven workers exposed to polycyclic aromatic hydrocarbons. Int J Occup Med Environ Health 29:915–926

Jurewicz J, Radwan M, Sobala W, Radwan P, Bochenek M, Hanke W (2018) Dietary patterns and their relationship with semen quality. Am J Mens Health 12:575–583

Kaarouch I, Bouamoud N, Madkour A, Louanjli N, Saadani B, Assou S et al (2018) Paternal age: Negative impact on sperm genome decays and IVF outcomes after 40 years. Mol Reprod Dev 85:271–280

Kadioglu TC, Aliyev E, Celtik M (2014) Microscopic varicocelectomy significantly decreases the sperm DNA fragmentation index in patients with infertility. Biomed Res Int:695713

Kamischke A, Behre HM, Bergmann M, Simoni M, Schäfer T, Nieschlag E (1998) Recombinant human follicle stimulating hormone for treatment of male idiopathic infertility: a randomized, double-blind, placebo-controlled, clinical trial. Hum Reprod 13:596–603

Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T (1997) Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. Fertil Steril 68:519–524

Köksal IT, Tefekli A, Usta M, Erol H, Abbasoglu S, Kadioglu A (2000) The role of reactive oxygen species in testicular dysfunction associated with varicocele. BJU Int 86:549–552

Krawetz SA (2005) Paternal contribution: new insights and future challenges. Nat Rev Genet 6(8):633–642

Kumar SB, Chawla B, Bisht S, Yadav RK, Dada R (2015) Tobacco Use Increases Oxidative DNA Damage in Sperm – Possible Etiology of Childhood Cancer. Asian Pac J Cancer Prev 16:6967–6972

La Vignera S, Vita R (2018) Thyroid dysfunction and semen quality. Int J Immunopathol Pharmacol 32:2058738418775241

La Vignera S, Condorelli R, Vicari E, D'Agata R, Calogero AE (2012) Effects of varicocelectomy on sperm DNA fragmentation, mitochondrial function, chromatin condensation, and apoptosis. J Androl 33:389–396

La Vignera S, Vita R, Condorelli RA, Mongioì LM, Presti S, Benvenga S et al (2017) Impact of thyroid disease on testicular function. Endocrine 58:397–407

Lacerda JI, Del Giudice PT, da Silva BF, Nichi M, Fariello RM, Fraietta R et al (2011) Adolescent varicocele: improved sperm function after varicocelectomy. Fertil Steril 95:994–999

Lafuente R, García-Blàquez N, Jacquemin B, Checa MA (2016) Outdoor air pollution and sperm quality. Fertil Steril 106:880–896

Leisegang K, Bouic PJ, Henkel RR (2016) Metabolic syndrome is associated with increased seminal inflammatory cytokines and reproductive dysfunction in a case-controlled male cohort. Am J Reprod Immunol 76:155–163

Lewis SE, Aitken RJ (2005) DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell Tissue Res 322:33–41

Li F, Yamaguchi K, Okada K, Matsushita K, Ando M, Chiba K et al (2012) Significant improvement of sperm DNA quality after microsurgical repair of varicocele. Syst Biol Reprod Med 58:274–247

Lopes S, Jurisicova A, Sun JG, Casper RF (1998) Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Hum Reprod 13:896–900

Lu X, Huang Y, Zhang H, Zhao J (2017) Effect of diabetes mellitus on the quality and cytokine content of human semen. J Reprod Immunol 123:1–2

Majzoub A, Esteves SC, Gosálvez J, Agarwal A (2016) Specialized sperm function tests in varicocele and

the future of andrology laboratory. Asian J Androl 18:205–212

Majzoub A, Agarwal A, Cho CL, Esteves SC (2017a) Sperm DNA fragmentation testing: a cross sectional survey on current practices of fertility specialists. Transl Androl Urol 6(Suppl 4):710–719

Majzoub A, Agarwal A, Esteves SC (2017b) Antioxidants for elevated sperm DNA fragmentation: a mini review. Transl Androl Urol 6(Suppl 4):S649–S653

Maresch CC, Stute DC, Alves MG, Oliveira PF, de Kretser DM, Linn T (2018) Diabetes-induced hyperglycemia impairs male reproductive function: a systematic review. Hum Reprod Update 24:86–105

Martínez-Soto JC, Domingo JC, Cordobilla B, Nicolás M, Fernández L, Albero P et al (2016) Dietary supplementation with docosahexaenoic acid (DHA) improves seminal antioxidant status and decreases sperm DNA fragmentation. Syst Biol Reprod Med 62:387–395

Meamar M, Zribi N, Cambi M, Tamburrino L, Marchiani S, Filimberti E et al (2012) Sperm DNA fragmentation induced by cryopreservation: new insights and effect of a natural extract from Opuntia ficus-indica. Fertil Steril 98:326–333

Mehraban D, Ansari M, Keyhan H, Sedighi Gilani M, Naderi G, Esfehani F (2005) Comparison of nitric oxide concentration in seminal fluid between infertile patients with and without varicocele and normal fertile men. J Urol 2:106–110

Mehta A, Bolyakov A, Schlegel PN, Paduch DA (2015) Higher pregnancy rates using testicular sperm in men with severe oligospermia. Fertil Steril 104:1382–1387

Ménézo YJ, Hazout A, Panteix G, Robert F, Rollet J, Cohen-Bacrie P et al (2007) Antioxidants to reduce sperm DNA fragmentation: an unexpected adverse effect. Reprod Biomed Online 14:418–421

Micillo A, Vassallo MR, Cordeschi G, D'Andrea S, Necozione S, Francavilla F et al (2016) Semen leukocytes and oxidative-dependent DNA damage of spermatozoa in male partners of subfertile couples with no symptoms of genital tract infection. Andrology 4:808–815

Miranda-Contreras L, Cruz I, Osuna JA, Gómez-Pérez R, Berrueta L, Salmen S et al (2015) Effects of occupational exposure to pesticides on semen quality of workers in an agricultural community of Merida state. Venezuela Invest Clin 56:123–136

Miyaoka R, Orosz JE, Achermann AP, Esteves SC (2018) Methods of surgical sperm extraction and implications for ART success. Panminerva Med. [ahead of print]

Mohammed EE, Mosad E, Zahran AM, Hameed DA, Taha EA, Mohamed MA (2015) Acridine orange and flow cytometry: which is better to measure the effect of varicocele on sperm DNA integrity? Adv Urol:814150

Moskovtsev SI, Lecker I, Mullen JB, Jarvi K, Willis J, White J et al (2009) Cause-specific treatment in patients with high sperm DNA damage resulted in significant DNA improvement. Syst Biol Reprod Med 55:109–115

Moskovtsev SI, Jarvi K, Mullen JB, Cadesky KI, Hannam T, Lo KC (2010) Testicular spermatozoa have statistically significantly lower DNA damage compared with ejaculated spermatozoa in patients with unsuccessful oral antioxidant treatment. Fertil Steril 93(4):1142–1146

Mostafa T, Anis TH, El-Nashar A, Imam H, Othman IA (2001) Varicocelectomy reduces reactive oxygen species levels and increases antioxidant activity of seminal plasma from infertile men with varicocele. Int J Androl 24:261–265

Mostafa T, As T, Imam H, El-Nashar AR, Osman IA (2009) Seminal reactive oxygen species—antioxidant relationship in fertile males with and without varicocele. Andrologia 41:125–129

Muratori M, Tamburrino L, Marchiani S, Cambi M, Olivito B, Azzari C et al (2015) Investigation on the origin of sperm DNA fragmentation: role of apoptosis, immaturity and oxidative stress. Mol Med 21:109–122

Naber KG, Kinzig M, Sorgel F, Weigel D (1993) Penetration of ofloxacin into prostatic fluid, ejaculate and seminal fluid. Infection 21:98–100

Nasr-Esfahani MH, Abasi H, Razavi S, Ashrafi S, Tavalaee M (2009) Varicocelectomy: semen parameters and protamine deficiency. Int J Androl 32:115–122

Ni K, Steger K, Yang H, Wang H, Hu K, Chen B (2014) Sperm protamine mRNA ratio and DNA fragmentation index represent reliable clinical biomarkers for men with varicocele after microsurgical varicocele ligation. J Urol 192:170–176

Ni K, Steger K, Yang H, Wang H, Hu K, Zhang T et al (2016) A Comprehensive investigation of sperm DNA damage and oxidative stress injury in infertile patients with subclinical, normozoospermic and astheno/oligozoospermic clinical varicocele. Andrology 4:816–824

Nijs M, De Jonge C, Cox A, Janssen M, Bosmans E, Ombelet W (2011) Correlation between male age, WHO sperm parameters, DNA fragmentation, chromatin packaging and outcome in assisted reproduction technology. Andrologia 43:174–179

Ochsendorf FR (1999) Infections in the male genital tract and reactive oxygen species. Hum Reprod Update 5:399–420

O'Connell M, McClure N, Lewis SE (2002) Mitochondrial DNA deletions and nuclear DNA fragmentation in testicular and epididymal human sperm. Hum Reprod 17(6):1565–1570

Omu AE, Al-Azemi MK, Kehinde EO, Anim JT, Oriowo MA, Mathew TC (2008) Indications of the mechanisms involved in improved sperm parameters by zinc therapy. Med Princ Pract 17:108–116

Pabuccu EG, Caglar GS, Tangal S, Haliloglu AH, Pabuccu R (2017) Testicular versus ejaculated spermatozoa in ICSI cycles of normozoospermic men with high sperm DNA fragmentation and previous ART failures. Andrologia 49(2)

Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ Jr, Agarwal A (2000) Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. Fertil Steril 73:459–464

Pasqualotto FF, Sundaram A, Sharma RK, Borges E Jr, Pasqualotto EB, Agarwal A. Semen quality and oxidative stress scores in fertile and infertile patients with varicocele. Fertil Steril 2008;89:602–607

Piomboni P, Gambera L, Serafini F, Campanella G, Morgante G, De Leo V (2008) Sperm quality improvement after natural anti-oxidant treatment of asthenoteratospermic men with leukocytospermia. Asian J Androl 10:201–206

Pourmand G, Movahedin M, Dehghani S, Mehrsai A, Ahmadi A, Pourhosein M et al (2014) Does L-carnitine therapy add any extra benefit to standard inguinal varicocelectomy in terms of deoxyribonucleic acid damage or sperm quality factor indices: a randomized study. Urology 84:821–825

Puerta Suarez J, Sanchez LR, Salazar FC, Saka HA, Molina R, Tissera A et al (2017) Chlamydia trachomatis neither exerts deleterious effects on spermatozoa nor impairs male fertility. Sci Rep 7:1126

Radwan M, Jurewicz J, Polańska K, Sobala W, Radwan P, Bochenek M et al (2016) Exposure to ambient air pollution--does it affect semen quality and the level of reproductive hormones? Ann Hum Biol 43:50–56

Ramasamy R, Yagan N, Schlegel PN (2005) Structural and functional changes to the testis after conventional versus microdissection testicular sperm extraction. Urology 65(6):1190–1194

Rima D, Shiv BK, Bhavna C, Shilpa B, Saima K (2016) Oxidative stress induced damage to paternal genome and impact of meditation and Yoga – can it reduce incidence of childhood cancer? Asian Pac J Cancer Prev 17:4517–4525

Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S et al (2012) The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod 27:2908–2917

Roque M, Esteves SC (2018) Effect of varicocele repair on sperm DNA fragmentation: a review. Int Urol Nephrol 50:583–603

Sakamoto Y, Ishikawa T, Kondo Y, Yamaguchi K, Fujisawa M (2008) The assessment of oxidative stress in infertile patients with and without varicocele. BJU Int 101:1547–1552

Saleh RA, Agarwal A, Nelson DR, Nada EA, El-Tonsy MH, Alvarez JG et al (2002) Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study. Fertil Steril 78:313–318

Sánchez-Peña LC, Reyes BE, López-Carrillo L, Recio R, Morán-Martínez J, Cebrián ME et al (2004) Organophosphorous pesticide exposure alters sperm chromatin structure in Mexican agricultural workers. Toxicol Appl Pharmacol 196:108–113

Schramm P (1986) Ofloxacin: concentration in human ejaculate and influence on sperm motility. Infection 14(Suppl 4):274–275

Sergerie M, Laforest G, Boulanger K, Bissonnette F, Bleau G (2005) Longitudinal study of sperm DNA fragmentation as measured by terminal uridine nick end-labelling assay. Hum Reprod 20:1921–1927

Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ Jr, Agarwal A (1999) The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod 14:2801–2807

Sharma R, Agarwal A, Harlev A, Esteves SC (2017) A meta-analysis to study the effects of body mass index on sperm DNA fragmentation index in reproductive age men. Fertil Steril 108:e138–e139

Shiraishi K, Matsuyama H (2017) Gonadotoropin actions on spermatogenesis and hormonal therapies for spermatogenic disorders. Endocr J 64:123–131

Showell MG, Mackenzie-Proctor R, Brown J, Yazdani A, Stankiewicz MT, Hart RJ (2014) Antioxidants for male subfertility. Cochrane Database Syst Rev:CD007411

Simon L, Emery BR, Carrell DT (2017) Review: Diagnosis and impact of sperm DNA alterations in assisted reproduction. Best Pract Res Clin Obstet Gynaecol 44:38–56

Smit M, Romijn JC, Wildhagen MF, Veldhoven JL, Weber RF, Dohle GR (2010) Decreased sperm DNA fragmentation after surgical varicocelectomy is associated with increased pregnancy rate. J Urol 183:270–274

Steele EK, McClure N, Maxwell RJ, Lewis SE (1999) A comparison of DNA damage in testicular and proximal epididymal spermatozoa in obstructive azoospermia. Mol Hum Reprod 5(9):831–835

Suganuma R, Yanagimachi R, Meistrich ML (2005) Decline in fertility of mouse sperm with abnormal chromatin during epididymal passage as revealed by ICSI. Hum Reprod 20(11):3101–3108

Sun XL, Wang JL, Peng YP, Gao QQ, Song T, Yu W et al (2018) Bilateral is superior to unilateral varicocelectomy in infertile males with left clinical and right subclinical varicocele: a prospective randomized controlled study. Int Urol Nephrol 50:205–210

Tavalaee M, Bahreinian M, Barekat F, Abbasi H, Nasr-Esfahani MH (2015) Effect of varicocelectomy on sperm functional characteristics and DNA methylation. Andrologia 47:904–909

Telli O, Sarici H, Kabar M, Ozgur BC, Resorlu B, Bozkurt S (2015) Does varicocelectomy affect DNA fragmentation in infertile patients? Indian J Urol 31:116–119

Tremellen K (2008) Oxidative stress and male infertility—a clinical perspective. Hum Reprod Update 14:243–258

Tunc O, Thompson J, Tremellen K (2009) Improvement in sperm DNA quality using an oral antioxidant therapy. Reprod Biomed Online 18:761–768

Tvrdá E, López-Fernández C, Sánchez-Martín P, Gosálvez J (2018) Sperm DNA fragmentation in donors and normozoospermic patients attending for a first spermiogram: Static and dynamic assessment. Andrologia 50. https://doi.org/10.1111/and.12986.. Epub ahead of print

Vani K, Kurakula M, Syed R, Alharbi K (2012) Clinical relevance of vitamin C among lead-exposed infertile men. Genet Test Mol Biomarkers 16:1001–1006

Varshini J, Srinag BS, Kalthur G, Krishnamurthy H, Kumar P, Rao SB et al (2012) Poor sperm quality and

advancing age are associated with increased sperm DNA damage in infertile men. Andrologia 44(Suppl 1):642–649

Vicari E (2000) Effectiveness and limits of antimicrobial treatment on seminal leukocyte concentration and related reactive oxygen species production in patients with male accessory gland infection. Hum Reprod 15:2536–2544

Werthman P, Wixon R, Kasperson K, Evenson DP (2008) Significant decrease in sperm deoxyribonucleic acid fragmentation after varicocelectomy. Fertil Steril 90:1800–1804

Zaazaa A, Adel A, Fahmy I, Elkhiat Y, Awaad AA, Mostafa T (2018) Effect of varicocelectomy and/or mast cells stabilizer on sperm DNA fragmentation in infertile patients with varicocele. Andrology 6:146–150

Zeyad A, Hamad M, Amor H, Hammadeh ME (2018) Relationships between bacteriospermia, DNA integrity, nuclear protamine alteration, sperm quality and ICSI outcome. Reprod Biol 18:115–121

Zhang J, Xue H, Qiu F, Zhong J, Su J (2019) Testicular spermatozoon is superior to ejaculated spermatozoon for intracytoplasmic sperm injection to achieve pregnancy in infertile males with high sperm DNA damage. Andrologia 51(2):e13175. https://doi.org/10.1111/and.13175

Zhao J, Zhang Q, Wang Y, Li Y (2014a) Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnant and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Fertil Steril 102:998–1005

Zhao J, Zhang Q, Wang Y, Li Y (2014b) Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Fertil Steril 102(4):998–1005.e1008

Zhou DD, Hao JL, Guo KM, Lu CW, Liu XD (2016) Sperm quality and DNA damage in men from Jilin Province, China, who are occupationally exposed to ionizing radiation. Genet Mol Res 15(1)

Zini A, Dohle G (2011) Are varicoceles associated with increased deoxyribonucleic acid fragmentation? Fertil Steril 96:1283–1287

Zini A, Blumenfeld A, Libman J, Willis J (2005) Beneficial effect of microsurgical subinguinal varicocelectomy on human sperm DNA integrity. Hum Reprod 20:1018–1021

Zylbersztejn DS, Andreoni C, Del Giudice PT, Spaine DM, Borsari L, Souza GH et al (2013) Proteomic analysis of seminal plasma in adolescents with and without varicocele. Fertil Steril 99:92–98

9 Cryopreservation of Sperm: Effects on Chromatin and Strategies to Prevent Them

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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. A number of defensive strategies against cryoinjuries have been proposed in the last decade. Most studies focused on supplementing cryoprotectant medium with various antioxidant molecules, all aimed at minimising oxidative damage and thus improving sperm recovery. Despite the promising results, identification of the ideal antioxidant treatment method is still hampered by the heterogeneity of the studies, which describe the use of different antioxidant regimens at different concentrations or in different combinations. For this reason, additional studies are needed to further investigate the use of antioxidants, individually and in combination, in the cryopreservation of human sperm, to determine the most beneficial conditions for optimal sperm recovery and preservation of fertility.

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Keywords

Semen cryopreservation · Sperm DNA damage · Male fertility preservation · Antioxidant supplementation · Cryoprotectant

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Sperm Cryopreservation

Cryopreservation is a technique that can keep sperm alive indefinitely, thus enabling male fertility to be conserved. 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 disease 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 the hope of future fertility; this is not only reassuring in relation to their possibility of fatherhood but is also a psychological support when dealing with the various stages of the treatment protocols, as it permits them to envision the prospect of their survival (Saito et al. 2005).

Advances in cancer treatments and increasingly sophisticated assisted reproduction techniques (ART) 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-ICSI era. Given the potential importance of reproduction for these patients, who are often young and childless, it is essential that this option is 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 linage of soldiers going off to war. Discussion of cryopreservation began when Rostand in 1946 and Polge in 1949 discovered that glycerol could act as a cryoprotectant, while in 1953 Bunge and Sherman reported three pregnancies 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 in a crystalline or vitreous state, in which its viscosity is so high as to limit its diffusion (Mazur 1984). The chemical and physical phenomena which 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 transmembrane 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 concentrations of solutes in intra- and extracellular aqueous solutions vary considerably. Such intra- and extracellular changes are the greatest biological problem encountered with freezing. In fact, 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 which 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 about 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 on thawing there may be mechanical damage to the membrane and subcellular organelles. In contrast, if the cell is cooled very slowly, 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, reverse osmosis takes place. As soon as the water passes from the solid to the liquid state, the concentration of extracellular solutes drops steadily, and the cells rehydrate to compensate for the different extra- and intracellular concentrations.

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 which 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 lead to high membrane fluidity (Clarke et al. 2003); their small cell size; and 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 try to obviate these problems. By replacing the water content, they keep the extracellular environment in the liquid phase even when the temperature drops below 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 environment 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 cryoprotectants:

Permeating cryoprotectants, which 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 <400 Da and cross the membrane very easily, creating an osmotic gradient and causing water to leave the cell by lowering the freezing point even further.

Non-permeating cryoprotectants, which do not cross the cell membrane. These include

sucrose, fructose, glucose, dextrose, starch, lipoproteins and polyvinylpyrrolidone (PVP). These large molecules with a molecular weight of >1000 Da increase the concentration of extracellular solutes, thus generating an osmotic gradient that causes water to leave the cell, causing 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 can 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 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 media and left to equilibrate at 37 °C for 10 minutes. 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 minutes 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 (about 20 °C/min). Following this phase, the straws are plunged in liquid nitrogen.

Slow freezing This was proposed by Behrman and Sawada (1966). It uses automatic equipment which 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 in 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 and mixed gently to avoid osmotic stress and the mixture incubated at 37 °C for 10 to 15 minutes 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 minutes and then incubated at 37 °C for 10 minutes; (2) straws are placed in a thermostatic bath at 37 °C for 10 minutes; and (3) straws are thawed at room temperature (22 °C) for 15 min.

Effect of Cryopreservation on 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). The latter authors showed that the freeze-thaw process induces a rigidifying effect on the sperm membrane and suggested that the adaptability of mammalian sperm to freeze-thaw-induced stress, and hence its ability to withstand cryopreservation, might depend on the fluidity of its membrane, which is in turn modulated by the membrane's lipid composition (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 Gutteridge 1984), are also vulnerable to peroxidation; this can not only damage the plasma membrane but also 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 (ROS) (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-DNA strand breakage. Various studies have examined how ROS form during cryopreservation and thawing. Mazzilli et al. (1995) studied 45 subjects for the presence of ROS in sperm selected by swim-up before and after cryopreservation in liquid nitrogen. After cryopreservation, 42.2% of samples showed an increase in ROS and 20% an increase in free radicals, which were absent prior to freezing. The study suggested that cryopreservation procedures can induce or increase ROS production in some semen samples. After thawing, samples with ROS showed reduced motility and viability in comparison with ROS-free samples. 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 hours after thawing. For this reason, sperm should be used rapidly after thawing (Gosálvez 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 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 processing; the second underwent swim-up (post-rise spermatozoa); and the third was stored according to standard cryopreservation

techniques in liquid nitrogen at −196 °C. In addition, an aliquot of the cryopreserved sample underwent a further swim-up procedure after thawing (post-rise spermatozoa after cryopreservation). The DNA damage observed in fresh samples increased in sperm thawed after cryopreservation. This increase may be indicative of the physical stresses the cryopreserved samples experienced, leading to chromatin deterioration in some of the spermatozoa in the native sample. On the other hand, the migrated sperm population (post-rise spermatozoa and post-rise after cryopreservation) exhibited a general improvement in DNA integrity compared with the unselected populations. These authors demonstrated that swim-up techniques select a subpopulation of highly motile cells with better sperm chromatin features, as evaluated by SCSA. Post-rise spermatozoa (from fresh and cryopreserved semen) are therefore characterised by superior and more homogeneous chromatin structure characteristics than those of unselected fresh (and, in the vast majority of cases, also cryopreserved) semen samples. The authors suggested that when performed correctly cryopreservation does not damage spermatozoa per se, but can enhance any 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. Poor-quality semen samples are more susceptible to DNA damage and cell death after cryopreservation than semen samples with normal parameters (Borges et al. 2007).

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, freshly prepared by density gradient spermatozoa and cryopreserved prepared spermatozoa. 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 DNA 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 acridine orange 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 which 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 the ROS (Donnelly et al. 2001a).

Confirmation of this hypothesis comes from another study by Donnelly et al. (2001b) carried out on semen samples from 40 infertile patients. For each sample, an aliquot of fresh semen was frozen unprepared, while other aliquots were prepared by Percoll density centrifugation or direct swim-up procedure. The authors compared fresh and frozen semen and prepared sperm (frozen with or without the addition of seminal plasma) from the same ejaculate. Sperm frozen unprepared in seminal fluid appeared more resistant to freezing damage than frozen prepared sperm. Moreover, the DNA of sperm prepared either by Percoll density centrifugation or by swim-up and frozen with additional seminal plasma appeared to be more resistant to freezing damage than the DNA of semen or prepared sperm (without seminal plasma) from the same ejaculate. These data suggest that the abundant presence of antioxidants in seminal plasma protects DNA integrity against freezing. Furthermore, De Paula et al. (2006) demonstrated by TUNEL assay an increase in post-cryopreservation apoptotic fragmentation in both 47 oligozoospermic and 30 normozoospermic subjects. According to these authors, cryopreservation induces fragmentation independently of sperm concentration, as the increase was similar in both groups. Another study by Ngamwuttiwong and Kunathikom (2007) found a decrease in sperm chromatin integrity on acridine orange testing in 20 men from infertile couples after 6 months of sperm cryopreservation.

Overall, 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 and 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 induces 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 caspases 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 observed in mature and immature sperm exhibiting cytoplasmic droplets, this mechanism differs in annexin V-positive and annexin V-negative sperm. The authors concluded that 'cryopreservation and thawing triggers activated caspase activity in spermatozoa by a mechanism that may be linked to the translocation of phosphatidylserine to the surface of the cell'. Unfortunately, the paper does not clearly explain if the cryopreserved and fresh samples derived from the same pools.

These interactions suggest that cryopreservation and thawing trigger the caspase activation through mechanisms possibly linked to membrane translocation of phosphatidylserine. However, more recent studies suggest that DNA fragmentation may 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 before and after cryopreservation. There was an increase in the percentage of DNA fragmentation, oxi-

dative 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 (Thomson et al. 2009). 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, ROS seem to play an important role in the pathophysiology of DNA damage in human sperm. Zribi et al. (2010) evaluated sperm DNA fragmentation by TUNEL and oxidative damage by flow cytometric analysis of 8OHdG in 15 semen samples pre- and post-cryopreservation. The increased fragmentation induced by cryopreservation was associated with a small increase in the percentage of sperm with oxidative damage.

More recently, Amor et al. (2018) reported that the cryopreservation process not only affects semen parameters but also induces DNA fragmentation and mitochondrial DNA damage in spermatozoa from both fertile and subfertile men. Freeze-thawing reduced sperm viability, total motility and membrane integrity in fertile men, with a significant mean increase in DNA fragmentation (from 14.8% to 27.5% with TUNEL) and in caspase-3 staining (from 5.8% to 7.3%). Similar results were seen in the subfertile group, with a significant increase in both DNA fragmentation (from 19.8% to 29.5%) and caspase-3 staining (from 7.5% to 10.2%). Finally, Lusignan et al. (2018) evaluated the impact of different freezing methods on sperm DNA and chromatin structure assessed by two assays, SCSA and TUNEL, in semen samples from normozoospermic, oligoasthenoteratozoospermic and teratozoospermic men. There was a significant increase in post-thaw TUNEL scores in all three categories for all the freezing methods, while there was no significant change in the DNA fragmentation index (DFI) on SCSA. The authors thus suggested that freeze-thawing had a negative impact on DNA quality regardless of the freezing method and that TUNEL was sensitive enough to detect such damage.

In conclusion, 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, as well as oxidative stress (Zribi et al. 2010).

Various studies have focused on the effects of cryopreservation on sperm DNA damage; however, there is still no consensus. Some studies found sperm DNA damage after cryopreservation (Thomson et al. 2009; Zribi et al. 2010; Amor et al. 2018), while others did not (Duru et al. 2001; Paasch et al. 2004) (Table 9.1).

Poor-quality semen samples seem to be more susceptible to DNA damage (Spanò et al. 1999; Oehninger et al. 2000; Kalthur et al. 2008) although De Paula et al. (2006) and Amor et al. (2018) reported that cryopreservation induces DNA fragmentation independently of semen quality. However, the use of different cryopreservation methods and of different techniques to assess sperm DNA damage (TUNEL, SCSA or Comet assay) confounds the issue. For this reason, more studies are needed to establish the true importance of such damage, especially in order to improve the results of ART.

Strategies to Prevent Cryopreservation Injuries

Although the cryopreservation of human semen is an important technique routinely employed in the clinical management of male infertility, it can induce changes in sperm function, and the risk of cryodamage is still a major issue. A number of defensive strategies against cryoinjuries have been proposed in the last decade, such as antifreeze proteins (AFPs) (Hezavehei et al. 2018), seminal plasma protein (Pini et al. 2018) and gangliosides (Gavella and Lipovac 2013), although most literature reports have focused on the role of antioxidants.

The cryopreservation process may result in adverse changes in membrane lipid composition,

Table 9.1 Literature data: effects of cryopreservation on DNA integrity

Year	Reference	Method	Results
1999	Spanò et al.	SCSA, AO	Human spermatozoa from 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 unaffected by cryopreservation; in contrast, there was a significant reduction in chromatin integrity after freeze-thawing of semen samples and selected sperm from infertile subjects
2001b	Donnelly et al.	Comet	Unprepared sperm in seminal fluid from infertile men seemed more resistant to freezing damage than prepared sperm. The DNA of spermatozoa prepared by Percoll density centrifugation or swim-up and frozen in seminal plasma from the same ejaculate also seemed more resistant to freezing
2001	Duru et al.	TUNEL	No significant effect on DNA fragmentation in fertile and infertile samples after freeze-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-stranded DNA fragmentation following cryopreservation
2007	Ngamwuttiwong and Kunathikom	SCSA	Cryopreservation of sperm from infertile patients reduced sperm chromatin integrity after 6 months
2008	Kalthur et al.	Comet, AO	DNA damage was higher in teratozoospermic than normozoospermic 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
2018	Amor et al.	TUNEL	Cryopreservation not only affected semen parameters but also induced DNA fragmentation and mitochondrial DNA damage in spermatozoa from both fertile and subfertile men
2018	Lusignan et al.	TUNEL, SCSA	Freeze-thawing had a negative impact on sperm DNA quality, independently of the freezing method tested. TUNEL assay was sensitive enough to detect DNA damage, while SCSA did not detect any significant change in the DNA fragmentation index

acrosome status, sperm motility and viability, as well as an increase in sperm DNA damage (Donnelly et al. 2001b; Medeiros et al. 2002; O'Connell et al. 2002; Hezavehei et al. 2018). Most of these deleterious effects are due to the reactive oxygen species (ROS) generated during cryopreservation (Agarwal and Majzoub 2017; Bui et al. 2018). Under normal conditions, spermatozoa and seminal plasma possess a number of antioxidant systems that scavenge ROS and prevent internal cellular damage, thus neutralising the detrimental effects of ROS. The imbalance between the presence of ROS and sperm antioxidant activity is considered an important cause of sperm cryodamage (Amidi et al. 2016).

Components of the human reproductive system contain antioxidants that are either endogenously formed or acquired from dietary sources. In males, antioxidants are found in the testis, epididymis, secretions of the male accessory organs and seminal plasma (Agarwal et al. 2014a). Based on their chemical structure, antioxidants may be enzymatic and non-enzymatic. Enzymatic (natural) antioxidants include catalase, superox-

ide dismutase (SOD) and glutathione reductase (GSH), while non-enzymatic antioxidants consist of radical scavengers such as vitamin E (α-tocopherol), vitamin C (ascorbic acid), cysteine, carnitine, melatonin, polyphenols and natural extracts (Tan et al. 2000; Stojanović et al. 2001; Meamar et al. 2012; Agarwal et al. 2014b).

Extensive research has been conducted to study the effect of antioxidant therapy in improving male fertility and pregnancy rates (Agarwal et al. 2004; Ahmadi et al. 2016; Majzoub and Agarwal 2018). Different types of cryoprotective media, mostly supplemented with antioxidants, have been designed in recent years to attempt to overcome the cell damage caused by cryopreservation. Antioxidants have generally proved beneficial in reversing the sperm dysfunction caused by oxidative stress, suggesting that antioxidant supplementation has a protective effect on ROS-induced sperm cryoinjury (Agarwal et al. 2014a; Amidi et al. 2016; Hezavehei et al. 2018).

Enzymatic Antioxidants

Catalase

Catalase is a ubiquitous antioxidant enzyme mostly found in cellular peroxisomes and involved in the detoxification of hydrogen peroxide (H_2O_2), by decomposing it to oxygen and water. Catalase originating mainly from the prostate gland is found in seminal fluid and motile spermatozoa (Jeulin et al. 1989). In 2008, Chi et al. investigated how the addition of catalase to a sperm preparation medium affected the functional parameters of the spermatozoa, finding reduced ROS and DNA fragmentation levels and an increased acrosome reaction rate in spermatozoa from normozoospermic men. Other studies have also found that catalase supplementation of cryomedia improves freeze-thaw outcomes. Li et al. (2010) found that both catalase (200 and 400 IU/mL) and ascorbate (300 mM) supplementation in cryomedia reduced ROS levels, thereby protecting against cryodamage. More recently, Moubasher et al. (2013) also demonstrated that catalase supplementation was associated with a higher percentage of progressive motility and improved sperm viability and DNA integrity.

Superoxide Dismutase

Superoxide dismutases (SODs) are metalloenzymes that convert superoxide to hydrogen peroxide. Murawski et al. (2007) found a significantly lower semen SOD activity in oligoasthenozoospermic cases when compared to normozoospermic men. SOD activity in seminal plasma is also positively associated with sperm concentration and overall motility (Yan et al. 2014). Co-supplementation of cryopreserved spermatozoa with catalase and SOD results in higher post-thaw motility and viability. It may also help prevent sperm membrane lipid peroxidation by ROS, thus allowing good sperm parameter recovery after freeze-thaw procedures (Rossi et al. 2001). Recent research has suggested that cell-permeable enzyme-mimetic agents may help reduce intracellular ROS levels. For example, manganese (III) meso-tetrakis (MnTE) is a mimetic agent which can convert superoxide to H_2O_2. Shafiei et al. (2015) suggested that the addition of MnTE or catalase to commercial optimised media improves post-thaw goat semen function.

Glutathione

The glutathione enzymatic family comprises reduced glutathione (GSH), glutathione peroxidase (GPx, isoforms Gpx 1 to GPx 6), glutathione S-transferase (GST) and glutathione reductase (GR). Glutathione is a sulphur-containing tripeptide present in both a reduced (GSH) and an oxidative (GSSG) form. The sulfhydryl groups of GSH protect cells against oxidants, electrophiles and free radicals (Agarwal et al. 2006). The addition of GSH to both freezing and thawing extenders improved post-thaw boar sperm quality, stabilised the nucleoprotein structure and improved both in vivo and in vitro fertilising ability (Estrada et al. 2014). The greatest improvement was seen when freezing and thawing media were supplemented with both GSH and L-ascorbic acid (Giaretta et al. 2015). The addition of GSH also improved the post-thaw quality of ram semen. Zeitoun and Al-Damegh

(2014) found that adding 1–2 mM glutathione to ram semen extender increased GPX and SOD activity, reduced free radicals and improved the post-thaw sperm survival rate.

Gadea et al. (2011) reported a reduction of up to 64% in GSH content after freeze-thawing, indicating that the antioxidant defence system is challenged by sperm cryopreservation. These authors also showed that the addition of GSH to freezing and thawing media reduced ROS levels and improved the motility of human spermatozoa. More recently, Ghorbani et al. (2016) also investigated GSH supplementation in human sperm cryopreservation, demonstrating that after thawing, cryovials supplemented with 5 mM of GSH showed greater sperm viability than the control samples. These cryovials also showed reduced sperm lipid peroxidation and DNA fragmentation and a lower H_2O_2 and O_2^- content than the controls.

Non-enzymatic Antioxidants

Vitamin E (a-Tocopherol/Trolox)

Vitamin E is a potent chain-breaking lipophilic antioxidant, residing on the cell membrane, which can break the covalent links formed by ROS between fatty acid side chains in membrane lipids. It quenches free hydroxyl radicals and superoxide anions, thereby reducing ROS-induced lipid peroxidation in the plasma membrane. The most active form, alpha-tocopherol, quenches hydrogen peroxide, superoxide anions and hydroxyl anions and breaks peroxidation chain reactions (Agarwal et al. 2004; Majzoub and Agarwal 2018).

Geva et al. (1996) were the first to demonstrate an improved fertilisation rate after 1 month of treatment with vitamin E (200 mg/day) for fertile normozoospermic men who had low fertilisation rates in previous IVF cycles. Greco et al. (2005a) showed that daily treatment with 1 g of vitamin C and 1 g of vitamin E improved the success rate of intracytoplasmic sperm injection (ICSI) and reduced sperm DNA fragmentation.

Taylor et al. (2009) found that supplementation of semen cryopreservation medium with 200 μmol of vitamin E significantly improved post-thaw motility but not viability or the degree of DNA fragmentation. The authors associated the positive effect of vitamin E supplementation with age: the effects were better in men over 40 years of age. Finally, Kalthur et al. (2011) demonstrated that supplementation of cryoprotective medium with vitamin E (5 mM) not only improved post-thaw sperm motility but also helped maintain sperm DNA integrity in normozoospermic and asthenozoospermic samples.

Trolox is a water-soluble vitamin E analogue with powerful antioxidant properties. Trolox supplementation (40 μM) significantly improved post-thaw human semen quality, especially progressive motility (Minaei et al. 2012). Nekoonam et al. (2016) supported the use of Trolox as a freezing extender supplement to improve the quality of cryopreserved human sperm. The authors investigated DNA integrity, mitochondrial function as expressed by its membrane potential (MMP) and phosphatidylserine externalisation, an early marker of apoptosis, in sperm cells following freeze-thawing in normozoospermic men and oligozoospermic patients. Sperm frozen in extender with Trolox had a higher MMP and lower DNA fragmentation and phosphatidylserine externalisation in both groups, though the most effective dose differed between normozoospermic and oligozoospermic semen samples (80 and 20 μM, respectively).

Vitamin C

Vitamin C (ascorbic acid) is a water-soluble vitamin freely available in fruits and vegetables, whose antioxidant properties offer many beneficial effects (Alahmar 2018). Ascorbate reduces H_2O_2-induced DNA damage, recycles inactive vitamin E and reduces lipid peroxidation (Donnelly et al. 1999; Sierens et al. 2002). Vitamin C is the principal antioxidant in the seminal plasma of fertile men, contributing up to 65% of its total chain-breaking antioxidant capacity. The concentration of ascorbate in seminal plasma is 10 times greater than in blood plasma (Agarwal et al. 2004). Several studies have reported a significant improvement in sperm quality after vitamin C supplementation (Akmal

et al. 2006; Kobori et al. 2014; Cyrus et al. 2015; Eslamian et al. 2017). As reported above, Greco et al. (2005b) demonstrated in ejaculated spermatozoa from 64 infertile men that DNA fragmentation was reduced by oral treatment with 1 g vitamin C and 1 g vitamin E daily for 2 months. Song et al. (2006) found that ascorbic acid levels in semen were directly correlated with the percentage of morphologically normal spermatozoa and negatively correlated with the DNA fragmentation index. Similarly, Fanaei et al. (2014) reported that vitamin C supplementation of culture media reduced lipid peroxidation and DNA damage while improving sperm motility and viability. Branco et al. (2010) demonstrated that 10 mM ascorbic acid added to semen samples from infertile men prior to adding cryomedia prevents DNA damage. Finally, Jenkins et al. (2011) found that supplementation of cryomedium with ascorbic acid-2-glucoside (AA2G), a stabilised form of ascorbate, protected the post-thaw motility of human sperm.

L-Carnitine

L-carnitine is a highly polar water-soluble quaternary amine. It is biologically important for the mitochondrial β-oxidation of long-chain fatty acids and the generation of ATP. In eukaryotic metabolism, its main function is to promote the translocation of fatty acids across the mitochondrial inner membrane as β-hydroxyl O-acyl esters of L-carnitine. Its concentration in epididymal plasma and spermatozoa is 2000-fold higher than in circulating blood. In the epididymal lumen, the initiation of sperm motility occurs in parallel to increasing L-carnitine levels (Enomoto et al. 2002). Carnitines also have antioxidant properties, thus protecting against ROS (Gülçin 2006).

In seminal plasma, L-carnitine plays an essential role in maintaining male fertility, and its beneficial effects on sperm parameters are well known (Agarwal and Said 2004; Ahmed et al. 2011). Combination therapy with L-carnitine and acetyl-L-carnitine improves sperm quality in men with asthenozoospermia, as demonstrated by Lenzi et al. (2004) in a double-blind placebo-controlled trial investigating the effects of L-carnitine supplements in 56 men with idiopathic oligoasthenoteratozoospermia, in which the intervention group received 2 g/day of L-carnitine and 1 g/day of L-acetyl carnitine for 6 months. Similarly, in a randomised double-blind trial of 59 men with idiopathic oligoasthenoteratozoospermia, Balercia et al. (2005) demonstrated that the administration of L-carnitine and acetyl-L-carnitine improved the sperm kinetic features and the total oxyradical scavenging capacity of the seminal fluid in patients with idiopathic asthenozoospermia.

Banihani et al. (2014) recently found that the use of cryopreservation medium supplemented with L-carnitine for cryopreservation of semen samples from infertile patients improved post-thaw sperm motility and viability but had no protective effect on sperm DNA oxidative damage in comparison with semen samples frozen and thawed without L-carnitine.

L-Cysteine

L-cysteine is a low molecular weight non-essential amino acid containing thiol. It penetrates the cell membrane to participate in intracellular GSH biosynthesis (Amidi et al. 2016). Uysal and Bucak (2007) reported that L-cysteine enhanced intracellular GSH biosynthesis and protected proteins, DNA and membrane lipids through the direct radical scavenging ability of GSH. L-cysteine protects the spermatozoa of various mammalian species against cryodamage, including boars (Chanapiwat et al. 2009), bulls (Sariözkan et al. 2009), buffalos (Topraggaleh et al. 2014), goats (Memon et al. 2012) and rams (Coyan et al. 2011). Zhu et al. (2017) recently found that the addition of L-cysteine to freezing extender for the cryopreservation of rabbit semen enhanced antioxidant GSH content and glutathione peroxidase activity, lowering ROS levels and lipid peroxidation.

Melatonin

Melatonin, a derivative of tryptophan, is mainly secreted by the pineal gland and contributes to the regulation of different physiological events, such as the seasonal and circadian rhythms of mammals (Reiter et al. 2009). It is a potent

endogenous free radical scavenger, independently of its many receptor-mediated effects (Tan et al. 2000). Human seminal fluid contains melatonin, and spermatozoa reportedly possess a melatonin receptor (Ortiz et al. 2011). Several studies have reported the beneficial effects of melatonin on spermatozoa. In adult rats with experimentally induced unilateral varicocele, it prevented testicular damage by stimulating antioxidant enzyme activity and reducing NO levels (Semercioz et al. 2003), while Sönmez et al. (2007) observed that it protected rat spermatozoa from the adverse effects of the pro-oxidative agent homocysteine.

Gavella and Lipovac (2000) investigated the ability of melatonin to suppress experimentally induced lipid peroxidation in semen samples from 41 infertile men. They found that it relieved the sperm mitochondrial oxidative stress caused by ROS, although it was 40-fold less efficient than Trolox. Melatonin has also been shown to protect spermatozoa from oxidative stress-induced apoptosis. Espino et al. (2010) evaluated its effect on H_2O_2-treated (10 μM) ejaculated human spermatozoa from 20 healthy donors, concluding that its free radical scavenging action subverted the pro-apoptotic action induced by H_2O_2. Several studies report that melatonin protects animal spermatozoa from the adverse effects of ROS during cryopreservation (Succu et al. 2011; Ashrafi et al. 2013). In humans, Karimfar et al. (2015) demonstrated that semen extender supplemented with melatonin (0.01 mM) significantly increased post-thaw motility and viability and reduced intracellular ROS and malondialdehyde levels in cryopreserved sperm.

Quercetin and Resveratrol

Resveratrol is a non-flavonoid and quercetin a flavonoid polyphenol. They are described as powerful antioxidants with a similar effect, resulting from their ability to inhibit ROS formation by enzymatic and non-enzymatic systems, especially NADPH-oxidase and NADH-dependent oxidoreductase (Stojanović et al. 2001).

Resveratrol is a natural phytoalexin with antioxidant properties which has different biological activities, including anti-inflammatory, antiviral and antitumorigenic activities. It is found in many plant species, especially grapes, and in red wine. Garcez et al. (2010) studied the effects of resveratrol supplementation in cryopreservation medium for human semen, demonstrating that it inhibited post-thaw oxidative damage, although it did not prevent post-thaw loss of motility (Garcez et al. 2010). Shabani Nashtaei et al. (2018) recently demonstrated in 22 normozoospermic semen samples that the addition of resveratrol before cryopreservation led to a significant reduction in the incidence of post-thaw sperm DNA fragmentation. The authors suggested that its protective effects against cryopreservation-induced oxidative stress may be mediated through activation of AMP-activated protein kinase (AMPK), an evolutionary conserved serine/threonine kinase that controls cell metabolism and acts as a key sensor of cell energy status through the stimulation of catabolic processes and inhibition of anabolic processes (Hardie et al. 2006).

Quercetin is a dietary flavonoid found in various vegetables, fruits, seeds and nuts, as well as in tea and red wine. It scavenges reactive species and hydroxyl radicals, thereby providing beneficial anticarcinogenic, anti-inflammatory and antimicrobial properties (Boots et al. 2008).

Quercetin helps protect against H_2O_2-mediated sperm damage in rats by reducing lipid peroxidation and increasing the sperm's antioxidant defences (Ben Abdallah et al. 2011). Supplementation of semen extender with quercetin also had beneficial antioxidant properties on the post-thaw characteristics of semen from bulls (Avdatek et al. 2018), horses (Seifi-Jamadi et al. 2016), boars (Kim et al. 2014) and rams (Silva et al. 2012). In humans, Zribi et al. (2012) showed that the addition of quercetin (50 μM) to the cryoprotective medium enhanced sperm motility and viability and reduced post-thaw DNA damage.

Although flavonoid compounds exhibit strong antioxidant properties in vitro, their effects are minimised in vivo due to their low water solubility, weak absorption and lower bioavailability. Moretti et al. (2016) tested the ability of quercetin-loaded liposomes compared to quercetin alone in preventing oxidative stress induced in vitro in

swim-up-selected human spermatozoa. Quercetin-loaded liposomes did not affect sperm viability and motility even at high concentrations, but were less efficient than quercetin alone at protecting spermatozoa from lipid peroxidation. Although quercetin alone appeared more effective at reducing oxidative stress, the authors suggested further investigations were needed on the use of liposomes to carry compounds into spermatozoa.

Natural Extracts (Opuntia ficus-indica and Genistein)

Opuntia ficus-indica (OFI) is a pear cactus whose extract contains a cocktail of antioxidants including ascorbic acid, polyphenols, carotenoids, taurine and several types of flavonoids, in particular quercetin. Meamar et al. (2012) evaluated whether the addition of OFI extract to cryopreservation medium would protect sperm from the deleterious effects of cryopreservation in comparison with the antioxidant resveratrol. They found that both substances had a slight but statistically significant protective effect against sperm DNA fragmentation.

Genistein is an isoflavone found in soya and other legumes. The structure of isoflavones, a class of phytoestrogens, resembles the steroid hormone 17β-oestradiol, and isoflavones can bind to the oestrogen receptor. However, their physiological effects are not confined to oestrogen modulation, and isoflavones have been reported to inhibit angiogenesis and cell proliferation and induce cancer cell differentiation. In addition, they may function as antioxidant species (Zielonka et al. 2003). Sierens et al. (2002) demonstrated that pretreatment with genistein at doses of 0.01–100 μmol/l significantly protected sperm DNA against oxidative damage. The addition of 50 μM or 100 μM of genistein to the cryoprotectant also had a significant protective effect on cryopreservation-induced DNA fragmentation in post-thaw human spermatozoa (Thomson et al. 2009). Martinez-Soto et al. (2010) confirmed that genistein has antioxidant properties in post-thaw spermatozoa, finding reduced cryopreservation-induced ROS production and DNA damage and improved sperm motility with a concentration of 1–10 μM.

In summary, cryopreservation may lead to changes in sperm structure and function secondary to the state of oxidative stress induced by the freeze-thaw process. Several studies focused on supplementing cryoprotectant medium with various antioxidant molecules, all aimed at minimising oxidative damage and thus improving sperm recovery. Despite the promising results, identification of the ideal antioxidant treatment method is still hampered by the heterogeneity of the studies, which describe the use of different antioxidant regimens at different concentrations or in different combinations. For this reason, additional studies are needed to further investigate the use of antioxidants, individually and in combination, in the cryopreservation of human sperm, to determine the most beneficial conditions for optimal sperm recovery and preservation of fertility.

References

Agarwal A, Majzoub A (2017) Role of antioxidants in assisted reproductive techniques. World J Mens Health 35:77–93

Agarwal A, Said TM (2004) Carnitines and male infertility. Reprod Biomed Online 8:376–384

Agarwal A, Nallella KP, Allamaneni SS, Said TM (2004) Role of antioxidants in treatment of male infertility: an overview of the literature. Reprod Biomed Online 8:616–627

Agarwal A, Gupta S, Sikka S (2006) The role of free radicals and antioxidants in reproduction. Curr Opin Obstet Gynecol 18:325–332

Agarwal A, Durairajanayagam D, du Plessis SS (2014a) Utility of antioxidants during assisted reproductive techniques: an evidence based review. Reprod Biol Endocrinol 24:112

Agarwal A, Virk G, Ong C, du Plessis SS (2014b) Effect of oxidative stress on male reproduction. World J Mens Health 32(1):17

Ahmadi S, Bashiri R, Ghadiri-Anari A, Nadjarzadeh A (2016) Antioxidant supplements and semen parameters: an evidence based review. Int J Reprod Biomed (Yazd) 14:729–736

Ahmed SD, Karira KA, Jagdesh, Ahsan S (2011) Role of L-carnitine in male infertility. J Pak Med Assoc 61:732–736

Akmal M, Qadri JQ, Al-Waili NS, Thangal S, Haq A, Saloom KY (2006) Improvement in human semen quality after oral supplementation of vitamin C. J Med Food 9:440–442

Alahmar AT (2018) The effects of oral antioxidants on the semen of men with idiopathic oligoasthenoteratozoospermia. Clin Exp Reprod Med 45:57–66

Amidi F, Pazhohan A, Shabani Nashtaei M, Khodarahmian M, Nekoonam S (2016) The role of antioxidants in sperm freezing: a review. Cell Tissue Bank 17:745–756

Amor H, Zeyad A, Alkhaled Y, Laqqan M, Saad A, Ben Ali H, Hammadeh ME (2018) Relationship between nuclear DNA fragmentation, mitochondrial DNA damage and standard sperm parameters in spermatozoa of fertile and sub-fertile men before and after freeze-thawing procedure. Andrologia 50:e12998

Arav A, Zeron Y, Leslie SB, Behboodi E, Anderson GB, Crowe JH (1996) Phase transition temperature and chilling sensitivity of bovine oocytes. Cryobiology 33:589–599

Ashrafi I, Kohram H, Ardabili FF (2013) Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. Anim Reprod Sci 139:25–30

Avdatek F, Yeni D, İnanç ME, Çil B, Tuncer BP, Türkmen R, Taşdemir U (2018) Supplementation of quercetin for advanced DNA integrity in bull semen cryopreservation. Andrologia, Feb 7

Bailey JL, Bilodeau JF, Cormier N (2000) Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J Androl 21:1–7

Balercia G, Regoli F, Armeni T, Koverech A, Mantero F, Boscaro M (2005) Placebo-controlled double-blind randomized trial on the use of l-carnitine, l-acetylcarnitine, or combined l-carnitine and l-acetylcarnitine in men with idiopathic asthenozoospermia. Fertil Steril 84:662–671

Banihani S, Agarwal A, Sharma R, Bayachou M (2014) Cryoprotective effect of l-carnitine on motility, vitality and DNA oxidation of human spermatozoa. Andrologia 46:637–641

Behrman SJ, Sawada Y (1966) Heterologous and homologous insemination with human semen frozen and stored in a liquid-nitrogen refrigerator. Fertil Steril 17:457–466

Ben Abdallah F, Zribi N, Ammar-Keskes L (2011) Antioxidative potential of Quercetin against hydrogen peroxide induced oxidative stress in spermatozoa in vitro. Andrologia 43:261–265

Benoff S (1997) Carbohydrates and fertilization: an overview. Mol Hum Reprod 3:599–637

Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U, Sakkas D (1993) Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. Biol Reprod 49:1083–1088

Boots AW, Haenen GR, Bast A (2008) Health effects of quercetin: from antioxidant to nutraceutical. Eur J Pharmacol 585:325–337

Borges E Jr, Rossi LM, Locambo de Freitas CV, Guilherme P, Bonetti TC, Iaconelli A, Pasqualotto FF (2007) Fertilization and pregnancy outcome after intracytoplasmic injection with fresh or cryopreserved ejaculated spermatozoa. Fertil Steril 87:316–320

Branco CS, Garcez ME, Pasqualotto FF, Erdtman B, Salvador M (2010) Resveratrol and ascorbic acid prevent DNA damage induced by cryopreservation in human semen. Cryobiology 60:235–237

Bui AD, Sharma R, Henkel R, Agarwal A (2018) Reactive oxygen species impact on sperm DNA and its role in male infertility. Andrologia 50:e13012

Bunge RG, Sherman JK (1953) Fertilizing capacity of frozen human spermatozoa. Nature 172:767–768

Bunge RG, Keettel WC, Sherman JK (1954) Clinical use of frozen semen: report of four cases. Fertil Steril 5:520–529

Chanapiwat P, Kaeoket K, Tummaruk P (2009) Effects of DHA-enriched hen egg yolk and L-cysteine supplementation on quality of cryopreserved boar semen. Asian J Androl 11:600–608

Chi HJ, Kim JH, Ryu CS, Lee JY, Park JS, Chung DY, Choi SY, Kim MH, Chun EK, Roh SI (2008) Protective effect of antioxidant supplementation in sperm-preparation medium against oxidative stress in human spermatozoa. Hum Reprod 23:1023–1028

Clarke GN, Liu DY, Baker HW (2003) Improved sperm cryopreservation using cold cryoprotectant. Reprod Fertil Dev 15:377–381

Coyan K, Başpınar N, Bucak MN, Akalın PP (2011) Effects of cysteine and ergothioneine on post-thawed Merino ram sperm and biochemical parameters. Cryobiology 63:1–6

Cross NL, Overstreet JW (1987) Glycoconjugates of the human sperm surface: distribution and alterations that accompany capacitation in vitro. Gamete Res 16:23–35

Cyrus A, Kabir A, Goodarzi D, Moghimi M (2015) The effect of adjuvant vitamin C after varicocele surgery on sperm quality and quantity in infertile men: a double blind placebo controlled clinical trial. Int Braz J Urol 41:230–238

De Paula TS, Bertolla RP, Spaine DM, Cunha MA, Schor N, Cedenho AP (2006) Effect of cryopreservation on sperm apoptotic deoxyribonucleic acid fragmentation in patients with oligozoospermia. Fertil Steril 86:597–600

Di Santo M, Tarozzi N, Nadalini M, Borini A (2012) Human sperm cryopreservation: update on techniques, effect on DNA integrity, and implications for ART. Adv Urol 2012:854837

Donnelly ET, McClure N, Lewis SE (1999) The effect of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. Mutagenesis 14:505–512

Donnelly ET, Steele EK, McClure N, Lewis SE (2001a) Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. Hum Reprod 16:1191–1199

Donnelly ET, McClure N, Lewis SE (2001b) Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. Fertil Steril 76:892–900

Duru NK, Morshedi MS, Schuffner A, Oehninger S (2001) Cryopreservation-Thawing of fractionated human spermatozoa is associated with membrane phosphatidylserine externalization and not DNA fragmentation. J Androl 22:646–651

Enomoto A, Wempe MF, Tsuchida H, Shin HJ, Cha SH, Anzai N, Goto A, Sakamoto A, Niwa T, Kanai Y, Anders MW, Endou H (2002) Molecular identification of a novel carnitine transporter specific to human testis. Insights into the mechanism of carnitine recognition. J Biol Chem 277:36262–36271

Eslamian G, Amirjannati N, Rashidkhani B, Sadeghi MR, Hekmatdoost A (2017) Nutrient patterns and asthenozoospermia: a case-control study. Andrologia 49:e12624

Espino J, Bejarano I, Ortiz A, Lozano GM, García JF, Pariente JA, Rodríguez AB (2010) Melatonin as a potential tool against oxidative damage and apoptosis in ejaculated human spermatozoa. Fertil Steril 94:1915–1917

Estrada E, Rodríguez-Gil JE, Rocha LG, Balasch S, Bonet S, Yeste M (2014) Supplementing cryopreservation media with reduced glutathione increases fertility and prolificacy of sows inseminated with frozen-thawed boar semen. Andrology 2:88–99

Fabbri R, Ciotti P, Di Tommaso B, Magrini O, Notarangelo L, Porcu E, Contro E, Venturosi S (2004) Tecniche di crioconservazione riproduttiva. Riv Ital Ostet Ginecol 3:33–41

Fanaei H, Khayat S, Halvaei I, Ramezani V, Azizi Y, Kasaeian A, Mardaneh J, Parvizi MR, Akrami M (2014) Effects of ascorbic acid on sperm motility, viability, acrosome reaction and DNA integrity in teratozoospermic samples. Iranian J Reprod Med 12:103–110

Gadea J, Molla M, Selles E, Marco MA, Garcia-Vazquez FA, Gardon JC (2011) Reduced glutathione content in human sperm is decreased after cryopreservation: effect of the addition of reduced glutathione to the freezing and thawing extenders. Cryobiology 62:40–46

Garcez ME, dos Santos Branco C, Lara LV, Pasqualotto FF, Salvador M (2010) Effects of resveratrol supplementation on cryopreservation medium of human semen. Fertil Steril 94:2118–2121

Gavella M, Lipovac V (2000) Antioxidative effect of melatonin on human spermatozoa. Arch Androl 44:23–27

Gavella M, Lipovac V (2013) Protective effects of exogenous gangliosides on ROS-induced changes in human spermatozoa. Asian J Androl 15:375–381

Geva E, Bartoov B, Zabludovsky N, Lessing J, Lerner-Geva L, Amit A (1996) The effect of antioxidant treatment on human spermatozoa and fertilization rate in an in vitro fertilization program. Fertil Steril 66:430–434

Ghorbani M, Vatannejad A, Khodadadi I, Amiri I, Tavilani H (2016) Protective effects of glutathione supplementation against oxidative stress during cryopreservation of human spermatozoa. Cryo Letters 37:34–40

Giaretta E, Estrada E, Bucci D, Spinaci M, Rodríguez-Gil JE, Yeste M (2015) Combining reduced glutathione and ascorbic acid has supplementary beneficial effects on boar sperm cryotolerance. Theriogenology 83:399–407

Giraud MN, Motta C, Boucher D, Grizard G (2000) Membrane fluidity predicts the outcome of cryopreservation of human spermatozoa. Hum Reprod 15:2160–2164

Gorczyca W, Traganos F, Jesionowska H, Darzynkiewicz Z (1993) Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. Exp Cell Res 207:202–205

Gosálvez J, Cortés-Gutierez E, López-Fernández C, Fernández JL, Caballero P, Nuñez R (2009) Sperm deoxyribonucleic acid fragmentation dynamics in fertile donors. Fertil Steril 92:170–173

Greco E, Romano S, Iacobelli M, Ferrero S, Baroni E, Minasi MG, Ubaldi F, Rienzi L, Tesarik J (2005a) ICSI in cases of sperm DNA damage: beneficial effect of oral antioxidant treatment. Hum Reprod 20:2590–2594

Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J (2005b) Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. J Androl 26:349–353

Gülçin I (2006) Antioxidant and antiradical activities of L-carnitine. Life Sci 78:803–811

Halliwell B, Gutterridge J (1984) Lipid peroxidation, oxygen radicals, cell damage and antioxidant therapy. Lancet 231:1396–1397

Hammadeh ME, Askari AS, Georg T, Rosenbaum P, Schmidt W (1999) Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men. Int J Androl 22:155–162

Hardie DG, Hawley SA, Scott JW (2006) AMP-activated protein kinase – development of the energy sensor concept. J Physiol 574:7–15

Hezavehei M, Sharafi M, Kouchesfahani HM, Henkel R, Agarwal A, Esmaeili V, Shahverdi A (2018) Sperm cryopreservation: a review on current molecular cryobiology and advanced approaches. Reprod Biomed Online 37:327–339

Jenkins TG, Aston KI, Carrell DT (2011) Supplementation of cryomedium with ascorbic acid-2-glucoside (AA2G) improves human sperm post-thaw motility. Fertil Steril 95:2001–2004

Jeulin C, Soufir JC, Weber P, Laval-Martin D, Calvayrac R (1989) Catalase activity in human spermatozoa and seminal plasma. Gamete Res 24:185–196

Thachil JV, Jewett MAS (1981) Preservation techniques for human semen. Fertil Steril 35:546–548

Kalthur G, Adiga SK, Upadhya D, Rao S, Kumar P (2008) Effect of cryopreservation on sperm DNA integrity in patients with teratosperm. Fertil Steril 89:1723–1727

Kalthur G, Raj S, Thiyagarajan A, Kumar S, Kumar P, Adiga SK (2011) Vitamin E supplementation in semen-freezing medium improves the motility and protects sperm from freeze-thaw–induced DNA damage. Fertil Steril 95:1149–1151

Karimfar MH, Niazvand F, Haghani K, Ghafourian S, Shirazi R, Bakhtiyari S (2015) The protective effects of melatonin against cryopreservation induced oxidative stress in human sperm. Int J Immunopathol Pharmacol 28:69–76

Karow AM Jr (1974) Cryopreservation: pharmacological considerations. In: Karow AM, Abouna GJ, Humphries AL (eds) Organ preservation for transplantation. Little Brown, Boston, pp 86–107

Kim TH, Yuh IS, Park IC, Cheong HT, Kim JT, Park JK, Yang BK (2014) Effects of quercetin and genistein on boar sperm characteristics and porcine IVF embryo developments. J Embrio Transfer 29:141–148

Kobori Y, Ota S, Sato R, Yagi H, Soh S, Arai G, Okada H (2014) Antioxidant cosupplementation therapy with vitamin C, vitamin E, and coenzyme Q10 in patients with oligoasthenozoospermia. Arch Ital Urol Androl 86:1–4

Lassalle B, Testart J (1994) Human zona pellucida recognition associated with removal of sialic acid from human sperm surface. J Reprod Fertil 101:703–711

Lasso JL, Noiles EE, Alvarez JG, Storey BT (1994) Mechanism of superoxide dismutase loss from human sperm cells during cryopreservation. J Androl 15:255–265

Lenzi A, Sgrò P, Salacone P (2004) A placebo-controlled double-blind randomized trial of the use of combined L-carnitine and L-acetylcarnitine treatment in men with asthenozoospermia. Fertil Steril 8:1578–1584

Li Z, Lin Q, Liu R, Xiao W, Liu W (2010) Protective effects of ascorbate and catalase on human spermatozoa during cryopreservation. J Androl 31:437–444

Lopes S, Sun JG, Jurisicova A, Meriano J, Casper RF (1998) Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil Steril 69:528–532

Lusignan MF, Li X, Herrero B, Delbes G, Chan PTK (2018) Effects of different cryopreservation methods on DNA integrity and sperm chromatin quality in men. Andrology 6:829

Mahadevan M, Trounson AO (1984) Effect of cooling, freezing and thawing rates and storage conditions on preservation of human spermatozoa. Andrologia 16:52–60

Majzoub A, Agarwal A (2018) Systematic review of antioxidant types and doses in male infertility: benefits on semen parameters, advanced sperm function, assisted reproduction and live-birth rate. Arab J Urol 16:113–124

Martinez-Soto JC, De Dioshourcade J, Gutiérrez-Adán A, Landeras JL, Gadea J (2010) Effect of genistein supplementation of thawing medium on characteristics of frozen human spermatozoa. Asian J Androl 12:431–441

Mazur P (1984) Freezing of living cells: mechanisms and implications. Am J Physiol 247(3 Pt 1):C125–C142

Mazzilli F, Rossi T, Sabatini L, Pulcinelli FM, Rapone S, Dondero F, Gazzaniga PP (1995) Human sperm cryopreservation and reactive oxygen species (ROS) production. Acta Eur Fertil 26:145–148

Meamar M, Zribi N, Cambi M, Tamburrino L, Marchiani S, Filimberti E, Fino MG, Biggeri A, Menezo Y, Forti G, Baldi E, Muratori M (2012) Sperm DNA fragmentation induced by cryopreservation: new insights and effect of a natural extract from Opuntia ficus-indica. Fertil Steril 98:326–333

Medeiros CM, Forell F, Oliveira AT, Rodrigues JL (2002) Current status of sperm cryopreservation: why isn't it better? Theriogenology 57:327–344

Memon AA, Wahid H, Rosnina Y, Goh YM, Ebrahimi M, Nadia FM (2012) Effect of antioxidants on post thaw microscopic, oxidative stress parameter and fertility of Boer goat spermatozoa in Tris egg yolk glycerol extender. Anim Reprod Sci 136:55–60

Minaei MB, Barbarestani M, Nekoonam S, Abdolvahabi MA, Takzare N, Asadi MH, Hedayatpour A, Amidi F (2012) Effect of Trolox addition to cryopreservation media on human sperm motility. Iran J Reprod Med 10:99–104

Moretti E, Mazzi L, Bonechi C, Salvatici MC, Iacoponi F, Rossi C, Collodel G (2016) Effect of Quercetin-loaded liposomes on induced oxidative stress in human spermatozoa. Reprod Toxicol 60:140–147

Moubasher AE, El Din AM, Ali ME, El-sherif WT, Gaber HD (2013) Catalase improves motility, vitality and DNA integrity of cryopreserved human spermatozoa. Andrologia 45:135–139

Murawski M, Saczko J, Marcinkowska A, Chwiłkowska A, Gryboś M, Banaś T (2007) Evaluation of superoxide dismutase activity and its impact on semen quality parameters of infertile men. Folia Histochem Cytobiol 45(Suppl 1):S123–S126

Nekoonam S, Nashtaei MS, Zangi BM, Amidi F (2016) Effect of Trolox on sperm quality in normozospermia and oligozospermia during cryopreservation. Cryobiology 72:106–111

Ngamwuttiwong T, Kunathikom S (2007) Evaluation of cryoinjury of sperm chromatin according to liquid nitrogen vapour method (I). J Med Assoc Thai 90:224–228

O'Connell M, McClure N, Lewis SE (2002) The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod 17:704–709

Oehninger S, Duru NK, Srisombut C, Morshedi M (2000) Assessment of sperm cryodamage and strategies to improve outcome. Mol Cell Endocrinol 169:3–10

Ortiz A, Espino J, Bejarano I, Lozano GM, Monllor F, García JF, Pariente JA, Rodríguez AB (2011) High endogenous melatonin concentrations enhance sperm quality and short-term in vitro exposure to melatonin improves aspects of sperm motility. J Pineal Res 50:132–139

Paasch U, Sharma RK, Gupta AK, Grunewald S, Mascha EJ, Thomas AJ Jr, Glander HJ, Agarwal A (2004) Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. Biol Reprod 71:1828–1837

Pini T, Leahy T, de Graaf SP (2018) Sublethal sperm freezing damage: Manifestations and solutions. Theriogenology 118:172–181

Polge C, Smith AU, Parkes AS (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164:666

Reiter RJ, Tan DX, Manchester LC, Paredes SD, Mayo JC, Sainz RM (2009) Melatonin and reproduction revisited. Biol Reprod 81:445–456

Rossi T, Mazzilli F, Delfino M, Dondero F (2001) Improved human sperm recovery using superoxide dismutase and catalase supplementation in semen cryopreservation procedure. Cell Tissue Bank 2:9–13

Rostand J (1946) Glycerine et resistance du sperm aux basses temperature. CR Acad Sci Paris 222:1524

Said TM, Gaglani A, Agarwal A (2010) Implication of apoptosis in sperm cryoinjury. Reprod Biomed Online 21:456–462

Saito K, Suzuki K, Iwasaki A, Yumura Y, Kubota Y (2005) Sperm cryopreservation before cancer chemotherapy helps in the emotional battle against cancer. Cancer 104:521–524

Sariözkan S, Bucak MN, Tuncer PB, Ulutaş PA, Bilgen A (2009) The influence of cysteine and taurine on microscopic-oxidative stress parameters and fertilizing ability of bull semen following cryopreservation. Cryobiology 58:134–138

Seifi-Jamadi A, Kohram H, Shahneh AZ, Ansari M, Macías-García B (2016) Quercetin ameliorate motility in frozen-thawed Türkmen stallions sperm. J Equine Vet Sci 45:73–77

Semercioz A, Onur R, Ogras S, Orhan I (2003) Effects of melatonin on testicular tissue nitric oxide level and antioxidant enzyme activities in experimentally induced left varicocele. Neuro Endocrinol Lett 24:86–90

Shabani Nashtaei M, Nekoonam S, Naji M, Bakhshalizadeh S, Amidi F (2018) Cryoprotective effect of resveratrol on DNA damage and crucial human sperm messenger RNAs, possibly through 5' AMP-activated protein kinase activation. Cell Tissue Bank 19:87–95

Shafiei M, Forouzanfar M, Hosseini SM, Esfahani MH (2015) The effect of superoxide dismutase mimetic and catalase on the quality of postthawed goat semen. Theriogenology 83:1321–1327

Sherman JK (1963) Improved methods of preservation of human spermatozoa by freezing and freeze-drying. Fertil Steril 14:49–64

Sherman JF (1990) Cryopreservation of human semen. In: Keel B, Webster BW (eds) Handbook of the laboratory diagnosis and treatment of infertility. CRC Press, Boca Raton/Ann Arbor/Boston, pp 229–260

Sierens J, Hartley J, Campbell M, Leathem A, Woodside J (2002) In vitro isoflavone supplementation reduces hydrogen peroxide-induced DNA damage in sperm. Teratog Carcinog Mutagen 22:227–234

Silva EC, Cajueiro JF, Silva SV, Soares PC, Guerra MM (2012) Effect of antioxidants resveratrol and quercetin on in vitro evaluation of frozen ram sperm. Theriogenology 77:1722–1726

Song GJ, Norkus EP, Lewis V (2006) Relationship between seminal ascorbic acid and sperm DNA integrity in infertile men. Int J Androl 29:569–575

Sönmez M, Yüce A, Türk G (2007) The protective effect of melatonin and Vitamin E on antioxidant enzyme activities and epididymal sperm characteristics of homocysteine treated male rats. Reprod Toxicol 23:226–231

Spanò M, Cordelli E, Leter G, Lombardo F, Lenzi A, Gandini L (1999) Nuclear chromatin variations in human spermatozoa undergoing swim-up and cryopreservation evaluated by the flow cytometric sperm chromatin structure assay. Mol Hum Reprod 5:29–37

Stojanović S, Sprinz H, Brede O (2001) Efficiency and mechanism of the antioxidant action of trans-resveratrol and its analogues in the radical liposome oxidation. Arch Biochem Biophys 391:79–89

Succu S, Berlinguer F, Pasciu V, Satta V, Leoni GG, Naitana S (2011) Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner. J Pineal Res 50:310–318

Sun JG, Jurisicova A, Casper RF (1997) Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod 56:602–607

Tan DX, Manchester LC, Reiter RJ, Qi WB, Karbownik M, Calvo JR (2000) Significance of melatonin in antioxidative defense system: reactions and products. Biol Signals Recept 9:137–159

Taylor K, Roberts P, Sanders K, Burton P (2009) Effect of antioxidant supplementation of cryopreservation medium on post-thaw integrity of human spermatozoa. Reprod Biomed Online 18:184–189

Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA, Clark AM (2009) Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod 24:2061–2070

Topraggaleh TR, Shahverdi A, Rastegarnia A, Ebrahimi B, Shafiepour V, Sharbatoghli M, Esmaeili V, Janzamin E (2014) Effect of cysteine and glutamine added to extender on post-thaw sperm functional parameters of buffalo bull. Andrologia 46:777–783

Uysal O, Bucak MN (2007) Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. Acta Vet Brno 76:383–390

Wang X, Sharma RK, Sikka SC, Thomas AJ, Falcone T, Agarwal A (2003) Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. Fertil Steril 80:531–535

White IG (1993) Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. Reprod Fertil Dev 5:639–658

Yan L, Liu J, Wu S, Zhang S, Ji G, Gu A (2014) Seminal superoxide dismutase activity and its relationship with semen quality and SOD gene polymorphism. J Assist Reprod Genet 31:549–554

Zeitoun MM, Al-Damegh MA (2014) Effect of nonenzymatic antioxidants on sperm motility and survival relative to free radicals and antioxidant enzymes of chilled-stored ram semen. Open J Anim Sci 5:50

Zeron Y, Pearl M, Borochov A, Arav A (1999) Kinetic and temporal factors influence chilling injury to germinal vesicle and mature bovine oocytes. Cryobiology 38:35–42

Zhu Z, Ren Z, Fan X, Pan Y, Lv S, Pan C, Lei A, Zeng W (2017) Cysteine protects rabbit spermatozoa against reactive oxygen species-induced damages. PLoS One 12:e0181110

Zielonka J, Gebicki J, Grynkiewicz G (2003) Radical scavenging properties of genistein. Free Radic Biol Med 35:958–965

Zribi N, Feki Chakroun N, El Euch H, Gargouri J, Bahloul A, Ammar Keskes L (2010) Effects of cryopreservation on human sperm deoxyribonucleic acid integrity. Fertil Steril 93:159–166

Zribi N, Feki Chakroun N, Ben Abdallah F, Elleuch H, Sellami A, Rebai T, Fakhfakh F, Keskes LA (2012) Effect of freezing-thawing process and quercetin on human sperm survival and DNA integrity. Cryobiology 65:326–331

10 Effect on Sperm DNA Quality Following Sperm Selection for ART: New Insights

Nicoletta Tarozzi, Marco Nadalini, and Andrea Borini

Abstract

In the female reproductive tract, male gametes undergo a natural sperm selection process in order to discriminate spermatozoa on the basis of their quality to maximize the chances of successful reproduction. With the introduction of assisted reproductive technology (ART), scientists and clinicians developed diverse sperm selection strategies focusing on the isolation of competent spermatozoa. With increasing understanding of sperm functions and fertilization mechanism and evolution of available technologies, the initial simple sperm preparation protocols were complemented, and sometimes replaced, by new sperm-sorting techniques. In particular, while in the early years the focus was on obtaining motile spermatozoa, in later years, especially after the introduction of intracytoplasmic sperm injection (ICSI), the focus shifted to the isolation of functional and "healthy" spermatozoa, considering some other important factors, such as sperm DNA integrity. Sperm DNA damage, as well as chromatin structure alterations, in fact, is related to decreased reproductive ability of men, in natural as well as in assisted reproduction.

Keywords

Sperm DNA quality · Sperm selection · ART outcome

In this chapter, we will describe different sperm selection strategies and their respective effects on sperm DNA quality. In particular we will discuss established sperm selection techniques currently in use in the clinical setting, advanced sperm selection methodologies, and emerging approaches to select spermatozoa for ART (Table 10.1). These different approaches have been proposed with the aim to select a good quality sperm population, not only in terms of morphology and motility, but also in terms of chromatin structure and DNA integrity, which are closely related to human reproductive ability (Borini et al. 2006, Borini et al. 2017, Tarozzi et al. 2007, Tarozzi et al. 2009b).

Sperm Selection: Methods Currently in Use

Various selection approaches can be used to select the best sperm from the raw semen populations and reduce DNA fragmentation rate. At present, the (i) *sperm washing*, (ii) *swim-up*, and (iii) *density-gradient centrifugation* (DGC) procedures remain the most widespread processing

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Table 10.1 The effectiveness of different sperm processing techniques in the selection of DNA damage-free spermatozoa

Sperm selection strategies		Comment
Conventional	Sperm washing Swim-up (SU) Density-gradient centrifugation (DGC)	There is no consensus regarding the effect of these procedures on sperm DNA quality, especially when they are compared to each other In some recent papers, DNA damage was also reported to arise during these sperm preparation techniques, in a proportion of samples
Advanced	Sperm surface charge (Zeta potential, electrophoresis)	*Zeta potential*: improved quality of the selected sperm population in terms of normal chromatin condensation and DNA integrity. This method allows recovery of only a very low number of sperm cells and is not applicable for testicular and epididymal gametes *Electrophoresis*: effective to select spermatozoa that exhibited low levels of DNA damage. Not many investigations have been done on this topic
	Hyaluronic acid binding (PICSI)	In a large number of studies, it has been reported that sperm cells selected by this technique are more likely to have lower DNA fragmentation
	IMSI (MSOME)	The adoption of MSOME as a real-time selection tool per se seems far from being standardized: there are still some doubts about the association of nuclear sperm vacuoles with sperm DNA damage
	MACS	Represents a promising new technology for selection of sperm cells with high DNA integrity; frequently used in combination with other selection techniques (DGC or SU)
	Birefringence	Has the ability to highlight spermatozoa with a better nuclear status and low DNA fragmentation. Not many investigations have been done on this topic
Emerging	Microfluidics Synthetic peptides Raman spectroscopy	These techniques represent potential tools for the evaluation and selection of DNA damage-free spermatozoa, showing encouraging and promising results. Further research and development efforts are necessary to allow the clinical application of this technology for their potential use in ART

methods used in ART laboratories (Henkel and Schill 2003; Paasch et al. 2007). However, it should be noted that these procedures utilize various centrifugation steps, exposing healthy sperm to oxidative stress, which might cause sperm DNA damage (Aitken et al. 2013).

Sperm washing Sperm washing identifies an easy method based on the centrifugation of the semen samples in order to remove the seminal plasma and collect the sperm cells at the bottom of the test tube. Following complete liquefaction, culture medium is added to the ejaculate and centrifuged twice to remove the seminal plasma. The pellet composed of spermatozoa is resuspended in appropriate media and used for subsequent ARTs.

Swim-up (SU) The SU method is useful in selecting motile spermatozoa as it is based on the ability of spermatozoa to migrate into the upper culture medium, while slow and immotile sperm cells remain, along with most debris, in the semen pellet. This method may be performed by layering the culture medium directly over the semen or layering the culture medium over the pellet, which is obtained after washing and centrifugation of the sample.

Density-gradient centrifugation (DGC) DGC is the preferred technique to maximize the recovery of motile spermatozoa in cases of severe oligozoospermia, teratozoospermia, or asthenozoospermia. In this procedure, good-quality sperm can be separated from dead sperm, leukocytes, and other components of the seminal plasma by a discontinuous density gradient.

The comparison of different semen preparation techniques in relation to sperm DNA damage has been the focus of several research studies.

However, there is no consensus in the literature regarding the effect of the different procedures on sperm DNA damage (Donnelly et al. 2000; Sakkas et al. 2000; Zini et al. 2000; Younglai et al. 2001; Hammadeh et al. 2001; Marchetti et al. 2002). Several authors reported that the samples prepared by DGC or SU method, either alone or in combination, had less DNA fragmentation and lower reactive oxygen species (ROS) content than raw semen. However, no significant difference was found between the two approaches (Enciso et al. 2011; Jayaraman et al. 2012; Zhao et al. 2016). Amiri and colleagues found that the percentage of DNA-fragmented spermatozoa in samples prepared by the SU method was larger than those in samples processed with the DGC approach (Amiri et al. 2012). Similar results were obtained by the groups of Ahmad (Ahmad et al. 2007) and Xue (Xue et al. 2014). However, some studies have found that mean DNA fragmentation in samples processed by DGC was higher than in samples collected with the SU method (Marchesi et al. 2010; Ghaleno et al. 2014; Volpes et al. 2016; Kim et al. 2017; Oguz et al. 2018). Finally, a couple of studies on sperm telomere length and DNA fragmentation showed that either the SU or DGC method could screen out sperm with lower DNA fragmentation and with longer telomeres for fertilization (Santiso et al. 2010; Yang et al. 2015).

DNA damage was also reported to arise during sperm processing techniques involving centrifugation (Stevanato et al. 2008); moreover some reports indicated that the use of colloidal silicon-based discontinuous density gradient for sperm separation is sometimes related with the generation of oxidative DNA damage (Aitken et al. 2014). Regarding this topic, a recent study by Muratori et al. (2016) demonstrated that DGC selection of sperm samples for ARTs may induce DNA fragmentation. According to the authors, the sperm selection treatment gave rise to different effects: in a proportion of samples, DGC selection produced an increase in the percentage of sperm without DNA damage; however, in about 50% of samples, post-DGC sperm DNA damage levels were higher than pre-DGC values, suggesting the induction of a de novo DNA damage during the sperm selection procedure. Most importantly, when such an event occurred, the patients had about a 50% lower chance to obtain a pregnancy. So, the authors proposed a sperm DNA fragmentation test, before and after DGC, for all the patients who undergo assisted reproduction treatments. Finally, Muratori and colleagues stressed the importance of the use of alternative strategies for sperm selection for ART in case of increased DNA damage after gradient preparation procedures.

Advanced Methods for Sperm Selection

Sperm Surface Charge for Sperm Selection

Mature human spermatozoa are characterized by a net negative surface charge, acquired during their final differentiation. In particular, during epididymal transit, human spermatozoa acquire three forms of the highly sialylated and negatively charged, lipid-anchored gp20/CD52 glycopolypeptides (Kirchhoff and Schröter 2001). Is it thought that the level of expression of these proteins reflects normal spermatogenesis and sperm maturation (Giuliani et al. 2004).

Based on the differences in sperm membrane charge, it is possible to select spermatozoa by means of two methods: the so-called Zeta potential method and the electrophoretic technique.

Zeta potential The Zeta potential, or electrokinetic potential, is defined as the electrical potential at the slipping plane of a moved particle in suspension away from the interface: in mature spermatozoa, the Zeta potential is about −16 to −20 mV (Henkel 2012). This negative charge is used to select mature spermatozoa by allowing them to adhere to a centrifuge tube which has been positively charged by rubbing or rotating the tube on a latex glove. The non-adherent sperm fraction and other contaminants are removed, and selected adherent spermatozoa are recovered (Chan et al. 2006).

It was shown that the selected sperm population has better quality not only in terms of normal morphology, progressive motility, and ability to undergo hyperactivation but also of normal chromatin condensation and DNA integrity (Chan et al. 2006; Razavi et al. 2009). Compared with DGC, the Zeta potential method was also found to be more effective in the selection of spermatozoa with normal protamine content and DNA integrity (Khajavi et al. 2009; Kheirollahi-Kouhestani et al. 2009). Moreover, a later study showed the effectiveness of using the Zeta potential method after DGC in the selection of spermatozoa with low level of DNA damage and protamine deficiency (Zarei-Kheirabadi et al. 2012). Regarding ART outcome, only few works tested the Zeta potential method, and conclusive data are still missing. In 2016, in a double-blind randomized clinical trial carried on 203 ICSI cycles, Nasr-Esfahani et al. (2016) demonstrated a significant increase in top-quality embryos and pregnancy rate when Zeta potential method was performed after DGC compared with DGC alone. In the same study, the authors also suggested that the Zeta method alters the sex ratio, compared to the conventional DGC method.

The Zeta potential method is very simple to perform, does not require specific equipment, and is relatively inexpensive. Moreover, it has been shown that Zeta potential method may be performed after sperm cryopreservation, since the sperm freezing process does not cause alterations to membrane charge potential (Kam et al. 2007): this application of Zeta potential method is particularly important considering that cryopreservation itself may lead to deleterious effects on sperm DNA integrity (Di Santo et al. 2012). On the other hand, despite the improved quality of the selected sperm population, the Zeta potential method allows recovery of only a very low number of sperm cells, limiting its usefulness especially in oligozoospermic men. The Zeta potential method is not applicable for testicular and epididymal gametes, as these cells lack sufficient net electrical charge on the membrane surface. Finally, it should be considered that this method must be performed as soon as possible after sperm collection because spermatozoa become less negatively charged as they undergo capacitation (Chan et al. 2006).

Electrophoresis In 2005 Ainsworth and colleagues developed an electrophoresis-based technology to separate spermatozoa, according to size and electronegative charge, from immature, dysfunctional germ cells and leukocytes. The device consists of four separate compartments: two outer chambers and two inner chambers (incubation and collection). The outer chambers house the electrodes and are separated from the inner chambers by two polyacrylamide restriction membranes, which retain the cell suspension within the inner chambers. The two inner chambers (inoculation chamber, containing semen sample, and collection chamber) are separated by a polycarbonate membrane whose pore size allows the passage of spermatozoa and excludes precursor germ cells and leukocytes. After 5 minutes of application of the electric field (75 mA; 18–21 V), the selected sperm cells are recovered and ready for ARTs.

In their paper, Ainsworth and colleagues concluded that this technique was comparable to DGC: the selected population of spermatozoa contained viable, motile, and morphologically normal spermatozoa that exhibited low levels of DNA fragmentation. The same authors (Ainsworth et al. 2007) obtained the first live birth after ICSI using electrophoretically isolated spermatozoa. Furthermore, other subsequent investigations by the same authors demonstrated that electrophoretically selected spermatozoa capacitate and bind the zona pellucida normally; also, they seem to be free from the oxidative DNA damage associated with the centrifugation steps of DGC technique (Ainsworth et al. 2011; Aitken et al. 2011). For all these reasons, it has been suggested that electrophoretic separation may represent a promising sperm selection method for assisted conception purposes. In the pilot study of Fleming and colleagues (Fleming et al. 2008), the authors demonstrated that electrophoretic sperm separation is as effective as DGC in preparing sperm for ICSI and in vitro fertilization (IVF): both methods yielded

comparable rates of fertilization, cleavage, and high-quality embryos.

Not many investigations have been done on electrophoresis system and ART outcomes, which may be due to the complexity of the apparatus involved and the costs associated. On the other hand, key features of electrophoresis-based technology that make it attractive for ART clinics are short operation times compared to the DGC technique and the absence of centrifugation steps which may cause damage from ROS and oxidative DNA damage in male gametes.

Sperm Selection by Hyaluronic Acid Binding

The use of hyaluronic acid (HA) for sperm selection can be attributed to the pioneering work of Huszar Gabor and colleagues (reviewed in Huszar 2012). The *ratio* behind this approach is linked to the intrinsic characteristics of spermatozoa that have successfully completed the maturation process, becoming functionally competent. In fact, during spermiogenesis, simultaneously to the final steps of cytoplasmic extrusion, a sperm plasma membrane remodeling process occurs, assisting the formation of HA binding sites, necessary to bind HA contained in the cumulus oophorus-corona radiata complex present around the oocyte. Interestingly, this sperm plasma membrane remodeling seems to correlate also with nuclear maturity, closely associated with sperm DNA integrity and sperm aneuploidy (Cayli et al. 2003). Therefore, HA can be considered a "physiological selector" of mature male gametes that have completed plasma membrane remodeling, cytoplasmic extrusion, and nuclear maturation.

Considering both the selective ability of hyaluronan to bind only to mature spermatozoa and minimal biosafety risks for oocytes, embryos, and patients (hyaluronan is present in cervical mucus, cumulus cells, and follicular fluid), HA has been employed in the development of (i) a diagnostic tool for assessing sperm function and maturity, named sperm-hyaluronan binding assay (HBA), and (ii) two ready-to-use systems designed for HA-mediated ICSI sperm selection, the so-called physiological intracytoplasmic sperm injection (PICSI) and the SpermSlow method.

The HBA is a commercial diagnostic tool based on the use of hyaluronan-coated slides: they allow to assess the proportion of sperm that express HA receptors (bound spermatozoa) versus those that do not (unbound swimming sperm cells). The assay was designed to evaluate sperm cell quality, maturity, and fertilization potential, improving the clinical diagnosis of male infertility, and may be useful in the assignment of patients to ICSI or conventional IVF treatment (Cayli et al. 2004).

PICSI relies on a specially designed Petri dish that features three microdots of hyaluronan hydrogel attached to its base. Prepared sperm sample is placed on top of these HA spots: spermatozoa that bind HA can be easily identified, picked, and used for microinjection (Parmegiani et al. 2012).

SpermSlow is a viscous medium containing HA which may be used in ICSI procedure instead of commonly utilized polyvinylpyrrolidone. In SpermSlow technique, a droplet of treated semen sample is connected to a droplet of culture medium with a pipette tip, and, on the other side, the culture medium is also connected to a SpermSlow droplet. Sperm cells bound to HA exhibit slower movement and are collected from the middle culture medium to be used for microinjection (Parmegiani et al. 2012).

In a number of studies, it has been reported that sperm cells selected by HA are more likely to have enhanced desirable characteristics: not only an improved viability, motility, and morphology but also lower DNA fragmentation, decreased chromosomal aneuploidies, and decreased levels of apoptotic markers (Jakab et al. 2005; Huszar et al. 2006, 2007; Ye et al. 2006; Nasr-Esfahani et al. 2008, 2012; Tarozzi et al. 2009a; Parmegiani et al. 2010a). For example, in 2010, Parmegiani and colleagues evaluated the role of hyaluronan for sperm selection, by comparing sperm DNA fragmentation and sperm nucleus normalcy rate of HA-bound sperm cells versus spermatozoa in polyvinylpyrrolidone, as

defined on the basis of motile sperm organelle morphology examination (MSOME) criteria (Parmegiani et al. 2010a; see below for MSOME criteria). The authors utilized the SpermSlow procedure for HA-bound sperm isolation and an injecting pipette for the collection of cells. Sperm DNA damage was evaluated using sperm chromatin dispersion (SCD) test. In this study, Parmegiani and colleagues demonstrated that spermatozoa bound to HA show a significant reduction in DNA damage and a significant improvement in nucleus normalcy, compared with spermatozoa from polyvinylpyrrolidone-containing medium. On the other hand, another study carried out using MSOME showed no differences in morphology between HA-bound and HA-unbound spermatozoa (Petersen et al. 2010). In 2009 Tarozzi and colleagues evaluated the ability of hyaluronan to select spermatozoa with low DNA fragmentation employing terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Tarozzi et al. 2009a). The TUNEL assay was carried out on total sperm population and on HA-bound spermatozoa obtained by removing the unbound cells from the HA-coated slide surfaces utilized for HBA. This approach allowed the authors to quickly evaluate a large number of sperm cells per patient, clearly demonstrating the significant reduction in DNA fragmentation of the HA-bound spermatozoa. Moreover, the authors also showed that the power of hyaluronan to select spermatozoa with higher DNA integrity was more pronounced in low-quality semen samples, characterized by a higher degree of DNA damage and poor sperm parameters, supporting the concept of improving ICSI efficiency by means of hyaluronan selection.

Presently, while there seems to be an increase in sperm DNA integrity using HA selection systems, there is no consensus about the use of HA to improve ART outcomes. In 2007 Worrilow and colleagues found an improvement in fertilization rates and embryo quality and a decrease in miscarriage rates using PICSI versus conventional ICSI (Worrilow et al. 2007). Later, the same authors confirmed these higher success rates in a multicenter, double-blinded, randomized controlled trial (Worrilow et al. 2012); couples with an HA binding efficiency less than or equal to 65% revealed a statistically significant decrease in miscarriage rates. This was accompanied by an improvement in implantation rates and clinical pregnancy rates, but without statistical significance. Also, in a recent paper (Erberelli et al. 2017), the authors showed that, in couples characterized by male factor infertility, the HA binding system for sperm selection enhances ICSI pregnancy rates. On the other hand, several studies have shown inconsistent results (Tarozzi et al. 2009a; Van Den Bergh et al. 2009; Menezo et al. 2010; Majumdar and Majumdar 2013). As an example, in the paper of Tarozzi and colleagues, although the ability of HA in the selection of spermatozoa with a significant reduction of DNA damage was demonstrated, the authors highlighted no correlation between HA binding and fertilization, cleavage, good embryo quality, implantation, pregnancy, or miscarriage rates in couples undergoing conventional IVF (Tarozzi et al. 2009a). However, another study demonstrated that HA-bound spermatozoa used for ICSI were associated with a better embryo quality and implantation rate although fertilization, miscarriage, and pregnancy rates remained comparable with those obtained performing conventional ICSI (Parmegiani et al. 2010b). Other authors demonstrated that oocytes inseminated after HA sperm selection had significantly higher fertilization rate, while pregnancy and implantation rates were only moderately increased (Nasr-Esfahani et al. 2008). These conflicting results and the paucity of studies clearly demonstrating the effectiveness of HA in improving ART outcomes were reported in a Cochrane Database Review (McDowell et al. 2014) concluding that evidence was insufficient to define if spermatozoa selected by HA binding improve pregnancy outcomes and live birth in ART. This opinion was also shared by the authors of a recent review aimed to determine the efficacy of the PICSI technique versus conventional ICSI in couples with male factor infertility, with respect to live births, pregnancy, implantation, miscarriage, embryo quality, and fertilization rates (Avalos-

Durán et al. 2018): the authors concluded that there is no statistically significant difference between PICSI and ICSI approaches in any of the analyzed reproductive outcomes.

In summary, considering the role of HA as "physiological selector" of mature male gametes in natural conception and on the basis of current evidence suggesting the ability of HA to select spermatozoa with low DNA damage in vitro, the utility of these selection systems appears promising. However larger studies are urgently needed to come to a conclusion about hyaluronan binding efficacy to improve ART outcomes.

Intracytoplasmic Morphologically Selected Sperm Injection (IMSI)

One of the major concerns regarding ICSI is subjective morphological selection of a spermatozoon based on the embryologist's routine evaluation under at ×400 optical magnification (Souza Setti et al. 2010). According to some authors, this magnification is not adequate to detect the presence of fine nuclear defects, with the risk of selecting defective male gametes, lowering success chances or even transmitting genetic- and chromosomal-derived diseases (Berkovitz et al. 2006; Cassuto et al. 2014).

To overcome the detection limits of conventional microscopy, in 2001, Bartoov et al. (2001) pioneered the development of a new method of unstained, real-time, high magnification examination of spermatozoa (up to ×6600). This unique high magnification scoring of spermatozoa is known under the term MSOME and allows finer observation and analysis of sperm morphology. Besides conventional sperm morphology assessment, indeed, MSOME evaluates the presence, size, and location of sperm head vacuole, as well as detailed characteristics of shape, acrosome, neck, tail, and other minor sperm structures. According to the original MSOME criteria, nuclear chromatin is considered to be abnormal if the sperm head contains one or more vacuoles (diameter of 0.78 ± 0.18 μm) occupying more than 4% of the normal nuclear area (Bartoov et al. 1994, 2001, 2002).

Presence of nuclear sperm vacuoles has been described to be associated with sperm DNA damage (Cassuto et al. 2012), but there are still some doubts about it (Berkovitz et al. 2006; Komiya et al. 2013).

The clinical relevance of the presence of sperm nuclear vacuoles among conventional semen characteristics or other sperm quality markers, such as chromosomal abnormality and DNA integrity, was specified with a wealth of details by Hammoud et al. (2013). The advantage of the real-time analysis of fine nuclear morphology at a high magnification for selecting spermatozoa is believed to depend on the possibility to exclude sperm cells bearing these alterations for ARTs (Garolla et al. 2008; Avendano et al. 2009). 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).

As it happens for many laboratory-grown techniques, the adoption of MSOME as a real-time selection tool per se seems far from being standardized, with several existing definitions for sperm morphological normalcy. In fact, the criteria initially proposed by Bartoov to define a normal spermatozoon have been slightly but progressively modified in the last few years, according to the opinions or data of various authors (Vanderzwalmen et al. 2008; Cassuto et al. 2009; Mauri et al. 2010; Perdrix et al. 2012). In addition, there is still limited evidence regarding the clinical relevance of these sperm features.

Supposedly, the absence of a predictive value of MSOME high magnification scoring may reflect the fact that the morphology pattern seen in cells of a semen sample is not necessarily representative of the ability of the individual spermatozoon to fertilize the oocyte. It is also clear that the definition of sperm morphology and its assessment, as well as other functional aspects, is a very controversial field (Eliasson 2010).

Microinjection of sperm cells selected by the score of the MSOME criteria culminated in a modified ICSI technique, called IMSI (Bartoov et al. 2003). It soon emerged that fine morpho-

logical integrity of the human sperm nucleus is an important characteristic associated with pregnancy rate (Bartoov et al. 2003; Berkovitz et al. 2005). In detail, the pioneers of the IMSI technique reported a significant increase in pregnancy rate with IMSI (66%) compared to traditional ICSI procedure (30%). The IMSI implantation rate was even threefold higher (27.9% vs 9.5% respectively). In cases where no optimal sperm cells were available for IMSI, an increase in abortion rate from 10% to 57% (Berkovitz et al. 2005) was reported. Although different authors have recommended IMSI procedure to be systematically offered to all patients, in the general opinion, the routine use of IMSI is unwarranted, considering the unclear benefit for unselected males and even for specific indications (Bartoov et al. 2003; Hazout et al. 2006; Antinori et al. 2008; Lo et al. 2013). In addition, another reported benefit derived from the use of IMSI may be the reduction in the health-related risks in the IMSI-conceived children compared with traditional ICSI (Cassuto et al. 2014). It should be considered also that the ability to reveal fine, subtle malformations in sperm morphology may also be affected by "background noise" in the form of morphological polymorphisms and phenotypical differences without clinical impact.

However, studies on the comparison between the IMSI method and traditional low magnification ICSI highlighted conflicting results with regard to the different clinical outcomes, including fertilization, embryo characteristics, pregnancy, and live birth rates (Hazout et al. 2006; Antinori et al. 2008; Mauri et al. 2010). For example, in a few studies, it was suggested that IMSI may not be of advantage in improving fertilization and early embryo development (Nadalini et al. 2009; Mauri et al. 2010; De Vos et al. 2013). Other studies indicated that IMSI may have a positive impact on later stages such as implantation, as higher pregnancy rates and lower abortion rates were described in patients that underwent IMSI when compared with patients that underwent traditional ICSI (Bartoov et al. 2003; Berkovitz et al. 2006).

It should be pointed out that the most papers published on IMSI were based on weak study designs or small groups of patients. However, in a recent strict prospective sibling-oocyte study comparing ICSI and IMSI groups, performed on 350 treatments, De Vos and colleagues (2013) described comparable results in terms of fertilization, embryo quality, and clinical outcome. The same authors stressed the concept that a routine employment of IMSI procedure in unselected ART patients cannot be advocated. The first meta-analysis comparing outcome results from 357 IMSI cycles versus 349 traditional ICSI cycles from 3 studies demonstrated no significant difference in fertilization rate. However, a significantly improved implantation (odds ratio (OR) 2.72; 95% confidence interval (CI) 1.50–4.95) and pregnancy rate (OR 3.12; 95% CI 1.55–6.26) was observed in IMSI cycles. Moreover, the results showed a significantly decreased miscarriage rate (OR 0.42; 95% CI 0.23–0.78) in IMSI cycles as compared with ICSI cycles (Souza Setti et al. 2010). Nevertheless, the most relevant available information on the effectiveness and safety of IMSI and ICSI in couples undergoing ART resides in a Cochrane Review, which indicates that the results from properly randomized controlled trials do not support the clinical application of IMSI technique. Importantly, the evidence that IMSI improves the clinical outcome in terms of pregnancy, miscarriage, and live birth is of very low quality (Teixeira et al. 2013). In addition, it should be pointed out that IMSI is a rather time-consuming technique since selecting enough morphologically normal spermatozoa for oocyte injection according to MSOME score may take up to 2 hours for a single case (Antinori et al. 2008). It has been suggested that a lengthy step of assessment and selection of spermatozoa at high magnification might damage male gametes, consistent with the observation that after 2 hours on the microscope's heated stage, sperm nucleus vacuolization displays a significant increase (Peer et al. 2007). Whether modifications occurring in the nucleus during the procedure are associated with increased sperm DNA damage is presently unknown.

In conclusion, there is enough evidence in literature that indiscriminate routine application of MSOME criteria in order to select the best

spermatozoon is not recommended (Marci et al. 2013). Nevertheless, in cases of severe male factor infertility, such as patients with high sperm DNA fragmentation levels, selection of normal spermatozoa with a vacuole-free head with IMSI may give the chances to obtain clinical pregnancies. However, this hypothesis requires confirmation from future studies.

Magnetic-Activated Cell Sorting (MACS)

In 1977, the research group of Molday et al. reported that the magnetic separation of cells was feasible utilizing iron-containing polymeric microspheres tagged with fluorescent dyes and chemically coupled to antibodies or lectins (Molday et al. 1977). This innovative technique, combined with microscopy and flow cytometry, has been widely applied in research as a method of cell identification and separation. More recently, the development of separation columns, containing magnetized matrices capable to retain cells bound to iron microspheres (Miltenyi et al. 1990), patented and marketed under the name of MACS® (magnetic-activated cell sorting, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), resulted in the clinical implementation of the procedure. The device is designed to allow unlabeled cells to pass through a magnetic field column, while labeled cells are retained. In 2001, it was reported that colloidal super-paramagnetic microbeads (50 nm diameter) conjugated with Annexin V were highly effective in separating apoptotic sperm cells by MACS (Grunewald et al. 2001). The procedure was based on the externalization of the phospholipid phosphatidylserine (PS) to the outer leaflet sperm plasma membrane which is a typical feature of apoptosis and occurs early during the programmed sperm cell death (Vermes et al. 2005). Since externalized PS has high affinity to Annexin V, apoptotic sperm cells bind to Annexin V-conjugated colloidal super-paramagnetic microbeads. The magnetically labeled sperm sample is then passed through a magnetic field column where magnetically labeled apoptotic or dead sperm cells (Annexin V-positive fraction) are retained in the column, while the unlabeled vital, non-apoptotic sperm cells (Annexin V-negative fraction) are collected in the flow-through for further processing for ARTs (Manz et al. 1995; Grunewald et al. 2001).

MACS benefits are represented by its simplicity, fast performance, relatively low cost, and high specificity. MACS colloidal super-paramagnetic microbeads are biodegradable and do not affect cell viability (Miltenyi et al. 1990), and no detrimental consequences have been reported on sperm cells (Grunewald et al. 2009). However, research is still needed to ensure the complete absence of freely floating microbeads in the non-apoptotic sperm cell fraction to eliminate the risk of injection in the oocyte. Moreover, MACS has limitations regarding sperm concentration and loading volume due to the small size and volume of the column. Another limit of MACS technology is that this sorting method is usually performed in conjunction with semen sample preparation techniques, like DGC, involving multiple centrifugation steps, which may result in higher ROS production. On the other hand, the DGC is recommended in order to exclude seminal plasma, leukocytes, and immature germ cells (Said et al. 2008).

Most studies compared the effectiveness of MACS with classical processing methods (DGC or SU) by measurement of DNA fragmentation. A few studies also compared the effects of combined methods on sperm morphology and apoptotic markers (Said et al. 2008; Lee et al. 2010; Gil et al. 2013; Nadalini et al. 2014; Bucar et al. 2015; Cakar et al. 2016).

In particular, one study reported a comparison between DGC alone, MACS alone, DGC before MACS, DGC after MACS, and a washed control (Tavalaee et al. 2012). It was found that both MACS alone and DGC alone reduce DNA fragmentation by about 30%. Both methods of combining the two technique, DGC before MACS and MACS before DGC, produce a reduction in sperm DNA fragmentation (40% and 49%, respectively) and apoptotic sperm cells compared to MACS or DGC alone. The use of MACS before DGC, in particular, also involves slight

increase in morphologically normal sperm cells, removal of leftover microbeads, and removal of tail defects inflicted by the MACS column. Similar results were obtained in 2017 by the group of Berteli, indicating that the MACS before DGC protocol was the most effective being able to separate sperm cells with the lowest percentage of DNA fragmentation, the highest percentage of progressive motility, and the highest percentage of normal morphology (Berteli et al. 2017). Conversely, the group of Chi et al. (2016) reported that MACS after DGC was more useful for clinical selection of sperm with higher DNA integrity than MACS before DGC.

In conclusion, MACS represents a promising new technology for sperm selection, but its improvement in ART success is still under debate. Recently, a systematic review and meta-analysis, considering the potential beneficial effects of MACS on IVF outcomes, reported that the implantation and miscarriage rates did not vary between MACS and standard sperm selection methods; however, the authors did observe an improvement in pregnancy rates (Gil et al. 2013). In addition, most of the studies described in the review have different experimental design, inclusion criteria, and population size. To confirm or reject the usefulness of this technique in clinical practice, further controlled and randomized studies are required.

Sperm Head's Birefringence

In 2004, Baccetti demonstrated that it was possible to include a polarization apparatus in an inverted light microscope used for ICSI to analyze the human motile sperm head birefringence in order to highlight the structural normality of spermatozoa (Baccetti 2004). Birefringence is a feature based on how light passes through the protoplasmic compartment of spermatozoa allowing distinction between isotropic and anisotropic sperm head and highlighting possible sperm defects (Magli et al. 2012).

In a preliminary study taking into account the comparison between groups of normal and abnormal semen samples according to WHO criteria, the normal specimens showed a superior amount of birefringent sperm, demonstrating the probable usefulness of this method to select viable spermatozoa (Gianaroli et al. 2008). Morphologically normal, motile spermatozoa exhibited a greater incidence of birefringence than sperm cells of oligoasthenoteratozoospermic (OAT) patients and spermatozoa obtained by testicular sperm extraction (Gianaroli et al. 2008; Magli et al. 2012). According to the same groups of authors, OAT semen specimens showing spermatozoa with progressive motility also had greater incidence of birefringence than OAT semen specimens with absence of motility or spermatozoa obtained by testicular sperm extraction (Gianaroli et al. 2008; Magli et al. 2012).

Another study demonstrated the association of sperm head birefringence with higher DNA integrity (Crippa et al. 2009). A further study has suggested that sperm head's birefringence can be evaluated as total or partial depending on the completion of the acrosome reaction (Gianaroli et al. 2008). Partial birefringence represents a characteristic of the completion of the acrosome reaction; the selection of sperm with partial birefringence seems to enhance the possibility to select spermatozoa with higher DNA integrity (Gianaroli et al. 2008; Magli et al. 2012). Similar results were evidenced by the study of Petersen (2011), in which significantly lower amount of sperm DNA damage was found for partial birefringence compared to total birefringence (7.3% versus 19.5%, respectively) (Petersen et al. 2011). When sperm head's birefringence was combined with MSOME, it was found that sperm selected by coupling the two procedures shows lower amount of DNA fragmentation when compared to sperm cells selected through the use of just one of the two methods individually (Garolla et al. 2014).

Following selection of birefringent sperm before ICSI, Gianaroli et al. (2010) reported higher percentages of day 3 good-quality embryos, higher implantation rates, and higher competence to proceed at least beyond 16 weeks of gestation with respect to ICSI cycles with conventionally selected sperm (Gianaroli et al. 2010). Similar results were obtained by Vermey and colleagues, supporting the indication that

when spermatozoa were selected based on their birefringence, a higher embryo quality could be expected (Vermey et al. 2015).

In conclusion, birefringence has the ability to highlight spermatozoa with a better nuclear status, low DNA fragmentation index (DFI), completion of acrosomal reaction, and thus higher fertilization potential; however, the benefit is mostly seen in patients with abnormal sperm parameters. Further studies are required to clearly demonstrate its role in routine practice.

Emerging Methods for Sperm Selection

Microfluidics

Microfluidics may be defined as the study of fluid behavior at a sub-microliter level. The field of study of this science and technology finds application in a number of scientific areas, including chemistry, medicine, genetics, and cell and molecular biology (Smith and Takayama 2017). The biochemical and mechanical characteristics of microfluidics can have practical applications in ART and, in particular, in the field of human sperm analysis and selection: microfluidic technology utilizes the dynamic and biochemical characteristics of fluids at a microscale to enhance sperm isolation, manipulation, and analysis.

It should be emphasized that we actually know very little about the dynamics of sperm migration through the female reproductive tract. The achievement of the oocyte by a very small fraction of sperm suggests that there is a strong selection pressure acting on male gametes during their passage through the female reproductive tract. Recent progresses of microfluidic technology provide new means to clarify the specific selection pressure affecting the male gamete and to apply this information to the diagnosis of male infertility. Moreover, microfluidics has the potential to provide a more physiological and efficient system for the selection of spermatozoa to be used in ART (Suarez and Wu 2017).

The first reports on the use of microfluidic devices to sort and isolate spermatozoa with hypothetical therapeutic implications were in 2003 (Cho et al. 2003; Schuster et al. 2003). In these papers, it was described the use of a simple, small, and disposable microfluidic device that can isolate motile and morphologically normal spermatozoa from seminal plasma, nonmotile sperm, and other cellular debris. This selection method was based on the ability of motile spermatozoa to cross streamlines in a laminar fluid stream. The two studies have shown that this microfluidic system increased sperm motility to almost 100% and improved the selection of spermatozoa with normal morphology also in oligozoospermic samples.

Commonly used semen processing systems currently used in ART, such as DGC and SU, all provide efficient means of sperm selection and preparation, but each involves centrifugation, which has been reported to cause damage to sperm DNA (Twigg et al. 1998): the use of centrifugation would expose spermatozoa to a level of ROS that can create oxidative stress, directly correlated with sperm DNA fragmentation (Barroso et al. 2000). For these reasons, it was hypothesized that the passive microfluidic sperm sorting could lead to the selection of male gametes with a lower degree of DNA damage. Already in 2007, it was shown that microfluidic sperm processing could provide spermatozoa with significantly reduced DNA damage, in comparison with DGC, SU, and simple wash with serial centrifugation (Schulte et al. 2007). In 2014 Nosrati and colleagues presented a clinically applicable microfluidic device for the selection of spermatozoa. This microfluidic system, which consists of 500 parallel microchannels and could process up to 1 ml of semen, represents a one-step procedure for high DNA integrity sperm selection: clinical tests demonstrated more than 80% improvement in human sperm DNA integrity, significantly outperforming common semen processing systems currently used in ART (Nosrati et al. 2014). This finding was confirmed in two recent papers (Shirota et al. 2016; De Martin et al. 2017). In 2016 Shirota and colleagues used flow cytometric measurement and sperm chromatin structure assay for analysis of DNA fragmentation after sperm preparation using microfluidic sperm

selection or the centrifugation and SU procedure, showing a significantly lower sperm DNA fragmentation rate with the microfluidic system. In 2017 De Martin and colleagues developed a novel one-step procedure to select and recover sperm with mature chromatin, evaluated using the aniline blue assay, for ICSI.

Interesting new microfluidic systems are based on sperm chemotaxis. The process of chemotaxis is the movement of cells or organisms following a gradient of chemoattractants. Experimental data support the idea that progesterone, secreted by cumulus cells, is the major chemoattractant for human spermatozoa that are actively guided to the oocyte (Teves et al. 2006). Since only a small fraction of male gametes are chemotactically responsive, it was hypothesized that the sperm population with enhanced ability to migrate to the chemoattractant source is characterized by superior morphological and functional features. Based on this hypothesis, a number of chemotaxis chips have been developed and used to investigate sperm motility and chemotaxis (Xie et al. 2010; Zhang et al. 2015). However, the impact of the use of chemotaxis-selected spermatozoa on ART outcomes is currently unknown.

All these data and the numerous interdisciplinary groups worldwide working in the area of microfluidics are encouraging for the development of a new generation of microfluidic devices for sperm selection, with the potential to be used in ART.

Synthetic Peptides

It has been shown how short synthetic peptides can be created to mimic protein domains, targeting specific epitopes (Merrifield 2001).

In the work of Enciso et al. (2012), the authors studied the development of a new synthetic peptide that binds to fragmented DNA and could potentially be used to reveal sperm DNA damage, coupling diagnostic and therapeutic intervention in one step: this approach would allow the selection of DNA-free spermatozoa to be used directly for fertilization.

In particular, p53, a protein with DNA-binding properties, was selected for the development of a synthetic peptide for detection of DNA damage. The synthetic oligopeptide (DW1), corresponding to 21 amino acids of the human p53 biomolecule, was designed using a bioinformatic approach based on analysis of 3D protein structure; specifically, DW1 was modeled on the critical region of p53 associated with DNA binding and labeled with a terminal rhodamine B dye. In the study design, human sperm samples were treated to induce single- and double-stranded DNA breaks, and the ability of DW1 to detect these kinds of damage was measured and compared with results obtained using standard tests for DNA fragmentation analysis (TUNEL assay, comet assay, and sperm chromatin dispersion test). The authors demonstrated that the synthetic DW1 peptide has affinity for various DNA lesions, such as double- and single-stranded breaks; moreover, this peptide-based staining displays a close correlation with the percentage of cell possessing DNA damage, evaluated using the standard, established tests for sperm DNA fragmentation.

In summary, the authors showed that DW1 can be employed to evaluate DNA damage in individual sperm cells, representing an easy-to-use, quick, and inexpensive method that is alternative to sperm DNA damage standard tests. However, this approach currently requires the permeabilization of plasma membranes, preventing this oligopeptide from being employed for the selection of spermatozoa with intact DNA to be used in vivo, in IVF treatments.

So, further research is needed to develop and optimize the peptides that can cross the plasma membrane and target sperm DNA damage, allowing the application of this technology for the selection of DNA-free spermatozoa to be used directly for fertilization.

Raman Spectroscopy

Raman spectroscopy (RS) is a noninvasive technique that provides a “fingerprint” of the sample’s biomolecular constituents and chemical

composition. The basic principle of this technology has been first described more than 80 years ago. Advances in optic and information technology and the development of nanotechnologies have made it possible to transform this tool into an important technology to be used in advanced biomedical research.

The Raman effect is the condition for which a small percentage of photons produced by a source are absorbed by the sample and reissued at a different frequency and wavelength to that in the original source. These shifts are different depending on the atomic mass, quantity of valence electrons, and molecular bonds, so these changes are characteristic of the molecular constituents, their arrangement, and their state (Ellis et al. 2013). Therefore, Raman spectroscopy, by examining the inelastic scattering of photons, provides information on the vibrational energies of the sample's molecular constituents and in a time frame that is feasible for use on biological material. The combination of confocal microscopy with Raman spectroscopy (Raman micro-spectroscopy) allows to obtain 3D spatial resolution, making possible investigations in a specific cell's component as well as its localization in situ, into single live cell. Therefore, as the noninvasiveness of the technique is maintained, this analysis allows for not only the detailed fingerprinting of a cell's components but also their localization and tracking (Swain and Stevens 2007; Mallidis et al. 2014).

Raman investigations were initially descriptions of biologically important components (e.g., cytochrome C or hemoglobin). With greater sensitivity and advances in spatial resolution, more complex interaction or compounds and structures made up of multiple elements were assessed (e.g., antibodies and viruses), allowing even a detailed analysis of cells and tissue sections. Particularly used in cancer research, Raman spectroscopy has been introduced into different areas of biomedical research and is now making inroads into clinical practice. Oncology research was the portal by which this technique was introduced into reproductive medicine to assess various aspects of reproductive functions (reviewed in Mallidis et al. 2014), including evaluation of both male and female gametes. In particular, considerable progress has been made in the identification, characterization, and localization of sperm DNA damage.

Three relatively recent studies investigated human spermatozoa applying Raman microspectroscopy (Huser et al. 2009; Meister et al. 2010; Mallidis et al. 2011).

In 2009, Huser and colleagues used this technology in individual human sperm cells and analyzed how differences in the Raman spectra of sperm chromatin correlate with cell shape. These authors showed the presence of vibrational marker modes, in spectra of individual human spermatozoa, which can be used to assess the degree of DNA packaging in each cell. They also found that DNA packaging rate and the relative protein content per cell are distributed over a wide range for spermatozoa with both normal and abnormal shapes. These results indicate that Raman spectroscopy should be an effective tool in assessing the quality of spermatozoa.

In their spectral mapping, Meister et al. (2010) characterized the nucleus, the neck, and the mitochondria-rich middle piece of human spermatozoa. The authors quantified the effect of ultraviolet (UV) radiation on different organelles and recorded chemical changes within the subcellular structure as a function of UV light exposure time, also demonstrating that Raman micro-spectroscopy can be a fast diagnostic tool for assessing the mitochondrial and motility status of human sperm cells.

Mallidis et al. (2011) reported that Raman spectra provide a chemical map delineating every sperm head region. In particular, they compared Raman spectra from individual human sperm cells before and after exposition to UVB radiation, demonstrating the possibility to detect the localization of UVB-induced sperm DNA damage. The conclusion of these authors was that Raman technology can be an accurate tool for assessing sperm DNA structure and the location and sites of damage.

All three studies have highlighted the importance of the peak at 1092 cm^{-1}, the signal attributable to the PO_4 backbone of DNA. By assessment of changes in this peak, it was possible to evaluate nuclear DNA status and construct maps showing

the sites where DNA damage occurred. Furthermore, the accuracy and reproducibility of these results obtained with Raman technology were highlighted by the study of Sánchez and colleagues (2012). This study was performed to determine whether Raman micro-spectroscopy was able to identify different levels of oxidative sperm DNA damage and to correlate Raman profiles with flow cytometric assessments of nuclear sperm DNA damage (DFI), one of the most widely used and clinically applied methods for DNA fragmentation evaluation. The authors demonstrated that the estimation of the percentage of sperm with DNA damage using Raman technology correlated linearly with the flow cytometric assessment and concluded that Raman micro-spectroscopy may provide a means of assessing the nuclear DNA status of a living sperm.

All these elements of evidence, in conclusion, highlight the real potential of this technology, which can be used as an important diagnostic tool for the evaluation, in particular, of sperm DNA structure and damage and, in a future prospective, for the sperm selection in ART. To date, the main problem in using Raman technology in ART is that the method works on air-dried or fixed spermatozoa. Despite suggestions that alive spermatozoa have been scanned successfully (Liu et al. 2013), no data have been so far published to confirm and implement these claims.

Conclusions

Sperm selection procedures for ART should be fast, easy, and low budget and possess the capacity to select motile and morphologically normal spermatozoa avoiding the production of ROS. ROS generation and sperm DNA fragmentation have negative consequences on ART outcomes. Therefore, sperm DNA quality is an important component of spermatozoa selected for ART. Sperm preparation procedures involving multiple centrifugation steps result in higher ROS production and damaged sperm DNA. Thus, centrifugation should be avoided or limited during sperm-sorting procedures by using substitute methods.

Advanced sperm selection techniques have been shown to be able to enhance sperm DNA quality, morphology, and motility, but there are still some concerns on whether these are significant improvements over the conventional procedures based on centrifugation. Zeta potential method and electrophoresis-based technology can select sperm with improved quality and low levels of DNA fragmentation, but their effects on ART outcomes have been poorly studied, and conclusive data are still missing. Magnetic-activated cell sorting also improves overall sperm quality and DNA integrity, but it requires centrifugation steps. Its impact on ART outcomes therefore remains a matter of discussion. Regarding IMSI, selection of spermatozoa with a vacuole-free head after observation at a very high magnification could lead to lower levels of sperm DNA damage, but its clinical usefulness in ARTs remains to be confirmed in future studies on a large scale. Considering the role of HA in sperm binding, the utility of this selection system to retrieve spermatozoa with low DNA damage appears promising, even if larger studies are needed to come to a conclusion about its clinical efficacy. Also selection procedures based on sperm head's birefringence have the potential to detect spermatozoa with a better nuclear status and low DNA fragmentation, but again further studies are required to establish their role in IVF practice.

Taking into account emerging methods for sperm preparation for ART, Raman spectroscopy represents a potential diagnostic tool for the evaluation, in particular, of sperm DNA structure and damage and, in a future prospective, for the selection of human spermatozoa in ART. Similarly, synthetic peptides, as DW1, represent a promising, easy-to-use, quick, and inexpensive method alternative to standard sperm DNA damage tests. Nevertheless, further research and development efforts are necessary to allow the clinical application of this technology for the selection of DNA damage-free spermatozoa. Finally, although a new generation of microfluidic devices for sperm selection show encouraging and promising results, these sorting methods should be thoroughly investigated for their potential use in ART.

In conclusion, despite encouraging preliminary results obtained with new sperm selection techniques for ART, more research is warranted to address safety issues before widespread application of these approaches. In particular, one of the major requirements would be to introduce a sperm selection method based on a solid diagnostic background and be able to select healthy and DNA-intact sperm for direct use in ART treatments.

References

Ahmad L, Jalali S, Shami SA et al (2007) Sperm preparation: DNA damage by comet assay in normo- and teratozoospermics. Arch Androl 53(6):325–338

Ainsworth C, Nixon B, Aitken RJ (2005) Development of a novel electrophoretic system for the isolation of human spermatozoa. Hum Reprod 20:2261–2270

Ainsworth C, Nixon B, Jansen RP et al (2007) First recorded pregnancy and normal birth after ICSI using electrophoretically isolated spermatozoa. Hum Reprod 22:197–200

Ainsworth CJ, Nixon B, Aitken RJ (2011) The electrophoretic separation of spermatozoa: an analysis of genotype, surface carbohydrate composition and potential for capacitation. Int J Androl 34(5):e422–e434

Aitken RJ, Hanson AR, Kuczera L (2011) Electrophoretic sperm isolation: optimization of electrophoresis conditions and impact on oxidative stress. Hum Reprod 26:1955–1964

Aitken RJ, Bronson R, Smith T et al (2013) The source and significance of DNA damage in human spermatozoa; a commentary on diagnostic strategies and straw man fallacies. Mol Hum Reprod 19(8):475–485

Aitken RJ, Finnie JM, Muscio L et al (2014) Potential importance of transition metals in the induction of DNA damage by sperm preparation media. Hum Reprod 29(10):2136–2147

Amiri I, Ghorbani M, Heshmati S (2012) Comparison of the DNA fragmentation and the sperm parameters after processing by the density gradient and the swim up methods. J Clin Diagn Res 6:1451–1453

Antinori M, Licata E, Dani G et al (2008) Intracytoplasmic morphologically selected sperm injection: a prospective randomized trial. Reprod Biomed Online 16:835–841

Avalos-Durán G, Cañedo-Del Ángel AME, Rivero-Murillo J et al (2018) Physiological ICSI (PICSI) vs conventional ICSI in couples with male factor: a systematic review. JBRA Assist Reprod 22(2):139–147

Avendano C, Franchi A, Taylor S et al (2009) Fragmentation of DNA in morphologically normal human spermatozoa. Fertil Steril 91:1077–1084

Baccetti B (2004) Microscopical advances in assisted reproduction. J Submicrosc Cytol Pathol 36(3–4):333–339

Barroso G, Morshedi M, Oehninger S (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. Hum Reprod 15:1338–1344

Bartoov B, Eltes F, Pansky M et al (1994) Improved diagnosis of male fertility potential via a combination of quantitative ultramorphology and routine semen analyses. Hum Reprod 9(11):2069–2075

Bartoov B, Berkovitz A, Eltes F (2001) Selection of spermatozoa with normal nuclei to improve the pregnancy rate with intracytoplasmic sperm injection. N Engl J Med 345:1067–1068

Bartoov B, Berkovitz A, Eltes F et al (2002) Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. J Androl 23(1):1–8

Bartoov B, Berkovitz A, Eltes F et al (2003) Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. Fertil Steril 80(6):1413–1419

Berkovitz A, Eltes F, Yaari S et al (2005) The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. Hum Reprod 20(1):185–190

Berkovitz A, Eltes F, Ellenbogen A et al (2006) Does the presence of nuclear vacuoles in human sperm selected for ICSI affect pregnancy outcome? Hum Reprod 21:1787–1790

Berteli TS, Da Broi MG, Martins WP et al (2017) Magnetic-activated cell sorting before density gradient centrifugation improves recovery of high-quality spermatozoa. Andrology 5(4):776–782

Borini A, Tarozzi N, Bizzaro D et al (2006) Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Hum Reprod 21(11):2876–2881

Borini A, Tarozzi N, Nadalini M (2017) Sperm DNA fragmentation testing in male infertility work-up: are we ready? Transl Androl Urol 6(Suppl 4):S580–S582

Bucar S, Gonçalves A, Rocha E et al (2015) DNA fragmentation in human sperm after magnetic-activated cell sorting. J Assist Reprod Genetics 32(1):147–154

Cakar Z, Cetinkaya B, Aras D et al (2016) Does combining magnetic-activated cell sorting with density gradient or swim-up improve sperm selection? J Assist Reprod Genet 33:1059–1065

Cassuto NG, Bouret D, Plouchart JM et al (2009) A new real-time morphology classification for human spermatozoa: a link for fertilization and improved embryo quality. Fertil Steril 92:1616–1625

Cassuto NG, Hazout A, Hammoud I et al (2012) Correlation between DNA defect and sperm-head morphology. Reprod Biomed Online 24:211–218

Cassuto NG, Hazout A, Bouret D et al (2014) Low birth defects by deselecting abnormal spermatozoa before ICSI. Reprod Biomed Online 28:47–53

Cayli S, Jakab A, Ovari L et al (2003) Biochemical markers of sperm function: male fertility and sperm selection for ICSI. Reprod Biomed Online 7(4):462–468

Cayli S, Sakkas D, Vigue L et al (2004) Cellular maturity and apoptosis in human sperm: creatine kinase, caspase-3 and Bcl-XL levels in mature and diminished maturity sperm. Mol Hum Reprod 10(5):365–372

Chan PJ, Jacobson JD, Corselli JU et al (2006) A simple zeta method for sperm selection based on membrane charge. Fertil Steril 85:481–486

Chi HJ, Kwak SJ, Kim SG et al (2016) Efficient isolation of sperm with high DNA integrity and stable chromatin packaging by a combination of density-gradient centrifugation and magnetic-activated cell sorting. Clin Exp Reprod Med 43(4):199–206

Cho BS, Schuster TG, Zhu X et al (2003) Passively driven integrated microfluidic system for separation of motile sperm. Anal Chem 75:1671–1675

Crippa A, Magli M, Paviglianiti B et al (2009) DNA fragmentation and characteristics of birefringence in human sperm head. Hum Reprod 24:i95

De Martin H, Cocuzza MS, Tiseo BC et al (2017) Positive rheotaxis extended drop: a one-step procedure to select and recover sperm with mature chromatin for intracytoplasmic sperm injection. J Assist Reprod Genet 34(12):1699–1708

De Vos A, Van de Velde H, Bocken G et al (2013) Does intracytoplasmic morphologically selected sperm injection improve embryo development? A randomized sibling-oocyte study. Hum Reprod 28(3):617–626

Di Santo M, Tarozzi N, Nadalini M et al (2012) Human sperm cryopreservation: update on techniques, effect on DNA integrity and implications for ART. Adv Urol 2012:854837

Donnelly ET, O'Connell M, McClure N et al (2000) Differences in nuclear DNA fragmentation and mitochondrial integrity of semen and prepared human spermatozoa. Hum Reprod 15:1552–1561

Eliasson R (2010) Semen analysis with regard to sperm number, sperm morphology and functional aspects. Asian J Androl 12:26–32

Ellis DI, Cowcher DP, Ashton L et al (2013) Illuminating disease and enlightening biomedicine: Raman spectroscopy as a diagnostic tool. Analyst 138(14):3871–3884

Enciso M, Iglesias M, Galán I et al (2011) The ability of sperm selection techniques to remove single- or double-strand DNA damage. Asian J Androl 13(5):764–768

Enciso M, Pieczenik G, Cohen J et al (2012) Development of a novel synthetic oligopeptide for the detection of DNA damage in human spermatozoa. Hum Reprod 27:2254–2266

Erberelli RF, Salgado RM, Pereira DH et al (2017) Hyalurinan-binding system for sperm selection enhances pregnancy rates in ICSI cycles associated with male factor infertility. JBRA Assist Reprod 21(1):2–6

Figueira RC, Setti AS, Braga DP et al (2011) Prognostic value of triploid zygotes on intracytoplasmic sperm injection outcomes. J Assist Reprod Genet 28(10):879–883

Fleming SD, Ilad RS, Griffin AM et al (2008) Prospective controlled trial of an electrophoretic method of sperm preparation for assisted reproduction: comparison with density gradient centrifugation. Hum Reprod 23:2646–2651

Garolla A, Fortini D, Menegazzo M et al (2008) High-power microscopy for selecting spermatozoa for ICSI by physiological status. Reprod Biomed Online 17:610–616

Garolla A, Cosci I, Menegazzo M et al (2014) Sperm selected by both birefringence and motile sperm organelle morphology examination have reduced deoxyribonucleic acid fragmentation. Fertil Steril 101(3):647–652

Ghaleno LR, Valojerdi MR, Janzamin E et al (2014) Evaluation of conventional semen parameters, intracellular reactive oxygen species, DNA fragmentation and dysfunction of mitochondrial membrane potential after semen preparation techniques: a flow cytometric study. Arch Gynecol Obstet 289:173–180

Gianaroli L, Magli MC, Collodel G et al (2008) Sperm head's birefringence: a new criterion for sperm selection. Fertil Steril 90(1):104–112

Gianaroli L, Magli MC, Ferraretti AP et al (2010) Birefringence characteristics in sperm heads allow for the selection of reacted spermatozoa for intracytoplasmic sperm injection. Fertil Steril 93:807–813

Gil M, Sar-Shalom V, Sivira YM et al (2013) Sperm selection using magnetic activated cell sorting (MACS) in assisted reproduction: a systematic review and meta-analysis. J Assist Reprod Genet 30:479–485

Giuliani V, Pandolfi C, Santucci R et al (2004) Expression of gp20, a human sperm antigen of epididymal origin, is reduced in spermatozoa from subfertile men. Mol Reprod Dev 69:235–240

Grunewald S, Paasch U, Glander HJ (2001) Enrichment of non-apoptotic human spermatozoa after cryopreservation by immunomagnetic cell sorting. Cell Tissue Bank 2:127–133

Grunewald S, Reinhardt M, Blumenauer V et al (2009) Increased sperm chromatin decondensation in selected non-apoptotic spermatozoa of patients with male infertility. Fertil Steril 92:572–577

Hammadeh ME, Zavos PM, Rosenbaum P et al (2001) Comparison between the quality and function of sperm after semen processing with two different methods. Asian J Androl 3(2):125–130

Hammoud I, Boitrelle F, Ferfouri F et al (2013) Selection of normal spermatozoa with a vacuole-free head (×6300) improves selection of spermatozoa with intact DNA in patients with high sperm DNA fragmentation rates. Andrologia 45(3):163–170

Hazout A, Dumont-Hassan M, Junca AM et al (2006) High-magnification ICSI overcomes paternal effect resistant to conventional ICSI. Reprod Biomed Online 12:19–25

Henkel RR (2012) Sperm preparation: state-of-the-art—physiological aspects and application of advanced sperm preparation methods. Asian J Androl 14:260

Henkel RR, Schill WB (2003) Sperm preparation for ART. Reprod Biol Endocrinol 1:108–130

Huser T, Orme CA, Hollars CW et al (2009) Raman spectroscopy of DNA packaging in individual human sperm cells distinguishes normal from abnormal cells. J Biophotonics 2:322–332

Huszar G (2012) Sperm testing and ICSI selection by hyaluronic acid binding: the hyaluronic acid-coated glass slide and petri dish in the andrology and IVF laboratories. In: Nagy ZP, Varghese AC, Agarwal A (eds) Practical manual of in vitro fertilization. Springer, New York, pp 241–257

Huszar G, Ozkavukcu S, Jakab A et al (2006) Hyaluronic acid binding ability of human sperm reflects cellular maturity and fertilizing potential: selection of sperm for intracytoplasmic sperm injection. Curr Opin Obstet Gynecol 18:260–267

Huszar G, Jakab A, Sakkas D et al (2007) Fertility testing and ICSI sperm selection by hyaluronic acid binding: clinical and genetic aspects. Reprod Biomed Online 14:650–663

Jakab A, Sakkas D, Delpiano E et al (2005) Intracytoplasmic sperm injection: a novel selection method for sperm with normal frequency of chromosomal aneuploidies. Fertil Steril 84:1665–1673

Jayaraman V, Upadhya D, Narayan PK et al (2012) Sperm processing by swim-up and density gradient is effective in elimination of sperm with DNA damage. J Assist Reprod Genet 29:557–563

Kam TL, Jacobson JD, Patton WC et al (2007) Retention of membrane charge attributes by cryopreserved-thawed sperm and zeta selection. J Assist Reprod Genet 24:429–434

Khajavi NA, Razavi S, Mardani M et al (2009) Can Zeta sperm selection method, recover sperm with higher DNA integrity compare to density gradient centrifugation? Iranian J Reprod Med 7(2):73–77

Kheirollahi-Kouhestani M, Razavi S, Tavalaee M et al (2009) Selection of sperm based on combined density gradient and Zeta method may improve ICSI outcome. Hum Reprod 24:2409–2416

Kim SW, Jee BC, Kim SK et al (2017) Sperm DNA fragmentation and sex chromosome aneuploidy after swim-up versus density gradient centrifugation. Clin Exp Reprod Med 44(4):201–206

Kirchhoff C, Schröter S (2001) New insights into the origin, structure and role of CD52: a major component of the mammalian sperm glycocalyx. Cells Tissues Organs 168:93–104

Komiya A, Watanabe A, Kawauchi Y et al (2013) Sperm with large nuclear vacuoles and semen quality in the evaluation of male infertility. Syst Biol Reprod Med 59:13–20

Lee TH, Liu CH, Shih YT et al (2010) Magnetic-activated cell sorting for sperm preparation reduces spermatozoa with apoptotic markers and improves the acrosome reaction in couples with unexplained infertility. Hum Reprod 25(4):839–846

Liu F, Zhu Y, Liu Y et al (2013) Real-time Raman microspectroscopy scanning of the single live sperm bound to human zona pellucida. Fertil Steril 99:684–689

Lo MG, Murisier F, Piva I et al (2013) Focus on intracytoplasmic morphologically selected sperm injection (IMSI): a mini-review. Asian J Androl 15:608–615

Magli MC, Crippa A, Muzii L et al (2012) Head birefringence properties are associated with acrosome reaction, sperm motility and morphology. Reprod Biomed Online 24(3):352–359

Majumdar G, Majumdar A (2013) A prospective randomized study to evaluate the effect of hyaluronic acid sperm selection on the intracytoplasmic sperm injection outcome of patients with unexplained infertility having normal semen parameters. J Assist Reprod Genet 30:1471–1475

Mallidis C, Wistuba J, Bleisteiner B et al (2011) In situ visualization of damaged DNA in human sperm. Hum Reprod 26:1641–1649

Mallidis C, Sanchez V, Wistuba J et al (2014) Raman microspectroscopy: shining a new light on reproductive medicine. Hum Reprod Update 20:403–414

Manz R, Assenmacher M, Pfluger E et al (1995) Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix. Proc Natl Acad Sci U S A 92(6):1921–1925

Marchesi DE, Biederman H, Ferrara S et al (2010) The effect of semen processing on sperm DNA integrity: comparison of two techniques using the novel Toluidine Blue Assay. Eur J Obstet Gynecol Reprod Biol 151(2):176–180

Marchetti C, Obert G, Deffosez A et al (2002) Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. Hum Reprod 17:1257–1265

Marci R, Murisier F, Lo Monte G et al (2013) Clinical outcome after IMSI procedure in an unselected infertile population: a pilot study. Reprod Health 10:16

Mauri AL, Petersen CG, Oliveira JB et al (2010) Comparison of day 2 embryo quality after conventional ICSI versus intracytoplasmic morphologically selected sperm injection (IMSI) using sibling oocytes. Eur J Obstet Gynecol Reprod Biol 150:42–46

McDowell S, Kroon B, Ford E et al (2014) Advanced sperm selection techniques for assisted reproduction. Cochrane Database Syst Rev 10:CD010461

Meister K, Schmidt DA, Bründermann E et al (2010) Confocal Raman microspectroscopy as an analytical tool to assess the mitochondrial status in human spermatozoa. Analyst 135:1370–1374

Menezo Y, Junca AM, Dumont M et al (2010) "Physiologic" (hyaluronic acid-carried) ICSI results in the same embryo quality and pregnancy rates as with the use of potentially toxic polyvinylpyrrolidone (PVP). Fertil Steril 94(4 Suppl):S232

Merrifield B (2001) Life during a golden age of peptide chemistry — the concept and development of solid-phase peptide synthesis. Oxford University Press, Oxford, UK

Miltenyi S, Muller W, Weichel W et al (1990) High gradient magnetic cell separation with MACS. Cytometry 11:231–238

Molday RS, Yen SP, Rembaum A (1977) Application of magnetic microspheres in labelling and separation of cells. Nature 268:437–438

Muratori M, Tarozzi N, Cambi M et al (2016) Variation of DNA fragmentation levels during density gradient sperm selection for assisted reproduction techniques: a possible new male predictive parameter of pregnancy? Medicine (Baltimore) 95(20):e3624

Nadalini M, Tarozzi N, Distratis V et al (2009) Impact of intracytoplasmic morphologically selected sperm injection on assisted reproduction outcome a review. Reprod Biomed Online 19(l3):45–55

Nadalini M, Tarozzi N, Di Santo M et al (2014) Annexin V magnetic-activated cell sorting versus swim-up for the selection of human sperm in ART: is the new approach better than the traditional one? J Assist Reprod Genet 31(8):1045–1051

Nasr-Esfahani MH, Razavi S, Vahdati AA et al (2008) Evaluation of sperm selection procedure based on hyaluronic acid binding ability on ICSI outcome. J Assist Reprod Genet 25:197–203

Nasr-Esfahani MH, Deemeh M, Tavalaee M (2012) New era in sperm selection for ICSI. Int J Androl 35:475–484

Nasr-Esfahani MH, Deemeh MR, Tavalaee M et al (2016) Zeta sperm selection improves pregnancy rate and alters sex ratio in male factor infertility patients: a double-blind, randomized clinical trial. Int J Fertil Steril 10(2):253–260

Nosrati R, Vollmer M, Eamer L et al (2014) Rapid selection of sperm with high DNA integrity. Lab Chip 14:1142–1150

Oguz Y, Guler I, Erdem A et al (2018) The effect of swim-up and gradient sperm preparation techniques on deoxyribonucleic acid (DNA) fragmentation in subfertile patients. J Assist Reprod Genet 35(6):1083–1089

Paasch U, Grunewald S, Glander HJ (2007) Sperm selection in assisted reproductive techniques. Soc Reprod Fertil Suppl 65:515–525

Parmegiani L, Cognigni GE, Bernardi S et al (2010a) "Physiologic ICSI": hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. Fertil Steril 93:598–604

Parmegiani L, Cognigni GE, Ciampaglia W et al (2010b) Efficiency of hyaluronic acid (HA) sperm selection. J Assist Reprod Genet 27:13–16

Parmegiani L, Cognigni GE, Filicori M (2012) New advances in intracytoplasmic sperm injection (ICSI). Advances in embryo transfer. InTech, New York, pp 99–115

Peer S, Eltes F, Berkovitz A et al (2007) Is fine morphology of the human sperm nuclei affected by in vitro incubation at 37 degrees C? Fertil Steril 88(6):1589–1594

Perdrix A, Saidi R, Menard JF et al (2012) Relationship between conventional sperm parameters and motile sperm organelle morphology examination (MSOME). Int J Androl 35:491–498

Petersen CG, Massaro FC, Mauri AL et al (2010) Efficacy of hyaluronic acid binding assay in selecting motile spermatozoa with normal morphology at high magnification. Reprod Biol Endocrinol 8:149

Petersen CG, Vagnini L, Mauri AL et al (2011) Relationship between DNA damage and sperm head birefringence. Reprod Biomed Online 22(6):583–589

Razavi SH, Nasr-Esfahani MH, Deemeh MR et al (2009) Evaluation of zeta and HA-binding methods for selection of spermatozoa with normal morphology, protamine content and DNA integrity. Andrologia 42:13–19

Said TM, Agarwal A, Zborowski M et al (2008) Utility of magnetic cell separation as a molecular sperm preparation technique. J Androl 29:134–142

Sakkas D, Manicardi GC, Tomlinson M et al (2000) The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. Hum Reprod 15(5):1112–1116

Santiso R, Tamayo M, Gosálvez J et al (2010) Swim-up procedure selects spermatozoa with longer telomere length. Mutat Res 688:88–90

Schulte RT, Chung YK, Ohl DA et al (2007) Microfluidic sperm sorting device provides a novel method for selecting motile sperm with higher DNA integrity. Fertil Steril 88:S76

Schuster TG, Cho B, Keller LM et al (2003) Isolation of motile spermatozoa from semen samples using microfluidics. Reprod Biomed Online 7:75–81

Shirota K, Yotsumoto F, Itoh H et al (2016) Separation efficiency of a microfluidic sperm sorter to minimize sperm DNA damage. Fertil Steril 105:315–321

Smith GD, Takayama S (2017) Application of microfluidic technologies to human assisted reproduction. Mol Hum Reprod 23(4):257–268

Souza Setti SA, Ferreira RC, Paes de Almeida Ferreira Braga D et al (2010) Intracytoplasmic sperm injection outcome versus intracytoplasmic morphologically selected sperm injection outcome: a meta-analysis. Reprod Biomed Online 21:450–455

Stevanato J, Bertolla RP, Barradas V et al (2008) Semen processing by density gradient centrifugation does not improve sperm apoptotic deoxyribonucleic acid fragmentation rates. Fertil Steril 90(3):889–890

Suarez SS, Wu M (2017) Microfluidic devices for the study of sperm migration. Mol Hum Reprod 23(4):227–234

Swain RJ, Stevens MM (2007) Raman microspectroscopy for non-invasive biochemical analysis of single cells. Biochem Soc Trans 35:544–549

Tarozzi N, Bizzaro D, Flamigni C et al (2007) Clinical relevance of sperm DNA damage in assisted reproduction. Reprod Biomed Online 14(6):746–757

Tarozzi N, Nadalini M, Bizzaro D et al (2009a) Sperm hyaluronan-binding assay: clinical value in conventional IVF under Italian law. Reprod Biomed Online 19(Suppl 3):35–43

Tarozzi N, Nadalini M, Stronati A et al (2009b) Anomalies in sperm chromatin packaging: implications for assisted reproduction techniques. Reprod Biomed Online 18(4):486–495

Tavalaee M, Deemeh M, Arbabian M et al (2012) Density gradient centrifugation before or after magnetic-activated cell sorting: which technique is more useful for clinical sperm selection? J Assist Reprod Genet 29:31–38

Teixeira DM, Barbosa MA, Ferriani RA et al (2013) Regular (ICSI) versus ultra-high magnification (IMSI) sperm selection for assisted reproduction. Cochrane Database Syst Rev 7:CD010167

Teves ME, Barbano F, Guidobaldi HA et al (2006) Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. Fertil Steril 86(3):745–749

Twigg J, Irvine DS, Houston P et al (1998) Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. Mol Hum Reprod 4:439–445

Van Den Bergh MJ, Fahy-Deshe M, Hohl MK (2009) Pronuclear zygote score following intracytoplasmic injection of hyaluronan-bound spermatozoa: a prospective randomized study. Reprod Biomed Online 19(6):796–801

Vanderzwalmen P, Hiemer A, Rubner P et al (2008) Blastocyst development after sperm selection at high magnification is associated with size and number of nuclear vacuoles. Reprod Biomed Online 17:617–627

Vermes I, Haanen C, Steffens-Nakken H et al (2005) A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression of early apoptotic cells using fluorescein labeled Annexin V. J Immunol Methods 184(1):39–51

Vermey BG, Chapman MG, Cooke S et al (2015) The relationship between sperm head retardance using polarized light microscopy and clinical outcomes. Reprod Biomed Online 30(1):67–73

Volpes A, Sammartano F, Rizzari S et al (2016) The pellet swim-up is the best technique for sperm preparation during in vitro fertilization procedures. J Assist Reprod Genet 33:765–770

Worrilow KC, Huynh HT, Bower JB et al (2007) PICSI™ vs. ICSI: statistically significant improvement in clinical outcomes in 240 in vitro fertilization (IVF) patients. Fertil Steril 88(Suppl 1):S37

Worrilow KC, Eid S, Woodhouse D et al (2012) Use of hyaluronan in the selection of sperm for intracytoplasmic sperm injection (ICSI): significant improvement in clinical outcomes – multicenter, double-blinded and randomized controlled trial. Hum Reprod 28(2):306–314

Xie L, Ma R, Han C et al (2010) Integration of sperm motility and chemotaxis screening with a microchannel-based device. Clin Chem 56(8):1270–1278

Xue X, Wang WS, Shi JZ et al (2014) Efficacy of swim-up versus density gradient centrifugation in improving sperm deformity rate and DNA fragmentation index in semen samples from teratozoospermic patients. J Assist Reprod Genet 31(9):1161–1166

Yang Q, Zhang N, Zhao F et al (2015) Processing of semen by density gradient centrifugation selects spermatozoa with longer telomeres for assisted reproduction techniques. Reprod Biomed Online 31:44–50

Ye H, Huang GN, Gao Y et al (2006) Relationship between human sperm hyaluronan binding assay and fertilization rate in conventional in vitro fertilization. Hum Reprod 21:1545–1550

Younglai EV, Holt D, Brown P et al (2001) Sperm swim-up techniques and DNA fragmentation. Hum Reprod 16:1950–1953

Zarei-Kheirabadi M, Tavalaee M, Deemeh M et al (2012) Evaluation of ubiquitin and annexin V in sperm population selected based on density gradient centrifugation and zeta potential (DGC-Zeta). J Assist Reprod Genet 29:365–371

Zhang Y, Xiao RR, Yin T et al (2015) Generation of gradients on a microfluidic device: toward a high-throughput investigation of spermatozoa chemotaxis. PLoS One 10(11):e0142555

Zhao F, Yang Q, Shi S et al (2016) Semen preparation methods and sperm telomere length: density gradient centrifugation versus the swim up procedure. Sci Rep 13(6):39051

Zini A, Finelli A, Phang D et al (2000) Influence of semen processing technique on human sperm DNA integrity. Urology 56:1081–1084

Sperm DNA Damage in Cancer Patients

11

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Abstract

Fertility is a growing healthcare issue for a rising number of cancer survivors. In men, cancer itself and its treatment can negatively affect spermatogenesis by targeting the dividing spermatogonia and their cellular environment, ultimately leading to a reduction of testicular germ cells and sperm count. Experimental data and prospective longitudinal studies have shown that sperm production can recover after cancer treatment. But despite this, yet unpredictable, recovery in sperm production, cancer survivors are more at risk to produce sperm with aneuploidy, DNA damage, abnormal chromatin structure, and epigenetic defects even 2 years post-treatment. Sperm DNA alteration is of clinical concern, as these patients may father children or seek assisted reproduction technologies (ART) using gametes with damaged genome that could result in adverse progeny outcomes. Interestingly, large cohort studies revealed lower birth rate but no significant impact on the health of the children born from male cancer survivors (naturally or using ART). Nevertheless, a better understanding of how cocktail of chemotherapy and new anticancer agents affect spermatogenesis and sperm quality is needed to reduce side effects. Moreover, developing new fertility preservation strategies is essential as sperm cryopreservation before treatment is currently the only option but does not apply for prepubertal/young postpubertal patients.

Keywords

Cancer · Chemotherapy · Fertility · Progeny · Radiotherapy · Sperm chromatin · Sperm DNA · Sperm epigenome

Abbreviations

ART	Assisted reproductive technologies
CED	Cyclophosphamide equivalent dose
DFI	DNA fragmentation index
HDS	High DNA stainability
SCSA	Sperm chromatin structure assay
SSCs	Spermatogonial stem cells

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Introduction

Thanks to advances in the medical management of cancer, including early diagnosis and the development of combination chemotherapy treatments, survival rates have increased significantly (Kaatsch 2010; Torre et al. 2016). The number of cancer survivors is therefore constantly increasing, and their quality of life is becoming a major public health issue. Many of the treatments' side effects associated with the diagnosis of cancer have been described (Diller et al. 2009; Miller et al. 2016). A negative impact on reproductive health is part of these long-term effects and can affect the family plans of cancer survivors diagnosed and treated during childhood or at childbearing age. In men in particular, cancer treatments target spermatogenesis (Ragheb and Sabanegh 2010), and cancer survivors have more difficulty to become father (Hohmann et al. 2011; Tang et al. 2016; Yonemoto et al. 2009). Fertility can therefore be a major concern for cancer survivors, especially since 80% of these patients want to have genetically related children and think that their cancer experience will make them better parents (Reinmuth et al. 2008; Schover et al. 2002). It is important to note that data from the Childhood Cancer Survivor Study (CCSS), which uses the largest and most thoroughly characterized cohort of cancer survivors diagnosed during childhood, showed that 53.6% of pediatric cancer male survivors had been medically evaluated for infertility in comparison to 21.4% of siblings (Wasilewski-Masker et al. 2014). In addition, studies on the fertility of cancer survivors show a significant increase in the use of assisted procreation techniques (ART), regardless of age at diagnosis (Stahl et al. 2011; Stensheim et al. 2013). Unfortunately, less than half of survivors report having been informed on this subject during their diagnosis or at the end of treatment (Cherven et al. 2015). The regret of not being adequately informed about the potential risks of anticancer treatments on fertility was also raised predominantly in a focus group study with male survivors and their parents (Stein et al. 2014).

Germ cells are targets of anticancer drugs because of their high cell division activity (Meistrich et al. 1982). Treatments therefore almost always result in low sperm count (Meistrich 1986), and this depletion may be transient or permanent (Schilsky et al. 1980). Determining the risk of infertility after cancer remission remains complex because a combination of factors must be taken into consideration: the type of cancer, the fertility status at the time of diagnosis, the age at diagnosis, the dose, and the combination of treatments received but also probably genetic factors (Jaffe et al. 1988; Müller et al. 1988). Moreover, spermatogenesis recovery with an increase in the number of spermatozoa in the ejaculate might not necessarily be a guarantee of spermatic quality. In this chapter, evidences that motility, morphology, aneuploidy, quality of DNA, and chromatin as well as epigenetic marks can be altered in sperm from cancer survivors will be reviewed. These abnormalities might originate from the cancer itself, but it can also be caused by the treatment. We will also consider the possible consequences of these spermatic abnormalities on the fertility of cancer survivors as well as the health of their offspring, leading to a review of fertility preservation strategies for cancer survivors.

Impact of Cancer on Sperm

The consequences of cancer on spermatogenesis can be assessed in postpubertal patients, on the production of ejaculated spermatozoa. However, a recent study using testicular biopsies taken from prepubertal boys at the time of cancer diagnosis and before treatment gives us the first hint of the effect of childhood cancer on spermatogenesis (Stukenborg et al. 2018). Despite a great variability in the number of spermatogonia in the 12 biopsies collected, the study suggested that genetic abnormalities in hematological diseases (thalassemia majors, Fanconi anemia, and immunodeficiency caused by variant of FOXP3-gene) may be associated with reduced numbers of spermatogonia (Stukenborg et al. 2018). As spermatogonia fuel continuous sperm production

later in adulthood, a reduction in their number in the prepubertal testis could likely result in a low sperm count. In parallel, in pubertal men, it has been shown that the cancer itself can affect sperm production, and this depends on the nature of the cancer and its stage (O'Flaherty et al. 2008; Rueffer et al. 2001). For example, in the case of leukemia or lymphoma, studies have shown that the stage of the disease is positively correlated with the negative impact on spermatogenesis (Rueffer et al. 2001; Gandini et al. 2003). Also, Hodgkin's lymphoma seems to greatly affect fertility, since in the Rueffer et al. study (Rueffer et al. 2001), 70% of the 158 men, tested before starting anticancer treatments, have at least 1 altered sperm parameter compared to the World Health Organization (WHO) standards (Cooper et al. 2010). In the case of testicular cancer, sperm concentration, motility, and morphology are significantly decreased compared to controls which could be due to a predisposition to testicular cell-related pathologies (O'Flaherty et al. 2008).

Chromatin structure abnormalities, DNA breaks, and increased frequency of aneuploidy in spermatozoa can also be measured upstream of cancer treatments compared to controls. Besides, it has been shown that the integrity of sperm DNA is affected by cancer, regardless of the nature of the cancer tested (Bujan et al. 2014, 2013; Kumar et al. 2018; Martinez et al. 2017; Meseguer et al. 2008; O'Flaherty et al. 2010, 2008; Paoli et al. 2015; Stahl et al. 2009; Tamburrino et al. 2017; Tempest et al. 2008). For example, using the COMET assay or the chromatin dispersion test on sperm collected before treatment, from groups of 6 to 26 men diagnosed with various cancers, it has been shown that the fragmentation of sperm DNA is significantly increased compared to the control group (Meseguer et al. 2008; O'Flaherty et al. 2008). More specifically, sperm chromatin structure assay (SCSA) analysis on sperm from men diagnosed with testicular cancer shows a percentage of DNA fragmentation index (DFI), higher than the controls and comparable to a group of infertile men (O'Flaherty et al. 2008; Stahl et al. 2009). In addition, the COMET assay revealed a higher rate of sperm DNA breaks in men diagnosed with testicular cancer or Hodgkin's lymphoma compared to community controls (O'Flaherty et al. 2008). Finally, chromatin compaction, measured by chromomycin A3 labelling, is altered in sperm from men diagnosed with Hodgkin's lymphoma (O'Flaherty et al. 2008). Potential mechanisms of cancer-induced sperm DNA damage may include genetic mutations, changes in hormone levels, fever, inflammation, or general oxidative stress.

Cancer can therefore affect not only the quantity but also the quality of sperm. But it should be noted that, at the individual level, the analysis of chromatin integrity using the SCSA before treatment cannot predict the chances of producing intact spermatozoa after cancer remission (Fossa et al. 1997). Indeed, cancer treatments can also be harmful to sperm production and sperm integrity.

Impact of Cancer Treatment on Sperm

Radiotherapy

The risks of radiation and radiation therapy on male reproductive health through their negative impact on semen parameters have been intensely studied for decades. Various preclinical and clinical studies have indicated the effect of radiation on reduction in the number of type A spermatogonia leading to impairment in spermatogenesis output (Meistrich 2013). It has been estimated that after a single dose of ~10 Gy, only ~15% of patients recover sperm count (Jacob et al. 1998; Sanders et al. 1996). Further, fractionated radiation may be more damaging than a single dose as the former causes greater delays in spermatogenic recovery with lower total doses required to cause permanent azoospermia (Abuelhija et al. 2013; Sandeman 1966).

In addition to its effects on conventional semen parameters, the impact of radiation on sperm DNA damage is of more clinical concern, as these patients may seek ART using gametes with potentially damaged genome for reproduction that could result in adverse reproductive

outcomes. Various groups of investigators have evaluated the impact of radiation on sperm DNA integrity. However, few have included analyses in patients receiving only radiation therapy without chemotherapy, which, as seen in the next section of this chapter, can also have profound effect on sperm chromatin integrity. Paoli et al. reported that for men with testicular seminoma receiving a dose of radiotherapy around 2550 cGy, there is a rise of DFI at 3 and 6 months post-treatment (Paoli et al. 2015). No significant increases, however, were noted at 9, 12, and 24 months post-treatment (Paoli et al. 2015). This transient increase in DFI was also reported in two prospective longitudinal studies on testicular cancer patients treated with adjuvant radiotherapy (Bujan et al. 2013; Stahl et al. 2004). The effects of radiotherapy on the fraction of highly DNA-stainable (HDS) cells among men treated for testicular cancer were less consistent. Some investigators reported a significant decrease in HDS sperm, indicating improved chromatin condensation, with increasing time at 9, 12, and 24 months since the end of treatment (Paoli et al. 2015). Others reported a transient increase of HDS sperm but no subsequent improvement in sperm chromatin condensation (Bujan et al. 2013). Interestingly, the one study on childhood cancer survivors treated with radiotherapy only showed that sperm exhibited statistically significantly higher DFI than the controls (Romerius et al. 2010). The odds ratio (OR) for having DFI >20% in this group was high (OR, 4.9; 95% CI, 1.3–18), but DFI was not associated with dose of scattered testicular irradiation (Romerius et al. 2010).

Several observations have been reported that may illustrate the potential mechanisms of radiation-induced sperm DNA damage and its impact on the sperm genome and epigenome. In a retrospective analysis, Kumar et al. reported altered sperm chromatin integrity in radiation health workers is associated with increase in seminal plasma antioxidant level, probably an adaptive measure to tackle the oxidative stress to protect sperm genomic and functional integrity in exposed subjects (Kumar et al. 2014). In another study, the same group of investigators reported elevated global hypermethylation of spermatozoa (Kumar et al. 2013). Further prospective controlled analyses on a wider scope of cancer diagnoses that can benefit from radiation therapy are still required to fully illustrate the impacts of radiation on the temporal changes of sperm genomic and epigenomic integrity, actual reproductive risks, and developmental health of the offspring, particularly in the context of using ART.

Chemotherapy

Chemotherapy regimens may include cocktails of alkylating agents, antimetabolites, and antitumor metabolites that specifically target proliferating cells. Most of these chemotherapeutic agents are known to disrupt spermatogenesis and male germ cell function as they target actively dividing spermatogonia leading to decreased sperm counts (Meistrich 2013). The extent of injury post-chemotherapy depends on the dosage, duration, and type of agents used (Meistrich 2013).

The first evidence of alteration of spermatogenesis by chemotherapies was observed postmortem on histological sections in 27 out of 30 men treated with nitrogen mustard treatments (Spitz 1948). Since then, alkylating agents have been shown to be the most harmful to male fertility (Meistrich 2013). The use of high-dose alkylating agent treatments results in a high cancer cure rate, but in return, men are at higher risk of developing definitive azoospermia (van der Kaaij et al. 2007; Viviani et al. 1985; Pryzant et al. 1993; Paoli et al. 2016; Sanders et al. 1996). In fact, the chances of finding a sperm concentration in WHO standards after remission are reduced by 33% when cyclophosphamide is administered at dose greater than 9.5 g/m^2 (Pryzant et al. 1993). In addition, the administration of alkylating agents, in comparison with chemotherapy without alkylating agents, reduces by 30% the chances of having a follicle-stimulating hormone (FSH) concentration in the standards, FSH levels being inversely proportional to the production of spermatozoa (Gordetsky et al. 2012; van der Kaaij et al. 2007). The negative impact of alkylating

agents has been shown to be dose-dependent, but the nature of the molecule received may also alter the impact on spermatogenesis. For example, compared to cyclophosphamide, procarbazine is already harmful to sperm production at doses lower than 9.5 g/m^2 (Meistrich 2013). Thus, when the dose of alkylating agent is reduced (cyclophosphamide equivalent dose (CED) <10 g/m^2), the chances of recovery of spermatogenesis after cancer remission are greater but still remain unpredictable (Pryzant et al. 1993; Green et al. 2014). In the case of pediatric cancers as well, a CED of 4000 mg/m^2 is associated with a decreased number of spermatogonia (counted in testicular biopsies) and decreased sperm count among survivors (Green et al. 2014; Chow et al. 2016; Poganitsch-Korhonen et al. 2017; Stukenborg et al. 2018).

Today, chemotherapy protocols include some restrictions to limit side effects (Pritchard-Jones et al. 2013). Therefore, when possible, the use of alkylating compounds, known for their long-term harmful systemic effects, is reduced. In addition to lower toxicity, protocols are in favor of mixing the use of several drugs at lower doses (Pinto et al. 2011). However, in vitro studies on spermatogonial cells showed that combination of drugs induced higher toxicity than each drug alone (Beaud et al. 2017b; Marcon et al. 2010). Likewise, even a mixture without alkylating agents commonly used in hematologic cancer treatment (doxorubicin plus vincristine) could induced spermatogonial cell death at clinically relevant doses (Beaud et al. 2017b). Notably, such toxicity data on the male germ line are not available for each commonly used chemotherapeutic compound, and the impact of chemotherapy cocktails remains largely unknown. Using animal models, we have shown that adult male rats exposed to the combination of chemotherapeutic agents used to treat testicular cancer (bleomycin-etoposide-cisplatin: BEP regimen) or non-Hodgkin's lymphoma (cyclophosphamide-doxorubicin-vincristine-prednisone: CHOP regimen) have a significant decrease in sperm count but also an increased amount of DNA breaks in spermatozoa, leading to impaired fertility and adverse progeny outcome (Bieber et al. 2006; Delbes et al. 2009, 2007; Vaisheva et al. 2007). Although sperm production returned to control values after a recovery period, DNA damage persisted, suggesting impaired DNA repair ability in male germ cells (Delbes et al. 2010).

Similarly, prospective longitudinal studies on cancer survivors of reproductive age, treated with cocktails of chemotherapy for testicular cancer or various types of lymphoma, demonstrated that cancer treatment negatively affected sperm production in all cancer survivors (Bujan et al. 2014, 2013; O'Flaherty et al. 2010, 2012; Smit et al. 2010). The azoo- or oligozoospermia induced can be temporary or permanent (Chan 2009). Despite a possible recovery of spermatogenesis for some patients, as demonstrated by an increase in semen sperm density and motility 1 or 2 years post-chemotherapy, sperm DNA damage can carry on. More specifically, using the SCSA and the COMET or the TUNEL assay, 3–6 months post-chemotherapy, it was shown that the DFI and DNA breaks were statistically higher in sperm from survivors of testicular cancer or lymphoma than in a control group (Bujan et al. 2014, 2013; O'Flaherty et al. 2010, 2012; Paoli et al. 2015). For testicular cancer patients, 1 year post-treatment, DFI and DNA breaks returned to control values (Bujan et al. 2013; O'Flaherty et al. 2010, 2012; Paoli et al. 2015), but chromatin compaction measured by HDS remained altered even 2 years after post-treatment (O'Flaherty et al. 2012). Interestingly, survivors of lymphoma displayed high DFI even 2 years post-chemotherapy, but this was not associated with DNA breaks or abnormal chromatin compaction (Bujan et al. 2014). These data suggest that depending on the cocktail of chemotherapy administered, long-term consequences on sperm quality could vary. Mechanisms of chemotherapy-induced sperm DNA damage are yet unclear but may be the consequences of impaired DNA repair (Delbes et al. 2010) or changes in the expression of genes involved in chromatin remodelling during spermiogenesis (Maselli et al. 2013).

In parallel, an increased incidence of aneuploidy has also been measured in sperm of cancer survivors up to 6 months post-treatment

(Tempest et al. 2008; Rives et al. 2017). This might be due to the mutagenic impact of chemotherapies. Most studies show that sperm aneuploidy levels may return to values similar to those measured before treatment or similar to the control group within 1 or 2 years post-treatment (Thomas et al. 2004; Tempest et al. 2008; Martinez et al. 2017). However, the return to a basal level of aneuploidy depends on the treatment administered (Martinez et al. 2017). In fact, lymphoma survivors treated with ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) have a higher chance to display control levels of sperm aneuploidy 1 year post-treatment than those treated with CHOP or MOPP-ABV (MOPP: mechlorethamine, oncovin, procarbazine, prednisone) who still displayed high level of aneuploidy even 2 years post-chemotherapy (Martinez et al. 2017).

Epigenetic marks could also be affected in sperm from cancer survivors. Experimental data are available showing that 9-week treatment of BEP in adult male rat induced changes in sperm DNA methylation profiles (Chan et al. 2012) and histone distribution patterns (Bagheri-Sereshki et al. 2016; Maselli et al. 2012, 2013). In humans, only two studies have examined DNA methylation in sperm after chemotherapy treatment. On the one hand, a case study demonstrates a 10% progressive loss of methylation of the H19 paternal imprinted gene up to 5 months after temozolomide treatment (Berthaut et al. 2013). On the other hand, immunoprecipitation analysis of methylated DNA followed by high-throughput sequencing revealed several differently methylated regions in sperm from pediatric cancer survivors treated with cisplatin, compared to control spermatozoa (Shnorhavorian et al. 2017). The importance of altered epigenetic marks in the germ line is still unclear, but recent data suggest that the sperm epigenome can affect embryo development and the health of future generations (Wu et al. 2015). As well it has been suggested as a mechanism for transgenerational transmission. Better understanding the effects of anticancer drugs on the germ line epigenome is therefore very relevant and even more with the development of drugs targeting epigenetic pathways to cure cancer.

It is important to note that these data showing the long-term damage in sperm DNA and chromatin structure have been generated on postpubertal individuals, while studies are lacking on the long-term impact of prepubertal chemotherapy on sperm chromatin quality. Although the prepubertal testis does not produce mature spermatozoa, it does contain diploid spermatogonia from which haploid spermatozoa will be derived. Little data using prepubertal animal models to elucidate mechanisms of action are available. Mainly, exposures to single agents such as doxorubicin, etoposide, or cisplatin have been shown to primarily deplete the testis of germ cells and to have a long-term impact on Sertoli cells (Brilhante et al. 2012; Lirdi et al. 2008; Okada et al. 2009). Importantly, Vendramini et al. have shown that doxorubicin-exposed rat spermatogonia in prepubertal rats produced long-term damage to sperm DNA and that this might be the cause of compromised conceptus development and reduced pregnancy outcome (Vendramini et al. 2012). In humans, only two studies investigated DNA breaks and/or chromatin integrity in sperm from childhood cancer survivors, years after chemotherapy and/or radiotherapy ended (Romerius et al. 2010; Thomson et al. 2002). In their study of 33 childhood cancer survivors, Thomson et al. did not observe any difference in sperm DNA integrity measured by the TUNEL assay when compared to age-matched controls (Thomson et al. 2002). On the other hand, Romerius et al. used the SCSA and studied 99 childhood cancer survivors for whom they observed an increased DFI compared to aged-matched controls which was of borderline statistical significance (Romerius et al. 2010). Both studies grouped childhood cancer survivors with various diagnostics, heterogeneous treatments, and a range of age at diagnosis. Therefore, analyses were done without segregating the impact of prepubertal and postpubertal treatment. While it was thought that being prepubertal during anticancer therapy conferred protection against gonadal damage, more recent evidence of the impact on long-term sperm production has led some researchers to conclude that the prepubertal gonad is even more vulnerable to the cytotoxic

effects of chemotherapy than the adult testis (Revel and Revel-Vilk 2008). Our most recent data focused on survivors of childhood hematologic cancer (Beaud et al. 2017a). Although limited by the number of subjects (6 prepubertal and 7 post-pubertal survivors), the data indicate that, independently of the age of diagnosis, childhood cancer survivors have a higher risk of no or low sperm count, and when sperm are present, chances of DNA and chromatin abnormalities appear similar to those seen in the general population. Nevertheless, exposure to anthracyclines, and doxorubicin in particular, could have long-term consequences on sperm integrity (Beaud et al. 2017a). According to current knowledge, the importance of age at diagnosis in relation to puberty on potential long-term effect on sperm DNA and chromatin remains poorly understood.

Impact on Fertility and the Health of Progeny

Because cancer and its treatment can affect the DNA, chromatin, and epigenome of survivors' sperm, it is important to know if this ultimately affects their fertility and/or the health of their progeny (Tremblay et al. 2017). Indeed, preclinical studies suggested that sperm DNA fragmentation induced by testicular irradiation may result in a variety of checkpoint responses in early embryo development and transgenerational genomic instability in the offspring (Adiga et al. 2007, 2010; Shiraishi et al. 2002). Moreover, paternal exposure to genotoxic agents or endocrine disruptors can induce genetic or epigenetic mutations in gametes which can negatively impact the health of the offspring, even over several generations (Danchin et al. 2011; van Otterdijk and Michels 2016; Xin et al. 2015).

The most important impact of cancer and its treatment is the decrease in sperm count that can be permanent and is most probably due to the toxicity of the drugs on spermatogonia (Meistrich 2013; Tremblay et al. 2017). Another significant consequence is the decrease in birth rate that has been reported in three large cohort studies combining all types of cancers and compared to the general population (Chow et al. 2016; Green et al. 2003; Tang et al. 2016). This is in agreement with the experimental data, and these effects depend on the type of treatment and the dose received (Chow et al. 2016). Beyond the difficulty of conceiving, the cancer history does not seem to have a significant impact on the health of the offspring. Some evidences show a slight increase in the risk of congenital anomalies (Seppanen et al. 2016; Stahl et al. 2011), but this risk remains close to that of the general population and seems to improve with the evolution of cancer treatments (Seppanen et al. 2016). As cancer survivors are more likely to use ART (Stensheim et al. 2013), it is important to note that the large cohort studies evaluated children born from ART separately and did not observe a higher risk of congenital abnormality for children conceived by in vitro fertilization or intracytoplasmic sperm injection (Seppanen et al. 2016; Stahl et al. 2011).

In parallel, the risk of de novo mutations, chromosomal abnormalities, or cancer development in children of male cancer survivors does not appear to be higher than in the general population, after adjusting for family heredity (Byrne et al. 1998; Kryukov et al. 2016; Winther et al. 2004; Hawkins et al. 1995). Even whole genome sequencing on families of two testicular cancer survivors did not show any genetic impact due to treatment (Kryukov et al. 2016). Finally, there does not appear to be a significant increase in the frequency of postnatal mortality among children of men with a history of cancer (Dere et al. 2013; Dufour et al. 2010; Tang et al. 2016). In addition, it has been shown that hospitalization rates for children of survivors up to the age of 15 are not higher than in the general population once heredity factors are removed (Winther et al. 2010). The study of 126,696 individuals born in Sweden of men with a history of cancer did not show a higher mortality rate in this population, regardless of when they were born in relation to the diagnosis of cancer, the type of cancer, or the age of the diagnosis of their father (Tang et al. 2016).

Therefore, because of the very low risk for the health of the progeny, the American Society for Reproductive Medicine Ethics Committee

considers that for patients who experience gonadotoxic therapy, concerns about the well-being of descendants are not sufficient to refuse them assistance for reproduction (NTP Monograph: Developmental Effects and Pregnancy Outcomes Associated With Cancer Chemotherapy Use During Pregnancy 2013). However, the scope of the epidemiological data remains limited in the measured health parameters, in addition to being restricted to the first generation. In order to know if there are transgenerational effects in the human population, it would be necessary to pursue cohort studies over several generations. The demonstration of epigenetic changes in the sperm of treated fathers could constitute a transgenerational transmission mechanism (Berthaut et al. 2013; Shnorhavorian et al. 2017).

Fertility Preservation Strategies

While spermatogenesis may recover in some cancer survivors over time, it is currently impossible to predict for whom and when it will resume. Sperm banking by cryopreservation prior to cytotoxic cancer treatment is therefore the best and currently the only feasible option for fertility preservation (Chan and Robaire 2011; Wallace et al. 2005a). However, when anticancer treatment does not cause infertility, there is still a debate about the use of cryopreserved sperm over fresh semen (Vakalopoulos et al. 2015). Indeed, a few years after the end of treatment, sperm DNA and chromatin integrity may be better than before treatment, at the time of banking, when it might have been affected by the disease (Paoli et al. 2016). In addition, sperm cryopreservation can induce oxidative stress in spermatozoa and cause DNA damage (Thomson et al. 2009; Lusignan et al. 2018). Sperm analysis including sperm DNA and chromatin integrity assays could be recommended before cryopreservation and after cancer recovery to assess which sperm to use. Moreover, we and others have developed clinically reliable strategies for sperm selection using magnetic-activated cell sorting (MACS) with annexin V to eliminate sperm that show apoptotic features associated with altered chromatin. The addition of annexin V-MACS to routine sperm preparation in the clinic has been shown to be efficient in enriching samples with high motility, viable, and non-apoptotic spermatozoa (Delbes et al. 2013; Said and Land 2011) with intact chromatin and DNA (Delbes et al. 2013; Tavalaee et al. 2012) and high fertilization potential (Lee et al. 2010). Such a strategy led to a successful pregnancy and live birth in a couple with recurrent ART failure, using cryopreserved sperm from a cancer patient survivor (Herrero et al. 2013).

Unfortunately, the sperm banking option only applies to pubertal patients whose sperm production is ongoing and who are able to provide a sperm sample (Chan and Robaire 2011). In addition, even young, newly pubescent boys (14–19 years old) cannot always provide a sperm sample, and when they can, the sperm is often of poor quality in terms of concentration, volume, or motility (Postovsky et al. 2003). In fact, for preadolescent boys with cancer, no clinically proven methods are available to preserve fertility. However, some centers do offer experimental protocols, such as testicular tissue cryopreservation before treatment, with the hope that the unexposed germ cells present in these biopsies can be used for future reproduction (Trost and Brannigan 2012). Indeed, transplantation of spermatogonial stem cells (SSCs) isolated from testicular biopsies has been proven efficient first in mice and more recently in primates and has therefore been proposed as a promising strategy to restore male fertility for cancer survivors (Wallace et al. 2005b; Mitchell et al. 2009; Chan and Robaire 2011; Goossens and Tournaye 2013; Struijk et al. 2013; Anderson et al. 2015; Jahnukainen et al. 2015; Raffoul et al. 2016; Brinster 2002; Hermann et al. 2012). In this procedure, testicular tissue removal is a relatively minor surgery, but it remains invasive and requires general anesthesia (Raffoul et al. 2016; Gupta et al. 2016). The biopsy is then frozen for later use in the patient's life. Freezing protocols are promising in humans (Keros et al. 2007) but

still require a better characterization of stem cell functionality after thawing (Anderson et al. 2015). Testicular biopsy programs for pediatric patients already exist, in view of future developments in this field (Sadri-Ardekani et al. 2016). Afterward, from the thawed testicular tissue, three main protocols are being investigated, although none are currently approved in humans:

1. Self-transplantation of testicular tissue once the patient is cured and reached adulthood, to allow the immature tissue to produce sperm and restore fertility (Mitchell et al. 2009; Chan and Robaire 2011). This technique is effective in mice but remains to be tested in humans (Anderson et al. 2015). In addition, biopsies carry a risk of contamination by cancer cells, especially in the case of blood cancers, and thus the reintroduction of cancer in survivors (Jahnukainen et al. 2015).
2. Self-transplantation of SSCs isolated from the testicular parenchyma, purified and amplified in vitro (Struijk et al. 2013), in order to colonize the seminiferous tubules and initiate spermatogenesis in the adult patient (Struijk et al. 2013). Again, there are several technical limitations such as effective purification of SSC to prevent reintroduction of cancer cells (Struijk et al. 2013; Goossens and Tournaye 2013; Jahnukainen et al. 2015).
3. Sperm maturation in vitro from isolated SSC and use in ART (Jahnukainen et al. 2015).

In 2016, Perrard et al. successfully produced sperm-like cells from fresh or frozen biopsies of men whose spermatogenesis was inhibited (Perrard et al. 2016). Although this option addresses the issue of reintroduction of cancer, it has not yet been tested with human prepubertal tissue.

In parallel, another interesting strategy for fertility preservation includes the improvement of existing protocols used in anticancer therapies to provide protection to the healthy cells. For example, inactivating spermatogenesis by suppression of gonadotropins using a GnRH antagonist during treatment has been investigated, but unfortunately, not only clinical trials have so far not shown a convincing level of benefit (Meistrich and Shetty 2008); it might be ineffective for prepubertal children as the proliferation of germ cells in prepubertal primates appears to be gonadotropin-independent (Kelnar et al. 2002). Co-treatment with radioprotectants has been effective in cases of cancer related to aging, to reduce the side effects in some organs without reducing the effectiveness of treatment against cancer (Gómez et al. 2013; Kemp et al. 1996). For example, co-treatment protocols are now included in oncology clinical practice guidelines to reduce neurotoxicity (Hershman et al. 2014). Radioprotective compounds have been suggested to exert protective action through their antioxidant properties and by increasing DNA repair capacity (Cabral et al. 2014; Lirdi et al. 2008). Data using a prepubertal rat model showed that carnitine and amifostine could be efficient in maintaining male germ cells against the cytotoxic impact of cisplatin (Lirdi et al. 2008), etoposide, or doxorubicin (Cabral et al. 2014; Okada et al. 2009). On one hand, carnitine pretreatment maintained sperm DNA integrity, embryo implantation rate, and litter size despite doxorubicin treatment (Cabral et al. 2018). On the other hand, amifostine pretreatment actually increased sperm DNA breaks and abnormal chromatin structure measured by the COMET assay and the SCSA, respectively, probably increasing embryonic loss rate (Vendramini et al. 2012). Although promising, these in situ protection methods still remain experimental, and the risks of the radioprotectant having negative impact on its own currently outweigh the potential benefit.

Overall, novel fertility preservation strategies, developed by evidence-based research, are urgently needed not only to help male cancer patients to preserve fertility but also to help reduce the risks of long-term adverse reproductive outcomes on sperm quality. This would in term improve the quality of life of many boys and men affected by cancer.

References

Abuelhija M, Weng CC, Shetty G, Meistrich ML (2013) Rat models of post-irradiation recovery of spermatogenesis: interstrain differences. Andrology 1(2):206–215. https://doi.org/10.1111/j.2047-2927.2012.00034.x

Adiga SK, Toyoshima M, Shiraishi K, Shimura T, Takeda J, Taga M, Nagai H, Kumar P, Niwa O (2007) p21 provides stage specific DNA damage control to preimplantation embryos. Oncogene 26(42):6141–6149. https://doi.org/10.1038/sj.onc.1210444

Adiga SK, Upadhya D, Kalthur G, Bola Sadashiva SR, Kumar P (2010) Transgenerational changes in somatic and germ line genetic integrity of first-generation offspring derived from the DNA damaged sperm. Fertil Steril 93(8):2486–2490. https://doi.org/10.1016/j.fertnstert.2009.06.015

Anderson RA, Mitchell RT, Kelsey TW, Spears N, Telfer EE, Wallace WHB (2015) Cancer treatment and gonadal function: experimental and established strategies for fertility preservation in children and young adults. Lancet Diabetes Endocrinol 3(7):556–567. https://doi.org/10.1016/s2213-8587(15)00039-x

Bagheri-Sereshki N, Hales BF, Robaire B (2016) The effects of chemotherapeutic agents, bleomycin, etoposide, and cisplatin, on chromatin remodeling in male rat germ cells. Biol Reprod 94(4):81. https://doi.org/10.1095/biolreprod.115.137802

Beaud H, Albert O, Robaire B, Rousseau M-C, Chan P, Delbès G (2017a) Abstracts from the ASA 42nd annual meeting. Andrology 5:78–79. https://doi.org/10.1111/andr.12364

Beaud H, van Pelt AM, Delbes G (2017b) Doxorubicin and vincristine affect undifferentiated rat spermatogonia. Reproduction 153:725. https://doi.org/10.1530/REP-17-0005

Berthaut I, Montjean D, Dessolle L, Morcel K, Deluen F, Poirot C, Bashamboo A, McElreavey K, Ravel C (2013) Effect of temozolomide on male gametes: an epigenetic risk to the offspring? J Assist Reprod Genet 30(6):827–833. https://doi.org/10.1007/s10815-013-9999-8

Bieber AM, Marcon L, Hales BF, Robaire B (2006) Effects of chemotherapeutic agents for testicular cancer on the male rat reproductive system, spermatozoa, and fertility. J Androl 27(2):189–200. https://doi.org/10.2164/jandrol.05103

Brilhante O, Okada FK, Sasso-Cerri E, Stumpp T, Miraglia SM (2012) Late morfofunctional alterations of the Sertoli cell caused by doxorubicin administered to prepubertal rats. Reprod Biol Endocrinol 10:79

Brinster RL (2002) Germline stem cell transplantation and transgenesis. Science 296(5576):2174–2176. https://doi.org/10.1126/science.1071607

Bujan L, Walschaerts M, Moinard N, Hennebicq S, Saias J, Brugnon F, Auger J, Berthaut I, Szerman E, Daudin M, Rives N (2013) Impact of chemotherapy and radiotherapy for testicular germ cell tumors on spermatogenesis and sperm DNA: a multicenter prospective study from the CECOS network. Fertil Steril 100(3):673–680. https://doi.org/10.1016/j.fertnstert.2013.05.018

Bujan L, Walschaerts M, Brugnon F, Daudin M, Berthaut I, Auger J, Saias J, Szerman E, Moinard N, Rives N, Hennebicq S (2014) Impact of lymphoma treatments on spermatogenesis and sperm deoxyribonucleic acid: a multicenter prospective study from the CECOS network. Fertil Steril 102(3):667–674.e663. https://doi.org/10.1016/j.fertnstert.2014.06.008

Byrne J, Rasmussen SA, Steinhorn SC, Connelly RR, Myers MH, Lynch CF, Flannery J, Austin DF, Holmes FF, Holmes GE, Strong LC, Mulvihill JJ (1998) Genetic disease in offspring of long-term survivors of childhood and adolescent cancer. Am J Hum Genet 62(1):45–52. https://doi.org/10.1086/301677

Cabral RE, Okada FK, Stumpp T, Vendramini V, Miraglia SM (2014) Carnitine partially protects the rat testis against the late damage produced by doxorubicin administered during pre-puberty. Andrology 2(6):931–942. https://doi.org/10.1111/andr.279

Cabral REL, Mendes TB, Vendramini V, Miraglia SM (2018) Carnitine partially improves oxidative stress, acrosome integrity, and reproductive competence in doxorubicin-treated rats. Andrology 6(1):236–246. https://doi.org/10.1111/andr.12426

Chan PTK (2009) Fertility after cancer in men. Can Urol Assoc J 3(3):223–224

Chan P, Robaire B (2011) Cancer in males: implications for sperm quality, fertility, and progeny outcome. In: Zini A, Agarwal A (eds) Sperm chromatin. Springer, New York, pp 351–360. doi:https://doi.org/10.1007/978-1-4419-6857-9_24

Chan D, Delbes G, Landry M, Robaire B, Trasler JM (2012) Epigenetic alterations in sperm DNA associated with testicular cancer treatment. Toxicol Sci 125(2):532–543. https://doi.org/10.1093/toxsci/kfr307

Cherven BO, Mertens A, Wasilewski-Masker K, Williamson R, Meacham LR (2015) Infertility education: experiences and preferences of childhood cancer survivors. J Pediatr Oncol Nurs 33:257. https://doi.org/10.1177/1043454215607342

Chow EJ, Stratton KL, Leisenring WM, Oeffinger KC, Sklar CA, Donaldson SS, Ginsberg JP, Kenney LB, Levine JM, Robison LL, Shnorhavorian M, Stovall M, Armstrong GT, Green DM (2016) Pregnancy after chemotherapy in male and female survivors of childhood cancer treated between 1970 and 1999: a report from the Childhood Cancer Survivor Study cohort. Lancet Oncol 17(5):567–576. https://doi.org/10.1016/S1470-2045(16)00086-3

Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, Haugen TB, Kruger T, Wang C, Mbizvo MT, Vogelsong KM (2010) World Health Organization reference values for human semen characteristics. Hum Reprod Update 16(3):231–245. https://doi.org/10.1093/humupd/dmp048

Danchin E, Charmantier A, Champagne FA, Mesoudi A, Pujol B, Blanchet S (2011) Beyond DNA: integrating inclusive inheritance into an extended theory of evolution. Nat Rev Genet 12(7):475–486. https://doi.org/10.1038/nrg3028

Delbes G, Hales BF, Robaire B (2007) Effects of the chemotherapy cocktail used to treat testicular cancer on sperm chromatin integrity. J Androl 28(2):241–249.; ; discussion 250–1. https://doi.org/10.2164/jandrol.106.001487

Delbes G, Chan D, Pakarinen P, Trasler JM, Hales BF, Robaire B (2009) Impact of the chemotherapy cocktail used to treat testicular cancer on the gene expression profile of germ cells from male Brown-Norway rats. Biol Reprod 80(2):320–327. https://doi.org/10.1095/biolreprod.108.072108

Delbes G, Vaisheva F, Luu T, Marcon L, Hales BF, Robaire B (2010) Reversibility of the effects of the chemotherapeutic regimen for non-Hodgkin lymphoma, cyclophosphamide, doxorubicin, vincristine, and prednisone, on the male rat reproductive system and progeny outcome. Reprod Toxicol 29(3):332–338. https://doi.org/10.1016/j.reprotox.2010.01.007

Delbes G, Herrero MB, Troeung ET, Chan PT (2013) The use of complimentary assays to evaluate the enrichment of human sperm quality in asthenoteratozoospermic and teratozoospermic samples processed with Annexin-V magnetic activated cell sorting. Andrology 1(5):698–706. https://doi.org/10.1111/j.2047-2927.2013.00106.x

Dere E, Anderson LM, Hwang K, Boekelheide K (2013) Biomarkers of chemotherapy-induced testicular damage. Fertil Steril 100(5):1192–1202. https://doi.org/10.1016/j.fertnstert.2013.09.017

Diller L, Chow EJ, Gurney JG, Hudson MM, Kadin-Lottick NS, Kawashima TI, Leisenring WM, Meacham LR, Mertens AC, Mulrooney DA, Oeffinger KC, Packer RJ, Robison LL, Sklar CA (2009) Chronic disease in the Childhood Cancer Survivor Study cohort: a review of published findings. J Clin Oncol 27(14):2339–2355. https://doi.org/10.1200/JCO.2008.21.1953

Dufour C, Oberlin O, De Vathaire F (2010) Offspring of childhood cancer survivors. Arch Pediatr 17(6):926–927. https://doi.org/10.1016/s0929-693x(10)70182-2

Fossa SD, De Angelis P, Kraggerud SM, Evenson D, Theodorsen L, Clausen OP (1997) Prediction of post-treatment spermatogenesis in patients with testicular cancer by flow cytometric sperm chromatin structure assay. Cytometry 30(4):192–196

Gandini L, Lombardo F, Salacone P, Paoli D, Anselmo AP, Culasso F, Dondero F, Lenzi A (2003) Testicular cancer and Hodgkin's disease: evaluation of semen quality. Hum Reprod 18(4):796–801

Gómez HL, Samanéz C, Campana F, Neciosup SP, Vera L, Casanova L, Leon J, Flores C, de Mendoza FH, Casteñeda CA, Pinto JA, Vallejos CS (2013) Addition of amifostine to the CHOP regimen in elderly patients with aggressive non-Hodgkin lymphoma: a phase II trial showing reduction in toxicity without altering long-term survival. Hematol Oncol Stem Cell Ther 5(3):152–157

Goossens E, Tournaye H (2013) Fertility preservation in boys: spermatogonial stem cell transplantation and testicular grafting. Gynecol Obstet Fertil 41(9):529–531. https://doi.org/10.1016/j.gyobfe.2013.07.013

Gordetsky J, van Wijngaarden E, O'Brien J (2012) Redefining abnormal follicle-stimulating hormone in the male infertility population. BJU Int 110(4):568–572. https://doi.org/10.1111/j.1464-410X.2011.10783.x

Green DM, Whitton JA, Stovall M, Mertens AC, Donaldson SS, Ruymann FB, Pendergrass TW, Robison LL (2003) Pregnancy outcome of partners of male survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. J Clin Oncol 21(4):716–721

Green DM, Liu W, Kutteh WH, Ke RW, Shelton KC, Sklar CA, Chemaitilly W, Pui CH, Klosky JL, Spunt SL, Metzger ML, Srivastava D, Ness KK, Robison LL, Hudson MM (2014) Cumulative alkylating agent exposure and semen parameters in adult survivors of childhood cancer: a report from the St Jude Lifetime Cohort Study. Lancet Oncol 15(11):1215–1223. https://doi.org/10.1016/S1470-2045(14)70408-5

Gupta AA, Donen RM, Sung L, Boydell KM, Lo KC, Stephens D, Pritchard S, Portwine C, Maloney AM, Lorenzo AJ (2016) Testicular biopsy for fertility preservation in prepubertal boys with cancer: identifying preferences for procedure and reactions to disclosure practices. J Urol 196(1):219–224. https://doi.org/10.1016/j.juro.2016.02.2967

Hawkins MM, Draper GJ, Winter DL (1995) Cancer in the offspring of survivors of childhood leukaemia and non-Hodgkin lymphomas†. Br J Cancer 71:1335. https://doi.org/10.1038/bjc.1995.259

Hermann BP, Sukhwani M, Winkler F, Pascarella JN, Peters KA, Sheng Y, Valli H, Rodriguez M, Ezzelarab M, Dargo G, Peterson K, Masterson K, Ramsey C, Ward T, Lienesch M, Volk A, Cooper DK, Thomson AW, Kiss JE, Penedo MC, Schatten GP, Mitalipov S, Orwig KE (2012) Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell 11(5):715–726. https://doi.org/10.1016/j.stem.2012.07.017

Herrero MB, Delbes G, Chung JT, Son WY, Holzer H, Buckett W, Chan P (2013) Case report: the use of annexin V coupled with magnetic activated cell sorting in cryopreserved spermatozoa from a male cancer survivor: healthy twin newborns after two previous ICSI failures. J Assist Reprod Genet 30(11):1415–1419. https://doi.org/10.1007/s10815-013-0086-y

Hershman DL, Lacchetti C, Dworkin RH, Lavoie Smith EM, Bleeker J, Cavaletti G, Chauhan C, Gavin P, Lavino A, Lustberg MB, Paice J, Schneider B, Smith ML, Smith T, Terstriep S, Wagner-Johnston N, Bak K, Loprinzi CL (2014) Prevention and management of chemotherapy-induced peripheral neuropathy in survivors of adult cancers: American society of clinical oncology clinical practice guideline. J Clin Oncol 32(18):1941–1967

Hohmann C, Borgmann-Staudt A, Rendtorff R, Reinmuth S, Holzhausen S, Willich SN, Henze G, Goldbeck L, Keil T (2011) Patient counselling on the risk of infertility and its impact on childhood cancer survivors: results from a national survey. J Psychosoc Oncol 29(3):274–285. https://doi.org/10.1080/07347332.2011.563344

Jacob A, Barker H, Goodman A, Holmes J (1998) Recovery of spermatogenesis following bone marrow transplantation. Bone Marrow Transplant 22(3):277–279. https://doi.org/10.1038/sj.bmt.1701332

Jaffe N, Sullivan MP, Ried H, Boren H, Marshall R, Meistrich M, Maor M, Cunha MD (1988) Male reproductive function in long-term survivors of childhood cancer. Med Pediatr Oncol 16(4):241–247. https://doi.org/10.1002/mpo.2950160404

Jahnukainen K, Mitchell RT, Stukenborg JB (2015) Testicular function and fertility preservation after treatment for haematological cancer. Curr Opin Endocrinol Diabetes Obes 22(3):217–223. https://doi.org/10.1097/MED.0000000000000156

Kaatsch P (2010) Epidemiology of childhood cancer. Cancer Treat Rev 36(4):277–285. https://doi.org/10.1016/j.ctrv.2010.02.003

Kelnar CJ, McKinnell C, Walker M, Morris KD, Wallace WH, Saunders PT, Fraser HM, Sharpe RM (2002) Testicular changes during infantile 'quiescence' in the marmoset and their gonadotrophin dependence: a model for investigating susceptibility of the prepubertal human testis to cancer therapy? Hum Reprod 17(5):1367–1378

Kemp G, Rose P, Lurain J, Berman M, Manetta A, Roullet B, Homesley H, Belpomme D, Glick J (1996) Amifostine pretreatment for protection against cyclophosphamide-induced and cisplatin-induced toxicities: results of a randomized control trial in patients with advanced ovarian cancer. J Clin Oncol 14(7):2101–2112

Keros V, Hultenby K, Borgström B, Fridström M, Jahnukainen K, Hovatta O (2007) Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. Hum Reprod 22(5):1384–1395

Kryukov GV, Bielski CM, Samocha K, Fromer M, Seepo S, Gentry C, Neale B, Garraway LA, Sweeney CJ, Taplin ME, Van Allen EM (2016) Genetic effect of chemotherapy exposure in children of testicular cancer survivors. Clin Cancer Res 22(9):2183–2189. https://doi.org/10.1158/1078-0432.ccr-15-2317

Kumar D, Salian SR, Kalthur G, Uppangala S, Kumari S, Challapalli S, Chandraguthi SG, Krishnamurthy H, Jain N, Kumar P, Adiga SK (2013) Semen abnormalities, sperm DNA damage and global hypermethylation in health workers occupationally exposed to ionizing radiation. PLoS One 8(7):e69927. https://doi.org/10.1371/journal.pone.0069927

Kumar D, Salian SR, Kalthur G, Uppangala S, Kumari S, Challapalli S, Chandraguthi SG, Jain N, Krishnamurthy H, Kumar P, Adiga SK (2014) Association between sperm DNA integrity and seminal plasma antioxidant levels in health workers occupationally exposed to ionizing radiation. Environ Res 132:297–304. https://doi.org/10.1016/j.envres.2014.04.023

Kumar K, Lewis S, Vinci S, Riera-Escamilla A, Fino MG, Tamburrino L, Muratori M, Larsen P, Krausz C (2018) Evaluation of sperm DNA quality in men presenting with testicular cancer and lymphoma using alkaline and neutral Comet assays. Andrology 6(1):230–235. https://doi.org/10.1111/andr.12429

Lee TH, Liu CH, Shih YT, Tsao HM, Huang CC, Chen HH, Lee MS (2010) Magnetic-activated cell sorting for sperm preparation reduces spermatozoa with apoptotic markers and improves the acrosome reaction in couples with unexplained infertility. Hum Reprod 25(4):839–846. https://doi.org/10.1093/humrep/deq009

Lirdi LC, Stumpp T, Sasso-Cerri E, Miraglia SM (2008) Amifostine protective effect on cisplatin-treated rat testis. Anat Rec (Hoboken) 291(7):797–808. https://doi.org/10.1002/ar.20693

Lusignan MF, Li X, Herrero B, Delbes G, Chan PTK (2018) Effects of different cryopreservation methods on DNA integrity and sperm chromatin quality in men. Andrology 6:829. https://doi.org/10.1111/andr.12529

Marcon L, Zhang X, Hales BF, Nagano MC, Robaire B (2010) Development of a short-term fluorescence-based assay to assess the toxicity of anticancer drugs on rat stem/progenitor spermatogonia in vitro. Biol Reprod 83(2):228–237. https://doi.org/10.1095/biolreprod.110.083568

Martinez G, Walschaerts M, Le Mitouard M, Borye R, Thomas C, Auger J, Berthaut I, Brugnon F, Daudin M, Moinard N, Ravel C, Saias J, Szerman E, Rives N, Hennebicq S, Bujan L (2017) Impact of Hodgkin or non-Hodgkin lymphoma and their treatments on sperm aneuploidy: a prospective study by the French CECOS network. Fertil Steril 107(2):341–350.e345. https://doi.org/10.1016/j.fertnstert.2016.10.001

Maselli J, Hales BF, Chan P, Robaire B (2012) Exposure to bleomycin, etoposide, and cis-platinum alters rat sperm chromatin integrity and sperm head protein profile. Biol Reprod 86(5):166, 161–110. https://doi.org/10.1095/biolreprod.111.098616

Maselli J, Hales BF, Robaire B (2013) The effects of chemotherapy with bleomycin, etoposide, and cisplatinum (BEP) on rat sperm chromatin remodeling, fecundity and testicular gene expression in the progeny. Biol Reprod 89(4):85. https://doi.org/10.1095/biolreprod.113.110759

Meistrich ML (1986) Relationship between spermatogonial stem cell survival and testis function after cytotoxic therapy. Br J Cancer Suppl 7:89–101

Meistrich ML (2013) Effects of chemotherapy and radiotherapy on spermatogenesis in humans. Fertil Steril 100(5):1180–1186. https://doi.org/10.1016/j.fertnstert.2013.08.010

Meistrich ML, Shetty G (2008) Hormonal suppression for fertility preservation in males and females. Reproduction 136(6):691–701. https://doi.org/10.1530/REP-08-0096

Meistrich ML, Finch M, da Cunha MF, Hacker U, Au WW (1982) Damaging effects of fourteen chemotherapeutic drugs on mouse testis cells. Cancer Res 42(1):122–131

Meseguer M, Santiso R, Garrido N, Fernandez JL (2008) The effect of cancer on sperm DNA fragmentation as measured by the sperm chromatin dispersion test. Fertil Steril 90(1):225–227. https://doi.org/10.1016/j.fertnstert.2007.06.026

Miller KD, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, Stein KD, Alteri R, Jemal A (2016) Cancer treatment and survivorship statistics, 2016. CA Cancer J Clin 66(4):271–289. https://doi.org/10.3322/caac.21349

Mitchell R, Saunders P, Sharpe R, Kelnar C, Wallace W (2009) Male fertility and strategies for fertility preservation following childhood cancer treatment. In: Wallace W, Kelnar C (eds) Endocrinopathy after childhood cancer treatment, vol 15. Karger Publishers, Suisse, pp 101–134

Müller J, Hertz H, Skakkebœk NE (1988) Development of the seminiferous epithelium during and after treatment for acute lymphoblastic leukemia in childhood. Horm Res 30:115–120. https://doi.org/10.1159/000181041

NTP Monograph: Developmental effects and pregnancy outcomes associated with cancer chemotherapy use during pregnancy (2013). NTP Monogr (2):i-214

O'Flaherty C, Vaisheva F, Hales BF, Chan P, Robaire B (2008) Characterization of sperm chromatin quality in testicular cancer and Hodgkin's lymphoma patients prior to chemotherapy. Hum Reprod 23(5):1044–1052. https://doi.org/10.1093/humrep/den081

O'Flaherty C, Hales BF, Chan P, Robaire B (2010) Impact of chemotherapeutics and advanced testicular cancer or Hodgkin lymphoma on sperm deoxyribonucleic acid integrity. Fertil Steril 94(4):1374–1379. https://doi.org/10.1016/j.fertnstert.2009.05.068

O'Flaherty CM, Chan PT, Hales BF, Robaire B (2012) Sperm chromatin structure components are differentially repaired in cancer survivors. J Androl 33(4):629–636. https://doi.org/10.2164/jandrol.111.015388

Okada FK, Stumpp T, Miraglia SM (2009) Carnitine reduces testicular damage in rats treated with etoposide in the prepubertal phase. Cell Tissue Res 337(2):269–280. https://doi.org/10.1007/s00441-009-0801-2

Paoli D, Gallo M, Rizzo F, Spanò M, Leter G, Lombardo F, Lenzi A, Gandini L (2015) Testicular cancer and sperm DNA damage: short- and long-term effects of antineoplastic treatment. Andrology 3(1):122–128. https://doi.org/10.1111/j.2047-2927.2014.00250.x

Paoli D, Rizzo F, Fiore G, Pallotti F, Pulsoni A, Annechini G, Lombardo F, Lenzi A, Gandini L (2016) Spermatogenesis in Hodgkin's lymphoma patients: a retrospective study of semen quality before and after different chemotherapy regimens. Hum Reprod 31(2):263–272. https://doi.org/10.1093/humrep/dev310

Perrard MH, Sereni N, Schluth-Bolard C, Blondet A, D Estaing SG, Plotton I, Morel-Journel N, Lejeune H, David L, Durand P (2016) Complete human and rat ex vivo spermatogenesis from fresh or frozen testicular tissue. Biol Reprod 95(4):89. https://doi.org/10.1095/biolreprod.116.142802

Pinto AC, Moreira JN, Simões S (2011) Combination chemotherapy in cancer: principles, evaluation and drug delivery strategies. INTECH Open Access Publisher

Poganitsch-Korhonen M, Masliukaite I, Nurmio M, Lahteenmaki P, van Wely M, van Pelt AMM, Jahnukainen K, Stukenborg JB (2017) Decreased spermatogonial quantity in prepubertal boys with leukaemia treated with alkylating agents. Leukemia 31(6):1460–1463. https://doi.org/10.1038/leu.2017.76

Postovsky S, Lightman A, Aminpour D, Elhasid R, Peretz M, Arush MW (2003) Sperm cryopreservation in adolescents with newly diagnosed cancer. Med Pediatr Oncol 40(6):355–359. https://doi.org/10.1002/mpo.10294

Pritchard-Jones K, Pieters R, Reaman GH, Hjorth L, Downie P, Calaminus G, Naafs-Wilstra MC, Steliarova-Foucher E (2013) Sustaining innovation and improvement in the treatment of childhood cancer: lessons from high-income countries. Lancet Oncol 14(3):e95–e103. https://doi.org/10.1016/S1470-2045(13)70010-X

Pryzant RM, Meistrich ML, Wilson G, Brown B, McLaughlin P (1993) Long-term reduction in sperm count after chemotherapy with and without radiation therapy for non-Hodgkin's lymphomas. J Clin Oncol 11(2):239–247. https://doi.org/10.1200/jco.1993.11.2.239

Raffoul L, Capito C, Sarnacki S (2016) Fertility considerations and the pediatric oncology patient. Semin Pediatr Surg 25(5):318–322. https://doi.org/10.1053/j.sempedsurg.2016.09.006

Ragheb AM, Sabanegh ES Jr (2010) Male fertility-implications of anticancer treatment and strategies to mitigate gonadotoxicity. Anticancer Agents Med Chem 10(1):92–102

Reinmuth S, Liebeskind AK, Wickmann L, Bockelbrink A, Keil T, Henze G, Borgmann A (2008) Having children after surviving cancer in childhood or adolescence – results of a Berlin survey. Klin Padiatr 220(3):159–165. https://doi.org/10.1055/s-2008-1073143

Revel A, Revel-Vilk S (2008) Pediatric fertility preservation: is it time to offer testicular tissue cryopreservation? Mol Cell Endocrinol 282(1–2):143–149. https://doi.org/10.1016/j.mce.2007.11.003

Rives N, Walschaerts M, Setif V, Hennebicq S, Saias J, Brugnon F, Auger J, Berthaut I, Szerman E, Daudin M, Bujan L (2017) Sperm aneuploidy after testicular cancer treatment: data from a prospective multicenter study performed within the French Centre d'Etude et de Conservation des Oeufs et du Sperme network. Fertil Steril 107(3):580–588.e581. https://doi.org/10.1016/j.fertnstert.2016.11.015

Romerius P, Ståhl O, Moëll C, Relander T, Cavallin-Ståhl E, Gustafsson H, Thapper KL, Jepson K, Spanò M, Wiebe T, Giwercman YL, Giwercman A (2010) Sperm DNA integrity in men treated for childhood cancer. Clin Cancer Res 16(15):3843–3850

Rueffer U, Breuer K, Josting A, Lathan B, Sieber M, Manzke O, Grotenhermen FJ, Tesch H, Bredenfeld H, Koch P, Nisters-Backes H, Wolf J, Engert A, Diehl V (2001) Male gonadal dysfunction in patients with Hodgkin's disease prior to treatment. Ann Oncol 12(9):1307–1311

Sadri-Ardekani H, McLean TW, Kogan S, Sirintrapun J, Crowell K, Yousif MQ, Hodges SJ, Petty J, Pranikoff T, Sieren L, Zeller K, Atala A (2016) Experimental testicular tissue banking to generate spermatogenesis in the future: a multidisciplinary team approach. Methods 99:120–127. https://doi.org/10.1016/j.ymeth.2016.02.013

Said TM, Land JA (2011) Effects of advanced selection methods on sperm quality and ART outcome: a systematic review. Hum Reprod Update 17(6):719–733. https://doi.org/10.1093/humupd/dmr032

Sandeman TF (1966) The effects of x irradiation on male human fertility. Br J Radiol 39(468):901–907. https://doi.org/10.1259/0007-1285-39-468-901

Sanders J, Hawley J, Levy W, Gooley T, Buckner C, Deeg H, Doney K, Storb R, Sullivan K, Witherspoon R, Appelbaum F (1996) Pregnancies following high-dose cyclophosphamide with or without high-dose busulfan or total-body irradiation and bone marrow transplantation. Blood 87(7):3045–3052

Schilsky RL, Lewis BJ, Sherins RJ, Young RC (1980) Gonadal dysfunction in patients receiving chemotherapy for cancer. Ann Intern Med 93(1):109–114

Schover LR, Brey K, Lichtin A, Lipshultz LI, Jeha S (2002) Oncologists' attitudes and practices regarding banking sperm before cancer treatment. J Clin Oncol 20(7):1890–1897

Seppanen VI, Artama MS, Malila NK, Pitkaniemi JM, Rantanen ME, Ritvanen AK, Madanat-Harjuoja LM (2016) Risk for congenital anomalies in offspring of childhood, adolescent and young adult cancer survivors. Int J Cancer 139(8):1721–1730. https://doi.org/10.1002/ijc.30226

Shiraishi K, Shimura T, Taga M, Uematsu N, Gondo Y, Ohtaki M, Kominami R, Niwa O (2002) Persistent induction of somatic reversions of the pink-eyed unstable mutation in F1 mice born to fathers irradiated at the spermatozoa stage. Radiat Res 157(6):661–667

Shnorhavorian M, Schwartz SM, Stansfeld B, Sadler-Riggleman I, Beck D, Skinner MK (2017) Differential DNA methylation regions in adult human sperm following adolescent chemotherapy: potential for epigenetic inheritance. PLoS One 12(2):e0170085. https://doi.org/10.1371/journal.pone.0170085

Smit M, van Casteren NJ, Wildhagen MF, Romijn JC, Dohle GR (2010) Sperm DNA integrity in cancer patients before and after cytotoxic treatment. Hum Reprod 25(8):1877–1883

Spitz S (1948) The histological effects of nitrogen mustards on human tumors and tissues. Cancer 1(3):383–398

Stahl O, Eberhard J, Jepson K, Spano M, Cwikiel M, Cavallin-Stahl E, Giwercman A (2004) The impact of testicular carcinoma and its treatment on sperm DNA integrity. Cancer 100(6):1137–1144. https://doi.org/10.1002/cncr.20068

Stahl O, Eberhard J, Cavallin-Stahl E, Jepson K, Friberg B, Tingsmark C, Spano M, Giwercman A (2009) Sperm DNA integrity in cancer patients: the effect of disease and treatment. Int J Androl 32(6):695–703. https://doi.org/10.1111/j.1365-2605.2008.00933.x

Stahl O, Boyd HA, Giwercman A, Lindholm M, Jensen A, Kjaer SK, Anderson H, Cavallin-Stahl E, Rylander L (2011) Risk of birth abnormalities in the offspring of men with a history of cancer: a cohort study using Danish and Swedish national registries. J Natl Cancer Inst 103(5):398–406. https://doi.org/10.1093/jnci/djq550

Stein DM, Victorson DE, Choy JT, Waimey KE, Pearman TP, Smith K, Dreyfuss J, Kinahan KE, Sadhwani D, Woodruff TK, Brannigan RE (2014) Fertility preservation preferences and perspectives among adult male survivors of pediatric cancer and their parents. J Adolesc Young Adult Oncol 3(2):75–82. https://doi.org/10.1089/jayao.2014.0007

Stensheim H, Klungsoyr K, Skjaerven R, Grotmol T, Fossa SD (2013) Birth outcomes among offspring of adult cancer survivors: a population-based study. Int J Cancer 133(11):2696–2705. https://doi.org/10.1002/ijc.28292

Struijk RB, Mulder CL, van der Veen F, van Pelt AM, Repping S (2013) Restoring fertility in sterile childhood cancer survivors by autotransplanting spermatogonial stem cells: are we there yet? Biomed Res Int 2013:1–13. https://doi.org/10.1155/2013/903142

Stukenborg JB, Alves-Lopes JP, Kurek M, Albalushi H, Reda A, Keros V, Tohonen V, Bjarnason R, Romerius P, Sundin M, Noren Nystrom U, Langenskiold C, Vogt H, Henningsohn L, Mitchell RT, Soder O, Petersen C, Jahnukainen K (2018) Spermatogonial quantity in human prepubertal testicular tissue collected for fertility preservation prior to potentially sterilizing therapy. Hum Reprod 33(9):1677–1683. https://doi.org/10.1093/humrep/dey240

Tamburrino L, Cambi M, Marchiani S, Manigrasso I, Degl'Innocenti S, Forti G, Maggi M, Baldi E, Muratori M (2017) Sperm DNA fragmentation in cryopreserved samples from subjects with different cancers. Reprod Fertil Dev 29(4):637–645. https://doi.org/10.1071/RD15190

Tang SW, Liu J, Juay L, Czene K, Miao H, Salim A, Verkooijen HM, Hartman M (2016) Birth rates among male cancer survivors and mortality rates among their offspring: a population-based study from Sweden. BMC Cancer 16. https://doi.org/10.1186/s12885-016-2236-y

Tavalaee M, Deemeh MR, Arbabian M, Nasr-Esfahani MH (2012) Density gradient centrifugation before or after magnetic-activated cell sorting: which technique is more useful for clinical sperm selection? J Assist Reprod Genet 29(1):31–38. https://doi.org/10.1007/s10815-011-9686-6

Tempest HG, Ko E, Chan P, Robaire B, Rademaker A, Martin RH (2008) Sperm aneuploidy frequencies analysed before and after chemotherapy in testicular cancer and Hodgkin's lymphoma patients. Hum Reprod 23(2):251–258. https://doi.org/10.1093/humrep/dem389

Thomas C, Cans C, Pelletier R, De Robertis C, Hazzouri M, Sele B, Rousseaux S, Hennebicq S (2004) No long-term increase in sperm aneuploidy rates after anticancer therapy: sperm fluorescence in situ hybridization analysis in 26 patients treated for testicular cancer or lymphoma. Clin Cancer Res 10(19):6535–6543. https://doi.org/10.1158/1078-0432.ccr-04-0582

Thomson AB, Campbell AJ, Irvine DS, Anderson RA, Kelnar CJH, Wallace WHB (2002) Semen quality and spermatozoal DNA integrity in survivors of childhood cancer: a case-control study. Lancet 360(9330):361–367

Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA, Clark AM (2009) Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod 24(9):2061–2070. https://doi.org/10.1093/humrep/dep214

Torre LA, Siegel RL, Ward EM, Jemal A (2016) Global cancer incidence and mortality rates and trends – an update. Cancer Epidemiol Biomarkers Prev 25(1):16–27. https://doi.org/10.1158/1055-9965.EPI-15-0578

Tremblay A, Beaud H, Delbes G (2017) Transgenerational impact of chemotherapy: would the father exposure impact the health of future progeny? Gynecol Obstet Fertil Senol 45:609. https://doi.org/10.1016/j.gofs.2017.09.004

Trost LW, Brannigan RE (2012) Oncofertility and the male cancer patient. Curr Treat Options Oncol 13(2):146–160. https://doi.org/10.1007/s11864-012-0191-7

Vaisheva F, Delbes G, Hales BF, Robaire B (2007) Effects of the chemotherapeutic agents for non-Hodgkin lymphoma, cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), on the male rat reproductive system and progeny outcome. J Androl 28(4):578–587. https://doi.org/10.2164/jandrol.106.002428

Vakalopoulos I, Dimou P, Anagnostou I, Zeginiadou T (2015) Impact of cancer and cancer treatment on male fertility. Hormones (Athens) 14(4):579–589. https://doi.org/10.14310/horm.2002.1620

van der Kaaij MA, Heutte N, Le Stang N, Raemaekers JM, Simons AH, Carde P, Noordijk EM, Ferme C, Thomas J, Eghbali H, Kluin-Nelemans HC, Henry-Amar M (2007) Gonadal function in males after chemotherapy for early-stage Hodgkin's lymphoma treated in four subsequent trials by the European Organisation for Research and Treatment of Cancer: EORTC Lymphoma Group and the Groupe d'Etude des Lymphomes de l'Adulte. J Clin Oncol 25(19):2825–2832. https://doi.org/10.1200/jco.2006.10.2020

van Otterdijk SD, Michels KB (2016) Transgenerational epigenetic inheritance in mammals: how good is the evidence? FASEB J 30(7):2457–2465. https://doi.org/10.1096/fj.201500083

Vendramini V, Robaire B, Miraglia SM (2012) Amifostine-doxorubicin association causes long-term prepubertal spermatogonia DNA damage and early developmental arrest. Hum Reprod 27(8):2457–2466. https://doi.org/10.1093/humrep/des159

Viviani S, Santoro A, Ragni G, Bonfante V, Bestetti O, Bonadonna G (1985) Gonadal toxicity after combination chemotherapy for Hodgkin's disease. Comparative results of MOPP vs ABVD. Eur J Cancer Clin Oncol 21(5):601–605. https://doi.org/10.1016/0277-5379(85)90088-4

Wallace WHB, Anderson RA, Irvine DA (2005a) Fertility preservation for young patients with cancer. Who is at risk and what can be offered? (vol 6, pg 209, 2005). Lancet Oncol 6(12):922–922

Wallace WHB, Anderson RA, Irvine DS (2005b) Fertility preservation for young patients with cancer: who is at risk and what can be offered? Lancet Oncol 6(4):209–218. https://doi.org/10.1016/S1470-2045(05)70092-9

Wasilewski-Masker K, Seidel KD, Leisenring W, Mertens AC, Shnorhavorian M, Ritenour CW, Stovall M, Green DM, Sklar CA, Armstrong GT, Robison LL, Meacham LR (2014) Male infertility in long-term survivors of pediatric cancer: a report from the childhood cancer survivor study. J Cancer Surviv 8(3):437–447. https://doi.org/10.1007/s11764-014-0354-6

Winther JF, Boice JD Jr, Mulvihill JJ, Stovall M, Frederiksen K, Tawn EJ, Olsen JH (2004) Chromosomal abnormalities among offspring of childhood-cancer survivors in denmark: a population-based study. Am J Hum Genet 74(6):1282–1285. https://doi.org/10.1086/421473

Winther JF, Boice JD Jr, Christensen J, Frederiksen K, Mulvihill JJ, Stovall M, Olsen JH (2010) Hospitalizations among children of survivors of childhood and adolescent cancer: a population-based cohort study. Int J Cancer 127(12):2879–2887. https://doi.org/10.1002/ijc.25286

Wu H, Hauser R, Krawetz SA, Pilsner JR (2015) Environmental susceptibility of the sperm epigenome during windows of male germ cell development. Curr Environ Health Rep 2:356–366

Xin F, Susiarjo M, Bartolomei MS (2015) Multigenerational and transgenerational effects of endocrine disrupting chemicals: a role for altered epigenetic regulation? Semin Cell Dev Biol 43:66–75. https://doi.org/10.1016/j.semcdb.2015.05.008

Yonemoto T, Ishii T, Takeuchi Y, Hagiwara Y, Iwata S, Tatezaki S (2009) Recently intensified chemotherapy for high-grade osteosarcoma may affect fertility in long-term male survivors. Anticancer Res 29(2):763–767

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