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Explaining infertility: a Sperm DNA Fragmentation review

Cristiana Daniela Pereira Lopes

Dissertação de Mestrado apresentada à
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Mestrado em Biologia Celular e Molecular
Departamento de Biologia
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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Authorship Statement

I, Cristiana Daniela Pereira Lopes, student number 201405455, currently undertaking Master's degree in Cell and Molecular Biology, edition 2018/2019, hereby declare that this dissertation was written by myself and using my own words, not contemplating plagiarism from any bibliographic source indicated and acknowledged as such. I am aware of any consequence as a result of plagiarism.

Porto, 26 th September 2019

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Resumo

A infertilidade masculina é um problema universal e pode ser uma consequência da existência de danos no ADN dos espermatozóides. Os danos no ADN espermático podem advir de várias causas, mas a mais preocupante é o *stress* oxidativo. A avaliação inicial do paciente do sexo masculino envolve uma análise convencional do sémen, que avalia a funcionalidade dos espermatozóides. Embora os danos no ADN espermático possam ter como consequência a incapacidade dos homens de conceber, a análise espermática normalmente não inclui um teste ao ADN espermático. Apesar da existência de técnicas de avaliação de fragmentação de ADN, estas são demoradas com protocolos extensos, reagentes complexos e equipamentos que não existem em laboratórios de andrologia comuns.

Para superar os problemas de infertilidade, os casais podem recorrer a tecnologias reprodutivas medicamente assistidas, como injeção intracitoplasmática de espermatozóides e fertilização *in vitro*. Durante esses procedimentos, muitos dos métodos de preparação e manipulação de espermatozóides executados no ambiente *in vitro* podem resultar na produção excessiva de espécies reativas de oxigénio, expondo os gametas e o crescimento de embriões a danos oxidativos significativos. Para contrabalançar os níveis mais altos de espécies reativas de oxigénio presentes nesses ambientes, são utilizados antioxidantes, no entanto, o regime antioxidante ideal a ser utilizado permanece desconhecido.

Assim, esta revisão destaca a importância do dano subjacente ao ADN espermático, mostrando as causas, consequências, testes aos danos no ADN espermático e também estratégias de tratamento.

Palavras-chave

Danos no ADN espermático, infertilidade, desenvolvimento embrionário, falha na implantação, *stress* oxidativo, tecnologias de reprodução assistida

Abstract

Male infertility is a widespread problem and can arise from multiple causes such as sperm DNA damages. There are multiple causes of sperm DNA damage but the most worrisome is sperm oxidative stress. The initial evaluation of the male patient involves a conventional semen analysis, which evaluates sperm functionality. Despite sperm DNA damage can have as a consequence the men's incapacity to conceive, these semen analysis does not include a sperm DNA damage assay. There are sperm DNA fragmentation evaluation techniques, however, they are time-consuming with extensive protocols, complex reagents, and equipment that does not exist in standard andrology laboratories.

In order to overcome infertility problems, couples may resort to medically assisted reproductive technologies, such as intracytoplasmic sperm injection and *in vitro* fertilization. During these procedures, many of the sperm preparation and manipulation methods performed in the *in vitro* environment can result in excessive production of reactive oxygen species exposing the gametes and growing embryos to significant oxidative damage. In order to counterbalance the higher levels of reactive oxygen species present in those environments, antioxidants have long been utilized, however, the optimal antioxidant regimen remains unknown.

Therefore, this review highlights the importance of sperm DNA damage by showing the causes, consequences, sperm DNA damage assays and also treatment strategies.

Key Words

Sperm DNA damage, infertility, embryo development, implantation failure, oxidative stress, assisted reproductive technologies

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List of Abbreviations

AB: Aniline blue

AO: Acridine Orange

ART: assisted reproductive technology

ATP: adenosine triphosphate

BER: base excision repair

ºC: temperature unit in Celsius

CMA3: chromomycin A3

ChK1: checkpoint kinase 1

Da: unit of atomic mass in Dalton

DFI: DNA fragmentation index

DNA: deoxyribonucleic acid

ds: double-strand

DSBR: double-strand break repair

EPI: epicatechin

HCO_3: bicarbonate

HNO_3: nitric oxide

HDS: high DNA stainability

HIV: human immunodeficiency virus

H_2O_2: hydrogen peroxide

HPG: hypothalamic-pituitary-gonadal

ICSI: intracytoplasmic sperm injection

IVF: *in vitro* fertilization

miRNA: micro ribonucleic acid

MMP: mitochondrial membrane permeability

MMR: mismatch repair

MPT: mitochondrial permeability transition

mPTP: mitochondrial permeability transition pore

MRE11: meiotic recombination 11

mRNA: messenger ribonucleic acid

NER: nucleotide excision repair

NHEJ: non-homologous end-joining

O_2: oxygen

O_2^-: superoxide anion

OH•: hydroxyl radicals

ORP: oxidation-reduction potential

OS: oxidative stress

PRM1: protamine 1

PRM2: protamine 2

PUFAs: polyunsaturated fatty acids

RNA: ribonucleic acid

ROS: reactive oxygen species

SC: Sertoli cells

SCD: sperm chromatin dispersion test

SCSA: sperm chromatin structure assay

SDF: sperm DNA fragmentation

ss: single-strand

TB: toluidine blue

TESE: testicular sperm extraction

TP1: transition protein 1

TP2: transition protein 2

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labelling

µmol: volume unit in micromole

CHAPTER I Reproductive Male System And Spermatogenesis Process

1.1. Reproductive Male System

One of the major differences between the male and female reproductive systems is the fact that most of the male genitals are outside the pelvic cavity (Barrett et al. 2019). The testicles are tubular compartments composed by seminiferous tubules in which the sperm is formed. To a normal function of this male genital organ, it is necessary a hormonal regulation that is ruled by the hypothalamic-pituitary-gonadal (HPG) axis (endocrine regulation). The endocrine regulation affects the testes being the effects mediated and controlled at the testicular level by local control mechanisms (John and Guyton 2011). The interaction between these two sections plays an important role in both quantitatively and qualitatively normal production of spermatozoa - spermatogenesis (Barrett et al. 2019) and male steroid hormones(John and Guyton 2011).

The testes are outside the body into the scrotum at 2°C lower temperature than normal body temperature (Sakkas and Alvarez 2010). An increase in the temperature of the testes can have a degenerative effect on most cells of the seminiferous tubules besides the spermatogonia, preventing the occurrence of spermatogenesis. The scrotum musculature contracts when the temperature is lower, pulling the testes close to the body to maintain the right temperature for the normal functionality of these organs(John and Guyton 2011). The male reproductive system is represented in Figure 1.

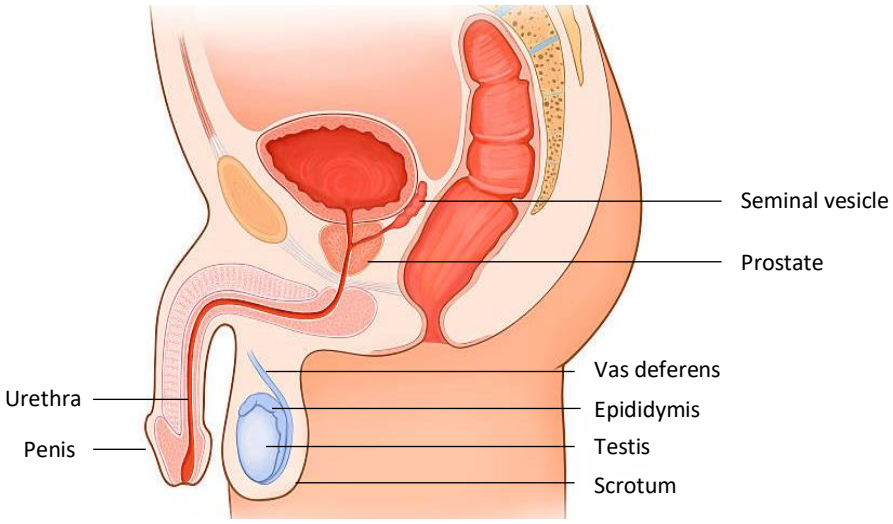

Figure 1: Representation of the male reproductive system structure. Adapted from: https://images.app.goo.gl/DXdtDvgVm1DaSFTT9. Accessed on: 30th November, 2019.

1.2. Spermatogenesis, Spermiogenesis And Spermiation Process

In the adult mammals, spermatogenesis (Figure 2) comprises three distinct phases: mitosis, meiosis, and spermiogenesis. In each of these there are specific morphological and biochemical changes of nuclear and cytoplasmic components (Bergmann 2005). During the first phase (spermatogonial stage) the diploid spermatogonia is situated at the periphery of the seminiferous tubule near the basal lamina being close to the blood vessels and interstitial tissue, ensuring the supply of the necessary nutrients as well as hormonal regulation of spermatogenesis. Spermatogenesis occurs from the periphery to the lumen of the seminiferous tubule, with the most undifferentiated cells in the basal region and the most differentiated cells closest to the lumen (Fox 2011).

Spermatogonia stem cells divide themselves producing type A and type B spermatogonia (Nieschlag et al. 2010). The type A spermatogonia self-renew throughout life providing a pool of stem cells available for spermatogenesis and the type B spermatogonia grows into primary spermatocytes which start meiotic division (O'Donnell et al. 2017).

When the first meiotic division ends, the primary spermatocytes produce secondary spermatocytes which are haploid. The meiosis continues and the secondary spermatocytes divide into haploid spermatids. During the meiotic division, the 46 chromosomes of the spermatocyte are divided, so each spermatid stays with 23 chromosomes (Sherwood 2014).

During spermiogenesis, there is no cell division but a conventional round cell becomes converted into a spermatozoon. This process is characterized by: (I) the development of an acrosomal space within the head, which houses specific enzymes required for fertilization and within the same local differentiation of acrosomal membranes that are pertinent for this process; (II) nuclear changes; (III) the development of the flagellum or sperm tail as a means of motility near the posterior aspect which is fuelled by the abundance of mitochondria in the mid-piece of the sperm cell; and (IV) the reorganisation of the cytoplasm and cell organelles (Allais-Bonnet and Pailhoux 2014; O'Donnell et al. 2017).

During this process, the spermatids remain connected with the Sertoli cells (SC) via cell junctions (De Jonge and Barrat 2006), and with each other by cytoplasmic bridges allowing substance exchange and synchronization of its maturation process (Fox

2011). These cells are only separated with its release in the seminiferous tubules lumen - spermiation (O'Donnell et al. 2017).

Once the fusion between the male and female sexual gamete happens during fertilization, the eventual fetus will be provided by half of the genetic characteristics from the father and half from the mother (Sherwood 2014).

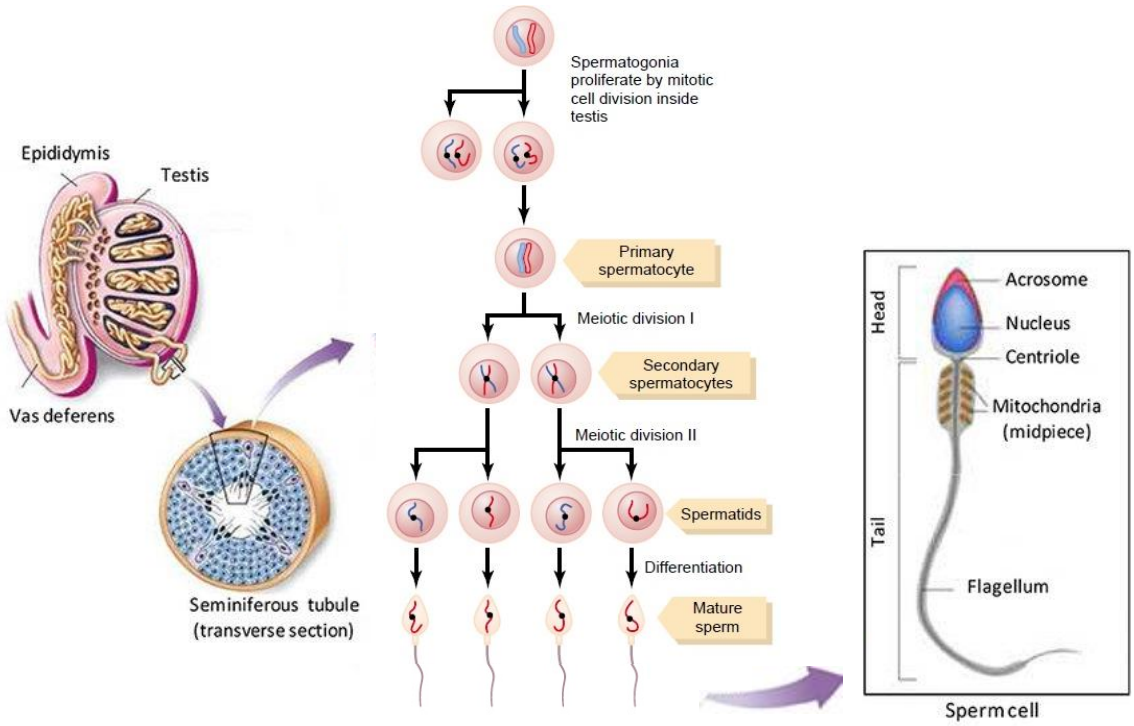

Figure 2: Illustration of spermatogenesis. Spermatogenesis occurs in the seminiferous tubules. Diploid primordial germ cells (also called spermatogonia) near the basal lamina of the seminiferous tubules suffer an initial mitotic division resulting diploid primary spermatocytes. About half the primary spermatocytes produced remain near the basal lamina to continue spermatogenesis to be continuous during male's reproductive lifespan. The other half migrate toward the lumen of the seminiferous tubules and begin to undergo meiosis I, resulting in haploid secondary spermatocytes. These secondary spermatocytes further undergo through meiosis II, producing haploid spermatids. Spermiogenesis results in mature spermatozoa. In this stage, specific regions of the spermatid differentiate into the head, mid-piece, and the tail of the sperm cell. Adapted from: Allais-Bonnet and Pailhoux 2014 and John and Guyton 2011.

1.3. Chromatin Reorganization In Maturing Spermatids

Spermatids have to pass through an extensive remodeling of cell elements and also elongation of the cell in order to differentiate into spermatozoa (Sherwood 2014).

During spermiogenesis, the chromatin of the haploid spermatids and their functioning genes begin to change over a period of several days (Montellier et al. 2013). Initially, the genome is packaged by histones (Annunziato 2008). Contrarily to somatic cells, where deoxyribonucleic acid (DNA) is complexed with histones into organizational units called nucleosomes, the DNA in sperm cells is disassembled from the nucleosomal structure (Aitken et al. 2009). Single-strand (ss) and double-strand (ds) breaks are naturally induced to allow unwinding of the nucleosomal structure and to avoid supercoiling. However, these strand breaks are normally repaired to prevent the persistence of DNA damage in mature spermatozoa (Bach and Schlegel 2016). Two small basic transition proteins, TP1 and TP2, are expressed and incorporated into the spermatid chromatin (Steger et al. 1998), replacing the majority of the somatic histones and the chromatin becomes more compact (Zini and Agarwal 2018). It is important to mention that only a small, but apparently well-defined fraction of the sperm genome remains histone-associated in mature sperm. High amounts of histones in sperm are related to decreased fertility and increased risk of embryonic failure after fertilization (Carrell and Hammoud 2009).

Protamines are the last proteins to be synthesized and their deposition into spermatid chromatin only begins after the majority of the histones have been successfully replaced by TP1 and TP2. Without this delay, the synthesis of the protamines would induce an early condensation of the spermatid DNA resulting in abnormally shaped sperm heads. These proteins contain multiple cysteine residues that form a series of inter- and intraprotamine disulfide bonds linking protamines to each other, during the passage through the epididymis (Zini and Agarwal 2018). These disulfide bonds are required to further stabilization resulting in the final nuclear maturation (Dadoune 2003; Fuentes-Mascorro et al. 2000).

The binding of these protamines to DNA completes the chromatin reorganization process, packaging the male’s haploid genome into a highly compact, genetically inactivated genome, programmed for reactivation once the sperm head enters an oocyte (Zini and Agarwal 2018).

CHAPTER II Sperm DNA Damage Etiology: Biological And Clinical Factors

2.1. Infertility: Male Factor

Infertility is commonly defined as the incapacity to conceive after at least 1 year of regular unprotected intercourse affecting 15-25% couples. Male factors are responsible for approximately half of those infertile couples (Agarwal et al. 2014; Kobayashi et al. 2012).

The major causes for male infertility include varicocele, genital tract obstruction, testicular failure, genetic conditions, infections, hormonal dysfunction, immunological conditions, ejaculatory/sexual dysfunction, cancer, systemic diseases, altered lifestyle and also environmental toxic exposure (Panner et al. 2018; Esteves et al. 2011). These factors can interfere with sperm quality and quantity, sperm viability, morphology and capacity of fertilizing an oocyte. However, when the decline in male fertility cannot be attributed to any specific cause, it is denominated as idiopathic infertility (Agarwal et al. 2016). Oxidative stress (OS) is believed to be an important and plausible cause of idiopathic male infertility (Agarwal et al. 2014) and is associated with defective human sperm function and the resultant loss of *in vivo* and *in vitro* fertilizing potential (Cassina et al. 2015).

Semen analysis consists in the analysis of standard World Health Organization-determined semen parameters (WHO 2010) like the ejaculate volume, morphology, sperm concentration and motility which are associated with the probability of natural conception. The presence of high numbers of immotile spermatozoa, low percentage of normal sperm morphology, reduced ejaculate volume, and reduced concentration of sperm in the seminal plasma severely affects the success of a conception (Nieschlag et al. 2010). Sperm DNA integrity is required for the proper delivery of the paternal genome to the offspring, and for this reason, it may be a better predictor of male fertilizing potential. However, sperm DNA assessment is not included in routine sperm analyses (Agarwal and Allamaneni 2004; Bungum et al. 2011).

2.2. Sperm DNA Integrity

Sperm DNA integrity is associated with fertility potential in reproductive biotechnologies (Larson-Cook et al. 2003). Several studies proved this assumption by reporting an increased sperm DNA fragmentation (SDF) in infertile males comparing with fertile men and also an abnormal DNA packing in normozoospermic men undergoing assisted reproduction treatment (Evenson et al. 1999; Spano et al. 2000; Saleh et al. 2003; Alkhayal et al. 2013; Oleszczuk et al. 2013).

Higher levels of sperm DNA fragmentation (SDF >30%) have effects on pre- and post-implantation embryos and are related to slower embryo cleavage, lower blastocyst formation, poor embryo morphology, lower implantation and pregnancy rates (Parinaud et al. 1993; Janny and Menezo 1994). Additionally, Robinson et al. (2012) demonstrated a significant high miscarriage rate for patients presenting high DNA damage.

Moreover, the fertility and genetic material of further generations can be affected by the presence of damages in the paternal sperm DNA (Ahmadi and Ng 1999; Tamburrino et al. 2012).

Knowing this - why is sperm DNA fragmentation testing not a standard parameter in the diagnosis of male infertility?

2.3. Types Of DNA Damage

DNA damage may occur in the form of single- or double-strand breaks (Enciso et al. 2009). dsDNA breaks can lead to very harmful lesions, such as chromosome fragmentation, chromosome domain loss, translocations or other genome rearrangements (Brugmans et al. 2007), and, if not correctly repaired, they can lead to genomic instability and cell death.

Oxidative attacks through hydroxyl radicals (OH•) are in the origin of dsDNA damage through the activation of sperm caspases and endonucleases (Badouard et al. 2008). Ionizing radiation is also a type of oxidative attack and results in the formation of ssDNA fragmentation thereafter (González-Marín et al. 2012).

Extensive dsDNA damages cannot be repairable and it is incompatible with a normal embryo and fetal development. dsDNA breaks induce a DNA damage response which may have different outcomes depending on the severity of the damage and on the cell type (García-Díaz et al. 2000).

2.4. Mechanisms Of Sperm DNA Fragmentation

DNA damage in spermatozoa can be detected in the cell nucleus and mitochondria (Sakkas and Alvarez 2010).

In the testes, abnormalities during spermatogenesis can result in cell apoptosis and DNA breaks. In addition, DNA can be damaged at the epididymis level by OS and at the vas deferens level, due to the increased levels of reactive oxygen species (ROS)

with respect to the epididymis (Agarwal et al. 2008; Aitken and Koppers 2011; Makker et al. 2009). The transport through the epididymis plays a major role in causing sperm DNA fragmentation (Sakkas and Alvarez 2010).

External factors like radiotherapy and chemotherapy or environmental toxicants can also affect the sperm DNA quality in the testes (Sotolongo et al. 2005; Sailer et al. 1997; Maione et al. 1997; Rubes et al. 2007; O'flaherty et al. 2008). Moreover, sperm DNA quality is affected by the method of semen collection, the season in which the semen is collected and pre-treatment procedures (washing protocols) (González-Marín et al. 2012).

2.4.1. Oxidative Stress

ROS are intracellular chemical species containing oxygen (O_2) although more chemically reactive than O_2, being capable of triggering numerous biological events. ROS present reactivity toward proteins, lipids and, DNA. Examples of ROS are hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radicals (Glasauer and Chandel 2013).

Equilibrated levels of ROS produced by the mitochondria have some important physiological functions like modulating gene and protein activities essentials for an efficient sperm proliferation, differentiation, and function (Du Plessis et al. 2015). However, the human spermatozoon is highly susceptible to ROS and for this reason, an excessive amount of ROS production to the antioxidant capacities of the male reproductive tract or seminal plasma results in oxidative stress (Aitken et al. 1992; Agarwal et al. 2008; Agarwal and Sekhon 2011).

OS is responsible for lipid peroxidation exacerbation of sperm plasma membrane, DNA damage and abortive apoptosis (Agarwal et al. 2014) and it is considered the major cause of defective sperm function in male infertility (Aitken et al. 2012). Sperm membranes are composed by higher concentrations of polyunsaturated fatty acids (PUFAs) than other cell types, which turn them to be highly vulnerable to oxidative damage, with a following loss of membrane and DNA integrity, impaired motility and the onset of apoptosis (Aitken et al. 2006).

When the somatic cells production of ROS is high, a mitochondrial membrane permeabilization (MMP) is created (Galluzzi et al. 2012), and it is accompanied by failure to synthesize adenosine triphosphate (ATP), and OS (Kroemer et al. 2007; Galluzzi et al. 2012).

MMP can be initiated due to an increase in the permeability of the mitochondrial inner membrane or at the outer membrane (Tait and Green 2010). Mitochondrial inner membrane permeabilization is responsible for the swelling of the matrix which in turn leads to the outer membrane rupture (Treulen et al. 2015). During this last event, named mitochondrial permeability transition (MPT) (Kroemer et al. 2007), there is the opening of a large quantity of conductance permeability transition pores that are responsible for mitochondrial inner membrane abruptly permeability to all solutes of molecular weight up to about 1500 Da (Bernardi and Forte 2007). The mitochondrial permeability transition pore (mPTP) (Gulbins et al. 2003) is a channel associated with MPT, whose components are located in the inner and outer mitochondrial membrane (Treulen et al. 2015).

Treulen et al. (2015) could conclude that MPT by mPTP opening induces the production of increased levels of ROS and the consequent existence of DNA fragmentation. Therefore the block of mPTP opening could be an effective solution to avoid DNA fragmentation by oxidative stress of mitochondrial origin in human spermatozoa (Treulen et al. 2015).

2.4.2. Induction Of Apoptosis During The Process Of Spermatogenesis

As already explained, the germ cells suffer multiple mitotic divisions before result in mature spermatozoa (Sakkas et al. 1999). Therefore, extensive quantity of germ cells are produced and for this reason germ cells are selected by apoptosis in order to not overcome with the supportive capacity of the Sertoli cells (Furuchi et al. 1996; Allan et al. 1992; Tapanainen et al. 1993; Bartke 1995; Billig et al. 1995; Sinha Hikim et al. 1997; Rodriguez et al. 1997). This selective mechanism induces the apoptosis in 50%–60% of all germ cells that enter meiosis I (González-Marín et al. 2012).

The germ cells are earmarked with apoptotic markers of the Fas type (Pentikäinen et al. 1999; Sakkas et al. 1999; Billig et al. 1996). Fas (Lee et al. 1997) is a membrane protein that mediates apoptosis (Krammer et al. 1994; Schulze-Osthoff et al. 1994; Suda et al. 1993). SC express Fas ligand (FasL) binding to Fas-positive germ cells and phagocyte them (Suda et al. 1993) (Figure 3a). With this mechanism, SC can limit the size of the germ cell population to numbers they can support (Rodriguez et al. 1997; Lee et al. 1997).

However, Fas-positive germ cells may undergo chromatin remodeling and differentiate into spermatozoa, particularly in men with poor sperm parameters due to failure on activation to undergo apoptosis or because of problems in activating Fas-mediated apoptosis (Figure 3b) (Sakkas et al. 1999; González-Marín et al. 2012).

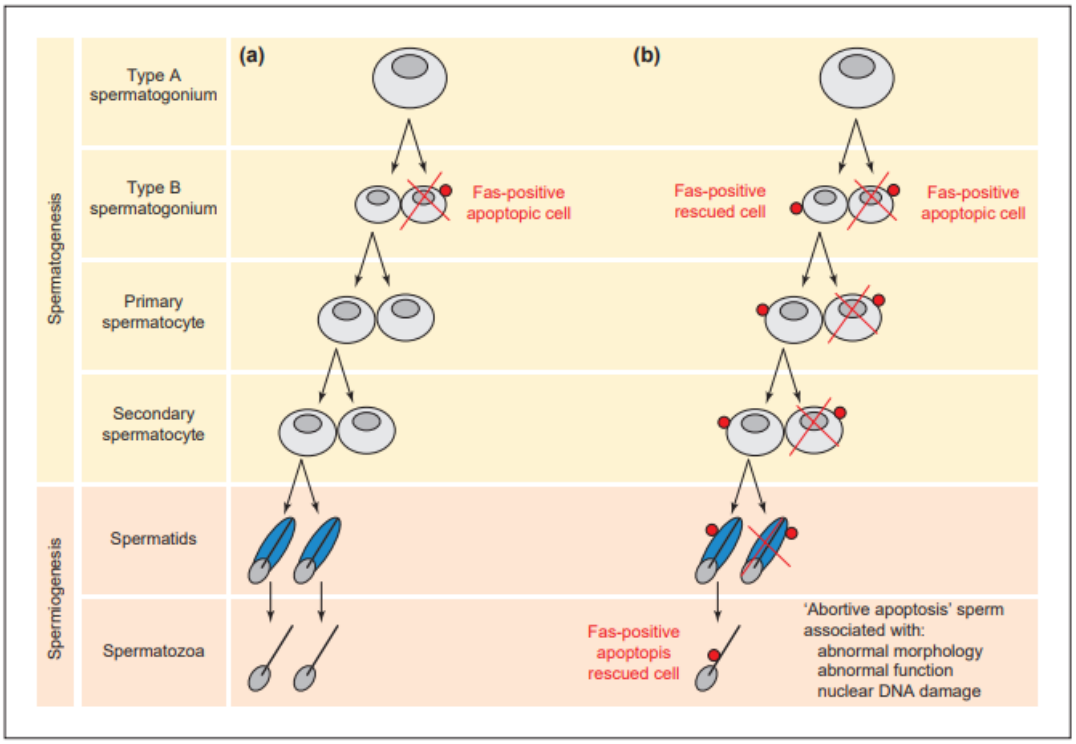

Figure 3: Induction of apoptosis during spermatogenesis by Fas in men with (a) normal and (b) abnormal sperm parameters. Retrieved from Sakkas et al. 2009.

2.4.3. Abnormal Spermatid Maturation

During chromatin packaging, endogenous nuclease activity is required to loosen the chromatin by histone hyper-acetylation and introduction of breaks, capable of both creating and ligating breaks in order to maintain the DNA integrity (González-Marín et al. 2012) and also to reassembly of the important unit of genome expression - the DNA lop domain. Unrepaired breaks may result in DNA fragmentation presence in ejaculated spermatozoa (González-Marín et al. 2012) due to DNA instability and sensitivity to denaturing stress (Lopes et al. 1998).

2.4.4. Post-Testicular Sperm DNA Fragmentation

Some DNA damages can be induced during the co-migration of both mature and immature sperm from the seminiferous tubules to the epididymis end portion (Ollero et al. 2001). The high packing of immature and mature sperm (Twigg et al. 1998) in the epididymis facilitates ROS-induced DNA damage in mature sperm (Lopes et al. 1998).

2.4.5. Extrinsic Factors

2.4.5.1. DNA Fragmentation Induced By Chemotherapy And Radiotherapy

Chemotherapy and radiotherapy used in cancer treatments have cytotoxic effects on the spermatogenic epithelium by affecting its proliferation in the pre-pubertal and adult testis (Morris 2002; De Palma et al. 2000; Howell and Shalet 2001). The effects of both therapies are dose- and time-dependent. Therefore, with the increase of dose applied, the toxicity also increases and the spermatogonial stem cells, which are more sensitive to cytotoxic therapy then spermatozoa, are ablated which may lead to possible transient or permanent azoospermia. As the effects of cytotoxic therapy are cumulative, even germ cells that can survive to this cytotoxic therapy carry DNA damage which can lead to developmental abnormalities and cancer within the offspring (Morris 2002).

Radiotherapy creates damages on the DNA avoiding cell replication and leading to its death (Gandini et al. 2006). This therapy is specifically useful in cancer cells which replicate more quickly. However, the radiotherapy effects can be also observed in normal cells especially in those with a high replication rate such as spermatogonia (Gandini et al. 2006).

Those therapies make necessary the establishment of centers for the banking of spermatozoa before cytotoxic therapy (Schover et al. 2002; Howell and Shalet 2001) or the search for cytotoxic therapies which do not impair sperm production (Radford et al. 1994). Sakkas and Alvarez (2010) suggested evaluating sperm DNA damage in cancer patients by using the Comet assay combined with Chromomycin A3 (CMA3) staining test to characterize sperm chromatin quality in cancer patients at the time of sperm banking.

2.4.5.2. The Effects Of Male Age On Sperm DNA Damage

The aging process affects human fertility due to hormonal and cellular changes. Nowadays, couples are conceiving later and, for this reason, the effect of age on fertility constitutes an important factor. The age of 40 is a turning point after this age men demonstrate declines in structural and functional parameters (Belloc et al. 2008; Evenson and Wixon 2006; Guerin et al. 2005). During aging, sperm DNA is more susceptible to damages caused by ROS or by spontaneous errors during DNA duplication, transcription, or post-transcriptional events (Paoli et al. 2018; Rattan 2006).

In men with advanced age, the body antioxidant capacity decreases contributing to a more common presence of DNA damages (Aitken 1989) in both sperm nucleus and mitochondria. Moreover, in older men, the apoptosis functions within spermatogenesis

might be defective determining sperm dysfunction and resulting in the production of more spermatozoa with fragmented DNA (Petersen et al. 2018).

The chromatin packaging process requires a correct ratio of protamine 1 and 2 (PRM1/PRM2), being in fertile men 0.8–1.2 (Grassetti et al. 2012), with any alteration to this ratio corresponding to a sign of infertility (Rogenhofer et al. 2013). Paoli et al. (2018) described an association between modifications in messenger ribonucleic acid (mRNA) levels and changes in protamine expression by detecting an altered PRM1/PRM2 mRNA profile in elderly men in comparison with young men (0.6 versus 0.9, respectively). This is consistent with the increased amount of sperm DNA damage observed in elderly subjects, suggesting the marked fragility of chromatin integrity. The protamine/mRNA ratio in ejaculated sperm could, therefore, be a prognostic marker to estimate sperm fertilizing potential and also the likelihoods of successful fertilization with *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) distinguishing fertile from sub fertile men (Paoli et al. 2018).

Advanced age is characterized by several cellular alterations such as heightened inflammatory response, stem cell exhaustion, cellular senescence, mitochondrial dysfunction, progressive shortening of telomeres, altered DNA damage response, changes in gene expression and epigenetic changes (Harries 2014). Although several microRNAs (miRNA) seem to be involved in these processes, little is yet known about their role in the aging process (Williams et al. 2017), especially the role of circulating miRNAs (Zhang et al. 2015).

2.4.5.3. Lifestyle factors

1) Obesity And Sperm DNA Quality

In obese men, toxic substances including, fat-soluble endocrine disruptors are accumulated in the adipose tissue, which might have an amplifying effect on these abnormalities (Sermondade et al. 2013; Katib 2015). Obesity also improves an increase in scrotal temperature, caused by the accumulation of fatty tissue around the scrotum, which may cause oxidative stress in the testicles with consequent adverse effects on sperm chromatin (Jung and Schill 2000; Fariello et al. 2012; Palmer et al. 2012; Crujeiras and Casanueva 2014). Additionally, fewer studies argued that increased levels of SDF in obese men may also be associated with low mitochondrial membrane potential (La Vignera et al. 2012; Fariello et al. 2012; Leisegang et al. 2014).

2) DNA Damage Induced By Environmental Toxicants

Air pollution can affect the DNA integrity. The exposure to high levels of metabolites of carcinogenic polycyclic aromatic hydrocarbons found in air pollution increases the DNA damage sperm of men who are homozygous null for glutathione-S-transferase M1. Without this gene men are incapable of detoxifying those metabolites, fragmenting the sperm DNA (Rubes et al. 2007).

3) Alcohol And Sperm Quality

Alcohol consumption induces an increase in β-endorphin levels that in turn could be involved in testicular damage, inducing sperm apoptosis (Sansone et al. 2018).

Talebi et al. (2011) argued that ethanol consumption affects nuclear maturity and DNA integrity from spermatozoa aspirated from cauda epididymis of rats. A significant decrease in sperm progressive and non-progressive motility of ethanol-consuming rats was also detected. A repeated ethanol treatment revealed a decrease in the mitochondrial membrane potential along with ROS generation in the testicular tissue (Jana et al. 2010).

4) Potential Effect Of Smoking On Semen Quality

Smoking is correlated with a decrease in sperm quality by DNA fragmentation (Silver et al. 2005; Sepaniak et al. 2004) due to absorption of heavy metals, carbon monoxide and nicotine throughout the body (Hamad et al. 2014; Jo et al. 2015; Abdul-Ghani et al. 2014).

Checkpoint kinase 1 (Chk1) controls the G2/M phase transition when DNA damage is detected (Ma et al. 2015; Albiges et al. 2014; Al-Kaabi et al. 2015). Following DNA damage, Chk is released from chromatin to the cytoplasm. Activated Chk phosphorylates some downstream effectors able to trigger cellular responses, such as alteration of energy consumption, transcription regulation, cell-cycle arrest or delay, and DNA repair or cell death if the damage is too difficult to repair (Zuazua-Villar et al. 2014).

Cui et al. (2016) observed that men who smoke present a significantly decrease in the Chk1 levels being correlated with sperm DNA fragmentation indices. A reduction of Chk1 expression leads to decreased sperm repair and increased sperm apoptosis, with a subsequent effect on semen quality (Cui et al. 2016).

5) Drugs Addiction And Male Fertility

Recreational drugs might have adverse effects on human reproduction. Cannabis interferes with the HPG axis, spermatogenesis, and sperm function, because of cannabinoid receptors existence in the anterior pituitary, Leydig cells, Sertoli cells and in testicular tissues (Du Plessis et al. 2015).

Studies with animal models were done in order to ascertain the effects of cocaine and ecstasy on fertility (Sansone et al. 2018). It was observed that cocaine provokes a reduction in the diameter of seminiferous tubules and, decreases the number of total germ cells (George et al. 1996). High oxidative stress levels were detected in mice following chronic administration of cocaine (Li et al. 1999), probably contributing to testicular damage. Similar effects have been described for ecstasy: Barenys et al. (2010) reported significantly decreased sperm concentration and motility, and increased rates of DNA damage and tubular degeneration in rats after administration with several dosages of ecstasy. In addition recent reports suggest that opioids as well as morphine addicted men also show increased rates of DNA fragmentation (Safarinejad et al. 2013; Ahmadnia et al. 2016).

6) Scrotal Hyperthermia

The testes have a temperature regulating mechanism in which there is a counter current heat exchange between the pampiniform plexus and the testicular artery. Any interruption to this system may cause problems with the spermatozoa production (Paul et al. 2009).

Heat stress causes an increase in the metabolism of the testes not allowing a sufficient increase in blood flow (Galil and Setchell 1988) or can reduce the oxygen transport capacity (Hockel and Vaupel 2001), causing a hypoxic status in the testis (Setchell 1998). Hypoxia is a condition characterized by a drop on the oxygen tension below the one required for normal cellular function in a given tissue. Some studies have shown that hypoxia leads to cell cycle arrest and apoptosis (Carmeliet et al. 1998; Iida et al. 2002).

Slight fluctuations in temperature are responsible for the existence of high levels of ROS generated within the testes and epididymis making the testicular responses to thermal stress insufficient (Paul et al. 2009).

CHAPTER III Sperm DNA Damage: Repairing Mechanisms And Possible Treatment Strategies

3.1. Repairing Sperm DNA Damage

When a cell is facing DNA damage the cell cycle slows down on its progression due to the activation of checkpoints until lesions are resolved. However, this may not be enough and unrepaired dsDNA breaks persist. In this case, cells can suffer apoptosis (Jurisicova and Acton 2004), destroying cells and impair viability; can tolerate the damage, however, mutations will probably occur in the next generation; or can repair the damage. To repair cell DNA damage, sophisticated cellular network can be deployed, where several proteins are mobilized in response to DNA damage (González-Marín et al. 2012).

There are plenty of known repair mechanisms: nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), and DNA double-strand break repair (DSBR) are the main mechanisms operating in the mammalian germline cells (González-Marín et al. 2012).

3.1.1. Repair During Spermiogenesis

In spermatogenic cells, repairing dsDNA breaks depends on the presence or deficiency of certain repairing proteins (Rübe et al. 2011). The chromatin composition differs within the various spermatogenic cells, therefore the chromatin structure strongly influences DNA repair processes (Kinner et al. 2008; Goodarzi et al. 2008; Pandita and Richardson 2009) requiring different repair proteins and repair mechanisms to restore the integrity of their genome. In higher eukaryotes, the non-homologous end-joining (NHEJ) pathway predominantly repairs dsDNA breaks (González-Marín et al. 2012).

NHEJ repairing pathway starts with the recognition and binding of dsDNA breaks by the heterodimer Ku (Ku70 and Ku80) which then recruit the DNA-dependent protein kinase. Following, the meiotic recombination 11 (MRE11) complex is recruited, which induces the removal of non-ligatable termini by an inward translocation followed by replication by DNA polymerases and ligation to create compatible ends. Defects in this repair system predispose to cancer and immune deficiency syndromes (Gunes et al. 2015).

3.1.2. DNA Repairing In The Fertilized Oocyte And During Early Embryonic Stage

If the sperm DNA damage was not repaired during spermiogenesis or if the damage was induced by extrinsic factors, it can be successfully repaired during fertilization (Brandriff and Pedersen 1981; Ashwood-Smith and Edwards 1996). Within this process the pronucleus is formed, DNA is replicated and pronuclear fusion occurs, which leads to the formation of a zygote. Sperm DNA fragmentation can be repaired by the oocyte repair machinery before the first cleavage division. When the oocyte machinery cannot completely repair deletions or sequence errors, these mutations may be transferred to the offspring. Moreover, the embryo may fail to develop or implant in the uterus or may be aborted naturally at a later stage. Sperm genetic anomalies are in the origin of about 80% of the structural chromosome aberrations in human (González-Marín et al. 2012). The extent of DNA or chromatin damage influences the oocyte capacity to repair that damage (Genesca et al. 1992). Ahmadi and Ng (1999) argued that when the percentage of sperm damages is less than 8%, the female gamete is able to repair it.

There are also mechanisms of DNA repair in the newly fertilized embryo, however, it is thought that this entirely depends on the maternal mRNAs and proteins deposited and stored in the oocyte before ovulation. Therefore, the embryo mechanisms of DNA repair may be compromised by the oocyte if it was not adequately equipped (Jurisicova et al. 1998).

The efficiency of DNA repair at those early stages plays an important role since the cell cycle is much shorter in embryonic cells than in adult cells (Mac Auley et al. 1993), and for this reason, the integrity of the genome is at greater risk during embryonic development. The precise replication of the genome during S-phase is of fundamental importance especially in the zygote, where, for this reason, the repairing mechanism acts during this phase (Arnaudeau et al. 2001). Failures of this repairing mechanisms make the embryo more susceptible to sperm DNA fragmentation, which can affect the embryo development even after the implantation of embryo with a normal karyotype (Tesarik et al. 2004; Borini et al. 2006).

3.2. Treatment Strategies For Men With High Sperm DNA Damage Levels

The best way to increase the pregnancy success rates involves the development of methods to improve sperm DNA quality (Kim 2018).

3.2.1. Sperm Cell Selection

Methods like density gradient centrifugation and swim-up could help select sperm with better DNA integrity than native semen (Tarozzi et al. 2007). In order to minimize damage to the spermatozoa, some precautions should be considered during sperm preparation: slow dilution of the samples, especially when using cryopreserved spermatozoa; a gradual change in temperature and tests performed at 37°C; a minimal use of centrifugation, and when necessary, this should be carried out at the lowest possible speeds; and controlled exposure to potentially toxic materials. Furthermore, since plastic glassware and media could present toxic substances, tests should be done in order to check for potential toxicity to spermatozoa (Gardner et al. 2012).

Magnetic cell separation allows the differentiation between apoptotic and nonapoptotic spermatozoa (Said et al. 2006) exhibiting minimal DNA damage (Gardner et al. 2012).

3.2.2. Lifestyle Modifications

Numerous physical agents are responsible for causing sperm DNA damage: airborne pollutants, radiation and heat, cigarette smoke; and also chemical agents such as sexually transmitted infections and anticancer drugs. Elevated body max index, increased male age and diabetes are included on biological factors that influence the status of sperm chromatin (Pacey 2010). Changing these kinds of habits by doing exercise and control the weight, avoiding environments with high temperatures, quit smoking and drinking would help on improving sperm quality (Kim 2018).

3.2.3. Antioxidant Therapy

The administration of antioxidants before an ICSI procedure in cases of high ROS production has not demonstrated differences in the fertility rate, cleavage rate, or embryo development, but had higher clinical pregnancy and implantation rates (Greco et al. 2005). Some authors suggested that antioxidant therapy is only efficient for limited levels

of ROS and for this reason, many patients still maintain with sperm DNA damages even after treatment (Tremellen et al. 2007).

The addition of antioxidants supplements to quench or neutralize excessive ROS, protects spermatozoa against the OS induced by the cryopreservation process (Moubasher et al. 2013; Zribi et al. 2012; Breininger et al. 2005). Several antioxidants have been investigated.

a) Quercetin

Quercetin is a bioflavonoid characterized to present antioxidant and anti-inflammatory properties (Shaik et al. 2006; Amália et al. 2007) being capable of significantly improve the sperm motility and zona binding ability, and reduces DNA fragmentation in cryopreserved stallion sperm (Gibb et al. 2013).

b) Catalase

Catalase is a common enzyme present in nearly all living organisms. This enzyme is able to protect cells from OS through catalyzing the decomposition of H_2O_2 to water and oxygen (Chelikani et al. 2004). Moubasher et al. (2013) described an increase in the percentages of progressive motility and sperm vitality and a significant decrease in the percentage of DNA damage after adding catalase to the media before cryopreservation.

c) Resveratrol

Resveratrol has a protective effect against freeze induced sperm DNA damage (Branco et al. 2010). In both infertile and fertile men, this polyphenol demonstrated to be capable to prevent post-thaw lipid peroxidation, thereby preventing the damaging effect of cryopreservation on sperm concentration and morphology (Garcez et al. 2010). On the other hand, studies in animals have confirmed a protective effect for resveratrol on sperm DNA integrity, however they did not show a significant effect on semen parameters and OS measures (Silva et al. 2012; Bucak et al. 2015).

d) Selenium

Selenium ensures a normal testicular development and spermatogenesis exhibiting a protecting effect on sperm DNA against OS. Dorostkar et al. (2012) reported a significantly improved post-thaw sperm motility, viability, membrane integrity, and semen total antioxidant capacity as well as reduced SDF in water buffaloes sperm after administration of selenium.

e) Vitamin E

This is perhaps the most commonly utilized antioxidant in cryoprotective media. This antioxidant reduces lipid peroxidation initiated by ROS at the level of plasma membranes having beneficent effects on post-thaw motility and DNA integrity (Kalthur et al. 2011).

f) Vitamin C

Vitamin C is highly concentrated in seminal plasma (Jacob et al. 1992). This acid ascorbic provides sperm protection against OS by neutralization of hydroxyl and superoxide radicals and also the highly reactive hydrogen peroxide (Fraga et al. 1991). A dose-dependent positive effect of Vitamin C was observed, with optimal results in sperm parameters (Thiele et al. 1995; Dawson et al. 1992). Lower vitamin C levels and higher ROS levels were detected in the seminal plasma of asthenozoospermic men (Lewis et al. 2013).

g) Carnitine

This antioxidant is water-soluble and can prevent lipid peroxidation (Mongioi et al. 2016) and it is also considered to be a source of energy improving the sperm motility (Agarwal and Majzoub 2017).

h) Coenzyme Q10

Coenzyme Q10 is an essential antioxidant presenting in almost all body tissues. This antioxidant is abundant in sperm mitochondria where it is involved in the process of energy production (Nadjarzadeh et al. 2014; Lewin and Lavon 1997). Increased fertilization rates in the subsequent assisted reproductive cycle were detected after

coenzyme Q10 media supplementation (Agarwal and Majzoub 2017). Giacone et al. (2016) suggested an antioxidant formula containing zinc, D-aspartic acid, and Coenzyme Q10. The authors observed that the antioxidant formula was able to maintain sperm motility in normozoospermic men and improved it significantly in asthenozoospermic patients, also significantly reducing sperm lipid peroxidation level in both normozoospermic men and asthenozoospermic patients ($p < 0.05$) (Giacone et al. 2016).

i) Epicatechin (EPI)

Epicatechin is a flavonoid compound belonging to the family of catechins and can be extracted from cocoa, green tea, grapes, and chocolate (Arts et al. 2000; Botten et al. 2015). Catechins despite having a large absorbable capacity (Botten et al. 2015), also presents anti-thrombotic, anti-inflammatory, anti-cancer antibacterial and anti-ischemic properties (Botten et al. 2015; Shay et al. 2015). Additionally, EPI has powerful scavenger properties related to a wide spectrum of ROS, including superoxide and hydroxyl radicals (Shi et al. 2000).

Recently, Tvrda et al. (2019) suggested that EPI is able to prevent the decline of spermatozoa quality as a consequence of ferrous ascorbate or cryo-promoted oxidative damage. The authors observed that higher concentrations of epicatechin (50 µmol/L and 100 µmol/L) had a particularly protecting effect on bovine male gametes against modifications caused by ROS overproduction through the prevention of oxidative degeneration. This flavonoid demonstrated to preserve sperm motility and metabolic activity. More studies should be done but epicatechin administration could become a suitable option to sperm vitality preservation and could potentially be used as a treatment for sperm DNA fragmentation due to ROS overproduction (Tvrda et al. 2019).

Imamovic Kumalic and Pinter (2014) conclude that vitamin C and vitamin E have reducing power on DNA fragmentation as well as zinc and selenium, and a significant improvement in clinical pregnancy and implantation rates following ICSI (Greco et al. 2005) when administrated by orality. When administered orally, Coenzyme Q10 demonstrated an attenuating effect in OS in seminal plasma, improvement of semen parameters and also antioxidant enzymatic activity (Agarwal and Majzoub 2017). However, additional research is needed since there are not known standardized treatment guidelines for male infertility patients with high levels of oxidative damage (Kim 2018).

3.2.4. Varicocele Repair

Varicocele is a disease characterized by abnormal dilatation of the pampiniform plexus veins causing high ROS production (Zini and Dohle 2011), damaging the nuclear and mitochondrial DNA causing base modifications, strand breaks, and chromatin cross-links, as well as increasing sperm DNA damage (Cho et al. 2016). Surgical repair can be used to treatment of varicocele and there are reports of improved semen parameters after this kind of treatment. Also this method is more cost-effective than IVF/ICSI. Among the surgical methods, microsurgical varicocelectomy through the sub inguinal approach was proven to be the most effective method (Kimura and Nagao 2014).

3.2.5. Testicular Sperm

Testicular sperm was found to have three to five lower proportions of SDF than when compared to ejaculated DNA (Esteves et al. 2015), presenting 8.3% of DNA damage being known that there is a tendency for the former to underlie better DNA integrity. On the other hand, ejaculated DNA presented 40.9% of DNA damage. Better reproductive outcomes were shown in patients where testicular sperm extraction (TESE) was done following ICSI even though high levels of sperm DNA damage were present in this sample after antioxidant treatment. This group of patients had inclusively higher birth rate and lower miscarriage rate; therefore, testicular sperm had better outcomes (Esteves et al. 2015). Based on this data, using testicular sperm from an infertile man with sperm DNA abnormalities might be more effective for performing ART cycles (Esteves 2018).

CHAPTER IV Sperm DNA Damage And Assisted Reproductive Technology

4.1. Assisted Reproductive Technology (ART)

The number of treatments with assisted reproduction increased during the last years due to the nowadays demographic tendency towards delaying the time of childbirth later in life (Nieschlag et al. 2010). ART have witnessed major advancements that allowed a good number of infertile couples to successfully conceive. However, only 35% of couples attempting ART get a live-birth delivery (Brezina and Zhao 2012).

The rapid development of assisted reproductive techniques to overcome the reproductive obstacles in conceiving a child has reduced the effort among clinicians to properly investigate, diagnose and treat the causes of male infertility (Culley et al. 2012). (Agarwal and Majzoub 2017).

The existence of a proper and trustful diagnosis (Aitken et al. 2004) is of most importance, since inadequate evaluation of the causes of male infertility results in a common situation in which the female partner is exposed to invasive, stressful and expensive ART procedures (Punab et al. 2016).

4.1.1. Oxidative Stress In ART Setting

Oxidative stress from both male and female gametes affects the ART clinical outcome (Agarwal and Majzoub 2017). ROS concentration in seminal plasma is negatively correlated with the fertilization rate in both IVF/ICSI procedures. The oxidant status showed to be an important factor during ART being crucial to a successful pregnancy (Agarwal and Majzoub 2017).

Sperm DNA integrity is threatened in specific occupational and environmental conditions that can lead to abnormal reproductive outcomes by changing the male genetic composition at either small scale (DNA regions) or even at larger scale (entire chromosomes) (Wyrobek 1993).The ROS production cannot be completely avoided, however, strategies have been developed to minimize their detrimental effects on ART outcomes. These strategies include *in vitro* use of antioxidants in the clinical laboratory setting during ART procedures with constant evolvement and optimization of the laboratory environment and techniques. A proper diagnosis and treatment of patients with antioxidants will just be possible with the discovery of the normal redox potential in the male reproductive tract and semen. Researchers at the Cleveland Clinic's American Center for Reproductive Medicine have proposed an oxidation-reduction potential assay (ORP) that allows for differentiating between normal men from male factor infertility patients by measuring OS. ORP identifies the OS present in a given semen sample,

providing a functional component not contained within the semen analysis. Detecting abnormal levels of OS can enhance clinical understanding related to poor sperm function and help optimize treatment strategies for male factor in fertility (Agarwal et al. 2016; Agarwal et al. 2016; Agarwal et al. 2017; Agarwal and Wang 2017).

4.1.1.1. Male Sources Of ROS In Assisted Reproduction Setting

During ART procedures, ROS can be originated endogenously from gamete or by exogenous environment factors (Du Plessis et al. 2008).

a) Endogenous Male Sources Of Reactive Oxygen Species

1) Immature Spermatozoa

An incomplete extrusion of cell cytoplasm during spermiogenesis may be in the origin of the development of immature sperm (Tanphaichitr et al. 2015; Gil-Guzman et al. 2001). Residual cytoplasm in sperm cells leads to excessive ROS generation (Aitken and Baker 2002) due to defects in sperm mitochondria which are associated with increased ROS production (Schatten et al. 2014) further promoting damages to sperm mitochondrial membranes triggering a vicious cycle of ROS production and mitochondrial dysfunction (Agarwal and Majzoub 2017).

2) Leukocytes

Leukocytospermia or increased semen leukocytes is associated with overproduction of ROS and consequently OS, possibly due to disturbs in the mitochondrial membrane potential (Mupfiga et al. 2013). This condition can be a consequence of infection of the prostate or seminal vesicles (Sandoval et al. 2013) and can also result from the removal of seminal plasma during the preparation of ART methods. Moreover, seminal leukocyte levels were inversely related to normal sperm morphology, motility and sperm concentration (Henkel et al. 2005).

b) Exogenous Sources Of Reactive Oxygen Species

1) Oxygen Concentration

Any aerobic environment promotes enzyme activity and release of ROS. Bavister (2004) agrees with this affirmation by arguing that embryos development in a medium at atmospheric oxygen concentration showed significantly higher levels of DNA fragmentation than embryos cultured in an environment of low oxygen tension. Kasterstein et al. (2013) reported that rats embryos when exposed to 5% O_2 concentrations showed significantly better blastomeres, top-quality embryos on day 3, implantation, pregnancy rates, and live birth than when exposed to 20% O_2 concentrations.

2) Culture Media

The composition of the media used during the culture of human oocytes and embryos influences the embryo quality, and consequently the ART success (Agarwal et al. 2006). The presence of metallic ions, such as iron or copper in the culture medium may lead to increased ROS generation (Guerin et al. 2001), fragmenting the DNA and affecting fertilization, blastocyst development, and cleavage (Bedaiwy et al. 2004). Iron chelator decreases lipid peroxidation and formation of ROS. Therefore, adding metal chelators to culture media may be a potential solution to reduce the ability of metals to react and produce ROS in the in vitro setting (Nasr-Esfahani and Johnson 1992). Alteration in pH and temperature in the culture media can disrupt the cellular homeostasis affecting protein synthesis, mitochondrial function, cellular metabolism and cytoskeletal regulation. pH variations can affect sperm motility, oocyte maturation, and embryo development (Will et al. 2011). Temperature maintenance is also important since increasing temperature can decrease pH levels (Ferguson et al. 1980) and cause ROS-induced cellular damage (Larkindale and Knight 2002).

3) Centrifugation

Centrifugation is routinely performed during sperm preparation to remove the seminal plasma and other constituents that can potentially be in the origin of ROS, such as immature sperm and dead cells (Shekarriz et al. 1995). However, longer centrifugation time affects the spermatozoa quality by exposing them to higher temperatures (Henkel and Schill 2003).

4) Effects Of Cryopreservation

Cryopreservation is a procedure that allows the preservation of cells, including male and female gametes, small multicellular organisms, and even more complex organisms such as embryos, at low temperatures (Di Santo et al. 2012). This technique plays an important role in cases of preservation of male fertility before radiotherapy or chemotherapy (Sanger et al. 1992), giving these couples the chance of conceiving children in the future and also in patients that are exposed to potentially toxic agents which may interfere with gametogenesis (Fabbri et al. 2004). For cases where patients have undergone testicular sperm extraction or percutaneous epididymal sperm aspiration, cryopreservation can be also used to avoid repeated biopsies or aspirations (Donnelly et al. 2001).

During this cryopreservation process, DNA damage can be induced due to reactive oxygen species (Said et al. 2010; Thomson et al. 2009; Lasso et al. 1994). By controlling the status of the sample in respect to the levels of reactive oxygen species (Ahmad et al. 2010; Kalthur et al. 2008; Donnelly et al. 2001), the technique used for cryopreservation or the cryoprotectant applied can reduce the risk of sperm DNA damages (Di Santo et al. 2012).

Several authors argued that cryopreservation decreases sperm motility, vitality and morphology (Satirapod et al. 2012; Lee et al. 2012; Di Santo et al. 2012; Thomson et al. 2010) and others did not detect any effect of cryopreservation (Isachenko et al. 2004; Duru et al. 2001; Høst et al. 1999). However, it is necessary to note that those cryopreservation studies have been performed with different techniques (García-Peiró et al. 2011). In 2014, Ribas-Maynou et al. detected a 10% single-stranded sperm DNA fragmentation (ssSDF) increased when using the comet assay. SsSDF is related to oxidative damage (Enciso et al. 2009; Ribas-Maynou et al. 2012). Therefore, these results are in agreement with the consideration that oxidative stress would be the main effector of DNA damage during cryopreservation (Mazzilli et al. 1995; Thomson et al. 2009). Breaks on dsDNA were not found by the comet assay. The increased ssSDF might have a clinical effect on pregnancy achievement, but the lack of double-strand sperm DNA fragmentation (dsSDF) would not generate an increase in the miscarriage risk (Ribas-Maynou et al. 2012).

4.2. Techniques To Evaluate Sperm DNA Damages

The standard semen analysis fails at describing the status of the paternal genetic material (Wyrobek 1993) and for this reason, several techniques to evaluate sperm chromatin quality and DNA fragmentation have been explored (Agarwal and Said 2003; Evenson et al. 2002).

The application of SDF assays is not easy due to several factors; the true nature of sperm DNA damage is not completely understood; the accuracy of SDF tests depends on the precision of the technique. Additionally, thresholds of many of the developed tests are not known yet, and the inter-laboratory variability that exists affects the consistency of test results. SDF results are influenced by laboratory or clinical conditions and also by the degree of sperm nuclear decondensation or the ejaculation abstinence period (Agarwal et al. 2016; Agarwal et al. 2016).

The predictive value of DNA fragmentation tests is influenced by many factors, such as: which test is used to analyze DNA fragmentation; the cause of the DNA damage; the extent of DNA fragmentation per cell; and the effect of SDF in introns or exons having different effects (Alvarez 2005).

There are two different methods to measure SDF: a) direct methods: methods that detect ss- and dsDNA breaks, such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and Comet assay; b) indirect methods: methods that measure the sensitivity of sperm DNA to denaturation under specific condition, such as the CMA3), sperm chromatin structure assay (SCSA), sperm chromatin dispersion (SCD), and staining of nuclear proteins including aniline blue (AB), toluidine blue (TB), acridine orange (AO) and Diff-Quik (Pourmasumi et al. 2019).

4.2.1. TUNEL Assay

TUNEL assay is characterized for recognizing “nicks” or free ends of DNA by applying fluorescent nucleotides (Sun et al 1997). The assessment of the samples can be done by using a flow cytometer or a standard fluorescence microscope. The *“*nicks*”* are detected and quantified due to incorporation of dUTP into ss- or dsDNA breaks through an enzymatic reaction, which creates a signal that increases with the number of DNA breaks (Agarwal et al. 2016; Rahiminia et al. 2018). On Figure 4 it can be seen a more intense fluorescence on sperm cells which have a higher number of “nicks” (Tunel (+)) when compared with the ones with low percentage of “nicks” (Tunel (-)).

In order to allow access to all *"nicks"*, it is used dithiothreitol before fixation to relax the entire chromatin structure; this alteration of the technique had an important role in reducing inter-laboratory discrepancies (Mitchell et al. 2011).

The concentration of ROS contributes to an increase in the number of TUNEL positive cells (Sergerie et al. 2005) as shown by Ramos and Wetzels (2001).

It was determined a threshold value for TUNEL assay of 20% to distinguish between fertile men and infertile patients (Sergerie et al. 2005).

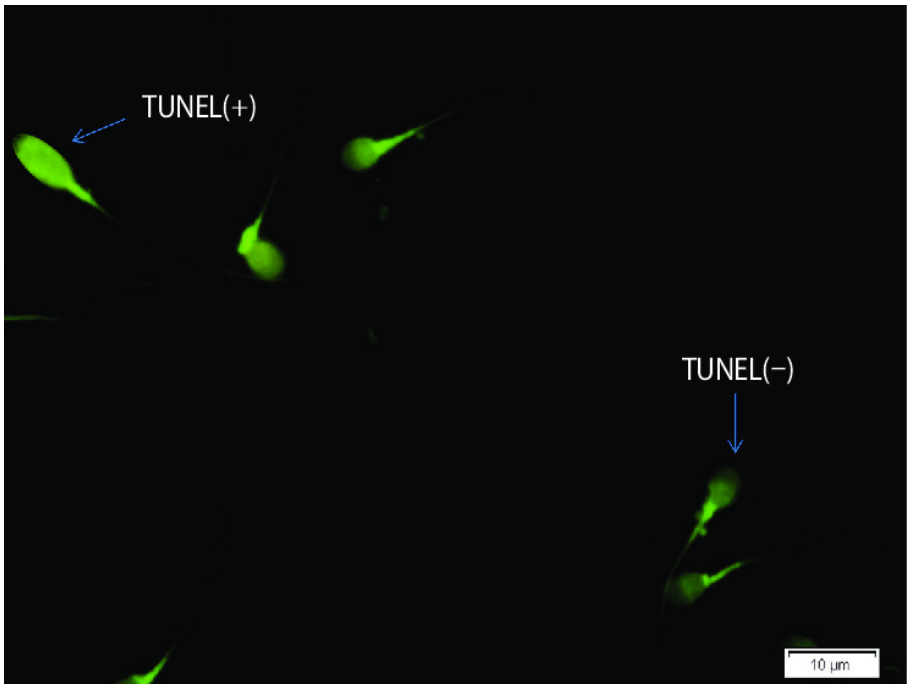

Figure 4: Sperm DNA fragmentation detected by using the TUNEL assay. Spermatozoa with fragmented DNA are identified as "TUNEL (+)" and "TUNEL (-)" are spermatozoa with an absence of fragmented DNA. Retrieved from Rahiminia et al. 2018.

4.2.2. Comet Assay

The comet assay (Singh et al. 1988) is an accurate and specific technique (Sipinen et al. 2010) that allows the quantification of the amount of DNA damage per spermatozoon. The assay consists in the migration of fragmented DNA of lysed cells under electrophoretic conditions in an agarose matrix. When observed under a microscope, the mass of DNA fragments, that comes from the sperm head migrating in the direction of the anode, has the form of a 'comet' tail (Hartmann et al. 2003) as it can be observed in Figure 5a and compared with Figure 5b in which is represented the result

of a comet assay when the sperm DNA is normal. The comet tail is stained, detecting DNA strand breaks and alkali-labile sites (Pereira et al. 2017); the staining intensity and length of the comet tail are directly related with the quantity of migrated DNA (Singh et al. 1988). Additionally to its capacity of identifying ss- and dsDNA breaks, this technique can also detect altered nucleotide bases (Agarwal et al. 2016).

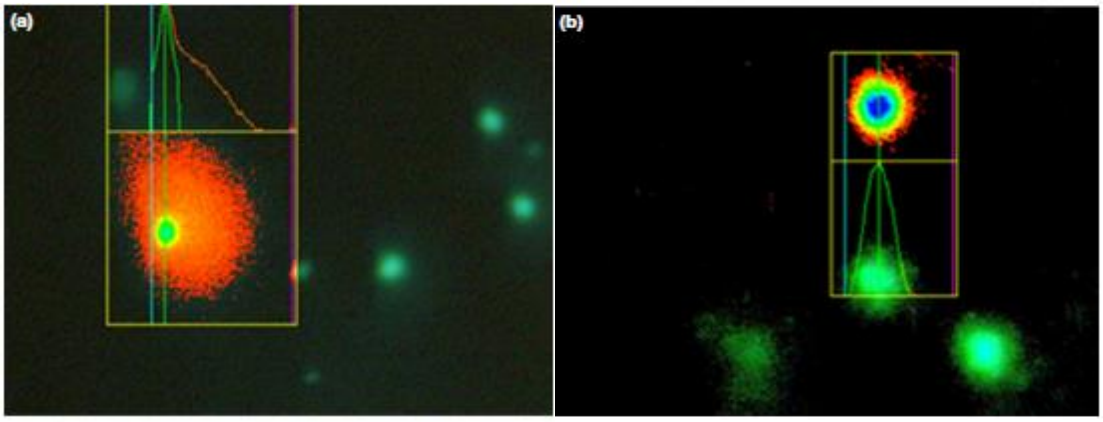

Figure 5: Comet assay of sperm samples using the software COMET IV®. (a) Sperm DNA is fragmented. (b) The sperm DNA is normal. Retrieved from Pereira et al. 2017.

Different types of DNA damages (Collins et al. 1993; Dusinska and Collins 1996) can be analyzed through neutral and alkaline electrophoresis; it can be applied in patients with severe oligozoospermia (Lewis et al. 2013); and it also indicates the extension of the damages (Pereira et al. 2017). However, this assay is time-consuming and to analyze the outcomes it is necessary highly specialized personnel (Agarwal et al. 2016).

4.2.3. CMA3 Staining

Decreased protamine expression is relatively common in infertile men and it seems to be related with decreased sperm counts, poor sperm motility, sperm dysmorphology, and low sperm penetration assay scores (Aoki et al. 2005; Carrell and Liu 2001; Aoki and Carrell 2003). Selecting spermatozoa with normal protamine levels for use in ART is important to ensure decreased imprinting errors in offspring (Allen and Reardon 2005). In order to assess the sperm protamine, CMA3 staining can be applied (Zandemami et al. 2012).

CMA3 is a fluorochrome and it is often used in sperm quality evaluation because it competes with the protamines for binding to the minor groove of DNA, detecting protamine deficiency in loosely packed chromatin which is correlated to the extent of nicked DNA (Kazerooni et al. 2009). In this technique the spermatozoa are fixed in methanol/glacial acetic, and then are treated with the CMA3 solution and observed with a fluorescence microscope. Abnormal chromatin packing is identified by the bright green fluorescence of the sperm head and normal chromatin packing with dull green staining (Figure 6). The decline in bright green staining in spermatozoa is attributed to the increase in protamine composition and to the high DNA packaging form, which restricted the reactive sites available (Lolis et al. 1996).

CMA3 is strongly correlated with other assays, presenting reliable results (Agarwal et al. 2016).

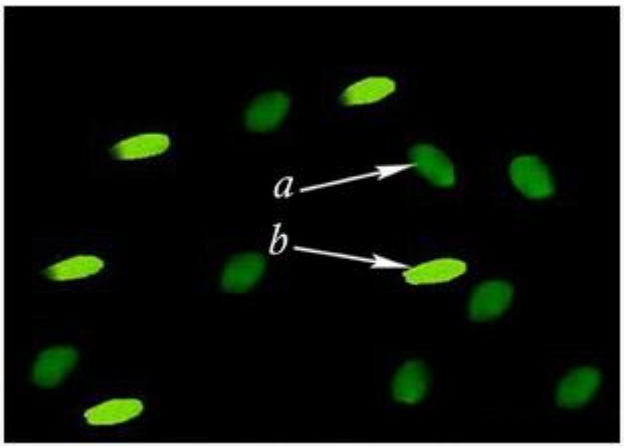

Figure 6: Chromomycin A3 staining of sperm chromatin illustrating: (a) normal spermatozoa and (b) spermatozoa with protamin deficiency. Retrieved from Kazerooni et al. 2009.

4.2.4. SCSA

Sperm chromatin structure assay is a flow cytometric technique that allows rapid identification of: the DNA fragmentation index (DFI); the percentage of cells showing denatured DNA; and percentage of cells with high DNA stainability (HDS, cells with defective chromatin condensation) in sperm samples (Vozdova et al. 2012).

The principle basis of SCSA consists in the exposition of sperm DNA to heat and acids using a flow cytometer in order to test the susceptibility of sperm DNA to denaturation (Lewis et al. 2013). The shift from green fluorescence to red fluorescence indicates the extent of DNA denaturation (Darżynkiewicz et al. 1975). Samples can

present up to 30% of DFI and 15% of HDS and still be considered normal (Larson-Cook et al. 2003; Kennedy et al. 2011).

The SCSA has, in comparison with traditional metrics of semen quality, an advantage of minimizing within-person variability and intra/inter-assay variation due to the existence of a standardized protocol for all users (Evenson et al. 1991; Evenson et al. 1999; Giwercman et al. 1999; Zini et al. 2001). The high efficiency of this assay has been demonstrated in toxicological and environmental studies and is considered a powerful tool in the clinical evaluation of the infertile male (Richthoff et al. 2002). The implementation of this assay to clinical andrology laboratories is limited to the requirement of a flow cytometer that displays high costs (Sergerie et al. 2005).

4.2.5. SCD Test

During this test, the sperm are exposed to a denaturing solution to eliminate nuclear proteins showing the damaged DNA. The main principle of SCD is that fragmented DNA does not produce a halo of dispersed DNA loops around the sperm nucleus (Muriel et al. 2005), contrarily to spermatozoa without DNA damage (Vandekerckhove et al. 2016; Agarwal et al. 2016). On Figure 7 it can be observed the different halos created when there is fragmentation of the DNA and when the integrity of DNA is not damaged.

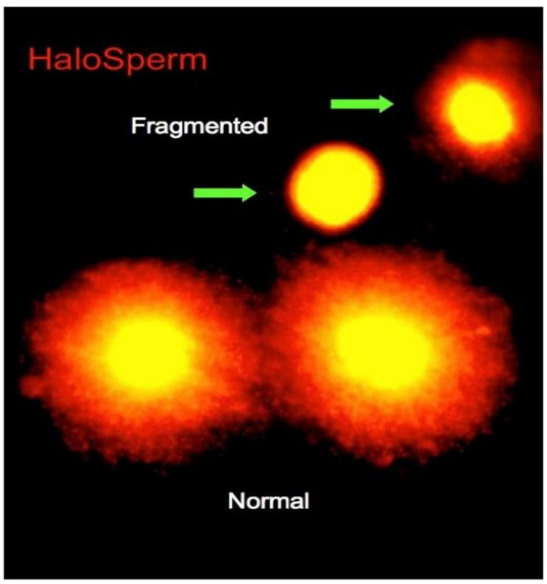

Figure 7: Sperm chromatin dispersion test of sperm samples. Sperm with fragmented DNA are pointed with arrows. Retrieved from Vandekerckhove et al. 2016.

The observation of the characteristic halo can be done by a bright-field microscope after staining with eosin and azure B solution, or by fluoresce microscope if there are used DNA-directed fluorochromes. A good categorization of the halos is important to correct SDF quantification, however this analysis is subjected to some inter-observer subjectivity (Agarwal et al. 2016). Fernández et al. (2005) determined a strong correlation between SCD test results and those from the SCSA confirming the validity of SCD assay.

4.2.6. AO Test

AO is a nucleic acid-selective cationic fluorescent dye that interacts with ss- and dsDNA by intercalation or electrostatic attraction, respectively. Following mild acid denaturation of sperm DNA, if it interacts with dsDNA (i.e., non-denatured) a green fluorescence is emitted as it is represented in Figure 8. However, if the interaction was with ssDNA (i.e., denatured), the excitation shifts to blue or red fluorescence. The red fluorescence is emitted when the sperm DNA damage is extensive (Figure 8) (Agarwal et al. 2016).

AO is an inexpensive, simple and fast test. However, the inter-laboratory variety with lack of reproducibility and the presence of heterogeneous slide staining with multiple intermediate colors make AO a less reliable test of SDF (Chohan et al. 2006; Shamsi et al. 2011).

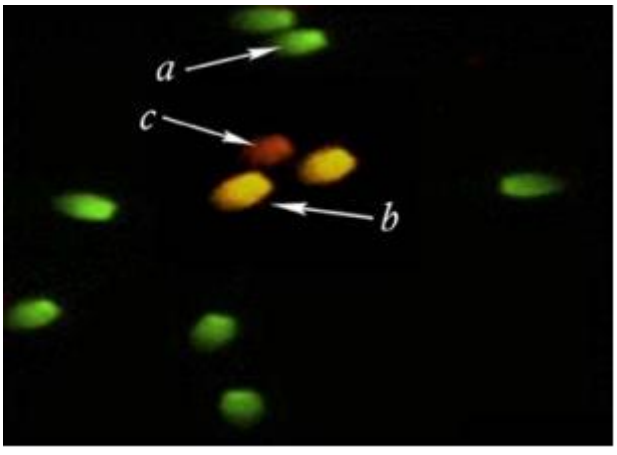

Figure 8: Acridine orange staining of sperm chromatin showing (a) normal, (b) moderately denaturated and (c) completely denaturated spermatozoa. Retrieved from Kazerooni et al. 2009.

4.2.7. AB Staining

AB staining is an acidic dye that interacts with histones in the nucleus of immature sperm, which stain blue (Auger et al. 1990). As this dye does not have an affinity with the protamine-rich nuclei of mature spermatozoa there is a negative reaction and it remains unstained (Figure 9). Increased AB staining of sperm indicates poor chromatin packing (Agarwal et al. 2016).

AB staining is characterized for being simple and inexpensive requiring a simple bright field microscope for analysis. While the results of AB staining correlate well with those of the AO test (Erenpreiss et al. 2001), heterogenous slide staining remains a prominent drawback of this technique (Agarwal et al. 2016).

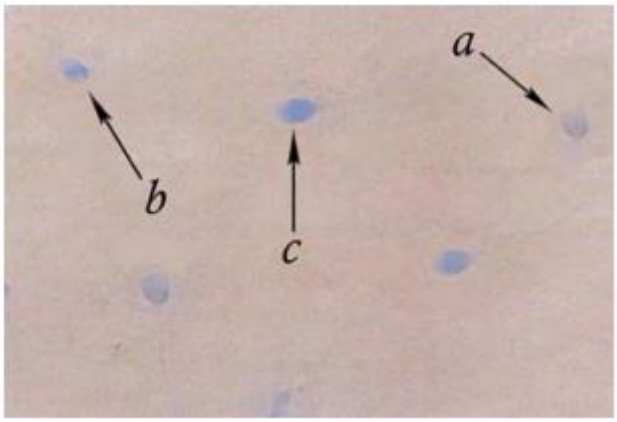

Figure 9: Aniline blue staining of sperm chromatin representing: (a) normal spermatozoa; (b) spermatozoa with a moderate level of remaining histones (c) and spermatozoa with remained histones. Retrieved from Kazerooni et al. 2009.

4.2.8. TB Test

Toluidine blue is a basic external thiazine metachromatic dye. External staining by TB is sensitive to the degree of DNA–protein binding and packaging regularity and therefore to DNA integrity. When in the presence of damaged chromatin this dye is incorporated producing a violet-blue intense coloration. However, in the presence of intact chromatin, the sperm nuclei present a pale blue stained. The results can be observed by using an ordinary microscope. However, the inter-observer variability is increased by intermediate coloration. Toluidine blue staining is characterized as rapid, simple, inexpensive and generally correlates well with other methods of sperm DNA testing (Erenpreiss et al. 2004).

4.2.9. Diff-Quik Kit

The usage of the techniques previously described is limited by elaborate protocols involving reagents and equipment (e.g. fluorescence microscopy and flow cytometer) which normally are not present on regular andrology laboratories. Furthermore, these techniques are time-consuming (Sousa et al. 2009). Additionally, it is difficult to assess the degree of the defects using several assays due to the particular compacted nature of sperm chromatin. It remains difficult to know what is evaluated by each assay (Sousa et al. 2009).

Mota and Ramalho-Santos (2006) have developed a simple and fast method to monitor sperm DNA status under field conditions: the Diff-Quik, which is often used in andrology laboratories to assess sperm morphology. During the analysis it is important to pay attention to the intensity of nuclear staining: normal sperm heads/ nuclei stain lightly, whereas heads/nuclei with fragmented/damaged DNA present a darker stain. Methanol is usually used to an efficient fixation, then the sample is sequentially exposed to eosin, an anionic/acid dye that stains positively charged/basic proteins red, and a thiazine dye (usually is Methylene Blue or its oxidation products, such as Azure B; or even a mix of several thiazines). Thiazine will stain DNA blue. This technique is easy, reproductible and routinely can indicate the status of the chromatin in human sperm (Sousa et al. 2009).

TUNEL and Diff-Quik assay were shown to be highly correlated. SDF was evaluated with both methods applied to the same samples. The authors verified similar proportion between TUNEL-positive cells and dark stained spermatozoa in those samples. The results are represented in Figure 10. When exposing male gametes to conditions that promote DNA fragmentation and chromatin decondensation *in vitro*, a significant increase in spermatozoa with dark nuclei was verified (Tavares et al. 2013).

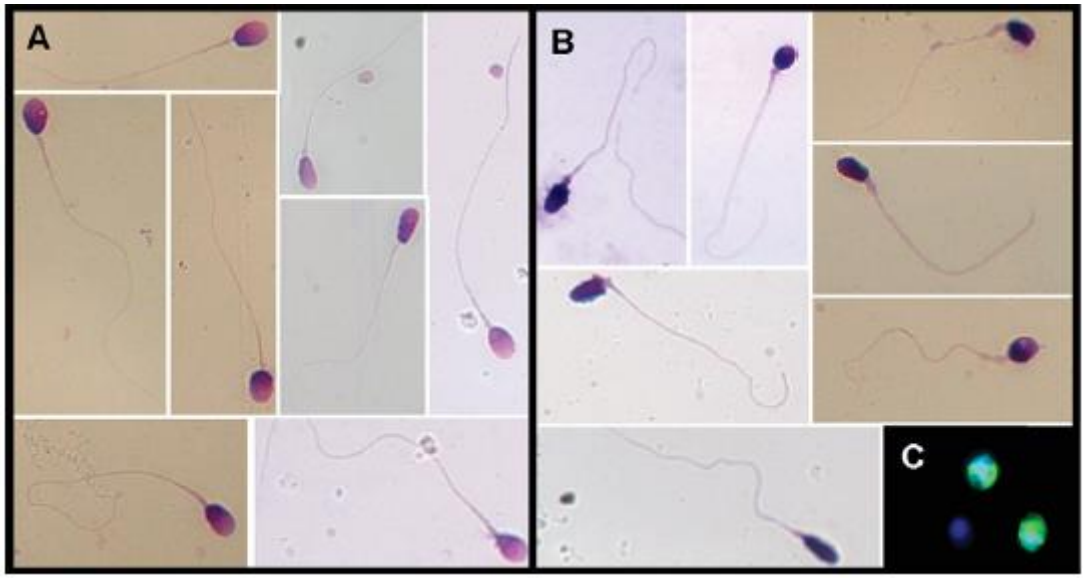

Figure 10: Evaluation of sperm chromatin. Images A and B were obtained by using the Diff-Quik assay: A) the spermatozoa presents normal staining patterns; B) the spermatozoa have abnormal dark staining heads. C) TUNEL assay, observed by fluorescence microscopy: sperm with DNA damage (TUNEL-positive sperm) are colored with green fluorescence, and sperm with intact DNA (TUNEL-negative sperm) present blue DAPI counterstain. Retrieved from Sousa et al. 2009.

The Diff-Quik method, as well as other assays, indicated a negative correlation between the abnormal chromatin status in native spermatozoa and embryo development rate, a relation among higher levels and lower quality embryos and negative clinical pregnancies among ART couples (Sousa et al. 2009).

CHAPTER V: Conclusion And Future Prospectives

The specialist health care to address male reproductive problems remains inadequate even though paternal genetic contribution through spermatozoa is vital for the offspring's health and development (Aitken et al. 2009). The male infertility diagnosis is based on semen analysis. However, whenever the results of these analyses are irregular, the patient is quickly guided to assisted reproductive procedures without additional tests to identify potentially reversible causes for the suboptimal result (Borini et al. 2006). High sperm DNA fragmentation rates are related to low pregnancy success and miscarriage and also affect embryo development even when using ART (Agarwal et al. 2016). The best way to increase the pregnancy success rates involves the development of methods to reduce sperm DNA damages, like antioxidant-therapy (Kim 2018). However, since the sedentary way of living can have serious reproductive impacts, changing some life-habits like smoking, doing more exercise, and reducing the alcohol and drugs consumption can improve the reproductive outcomes.

It is known that a major part of the existing methods to evaluate sperm DNA status require extensive protocols and/or expensive reagents and equipment (Tavares et al. 2013). However, there are efficient, low cost and low time-consuming techniques, such as the modified Diff-Quik staining method, that proved to have results consistent with the ones from TUNEL assay, which is one of the most efficient techniques (Sousa et al. 2009).

The application of the Diff-Quick technique to sperm DNA damage assay may be considered, since it can overcome the previously described limitations of the normally used methods contributing to a more complete male infertility diagnosis.

Also, the treatment strategies for male factor in fertility could be optimized by measuring the abnormal levels of ROS in the sample. The oxidation-reduction potential assay proposed by the Researchers at the Cleveland Clinic's American Center for Reproductive Medicine can be crucial to enhance clinical understanding related to poor sperm function (Agarwal et al. 2016; Agarwal et al. 2016; Agarwal et al. 2017; Agarwal and Wang 2017) and for this reason, more studies need to be done in order to develop this OS measurement assay to make possible its application to men presenting sperm DNA abnormalities.

Furthermore, controlling the ART setting conditions is necessary in order to ensure that the results are the less possible affected (Agarwal et al. 2016). Sperm nuclear DNA integrity is commonly affected during ART procedures which means that more efforts must continue to be done by ART technicians to minimize the adverse effects that may compromise the offsprins.

CHAPTER VI Bibliography

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