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The role of oxidative stress on cells in spermiogenesis

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SUMMARY

An increasing trend in parental age has been observed, together with this increase there is a growing risk for perinatal problems. One theory for this growing risk is the free radical theory. This states that there is a build-up of oxidative stress due to free radicals as humans age. This can have serious effects on male fertility, the main cause for male infertility is impaired spermatogenesis. Spermatogenesis is a cell differentiation process by which spermatogonial stem cells develop and differentiate into mature spermatozoa [8]. This complex process is disturbed by various factors, the main factor being oxidative stress [1,3]. For this review, literature has been researched in order to provide an overview on the role of oxidative stress on the spermatogenesis process with a focus on spermiogenesis and how oxidative stress can be measured with a novel technique, diamond magnetometry.

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1. INTRODUCTION

Worldwide, about 15% of couples of reproductive age experience infertility. In approximately 50% of these infertility cases, the male factor is the main contributing cause [1,2,3]. Male infertility is defined as the inability of a male to make a fertile female pregnant over a period of at least one year of unprotected intercourse [4]. About 1 in 20 men of reproductive age are affected by infertility. Spermatogenesis is the process where spermatozoa develop from germ cells in the testis, which under normal circumstances leads to fertile sperm. There can be several causes for impaired spermatogenesis, which is the main factor causing male infertility. Age, medications, genetic problems, systemic diseases, temperature, and environmental toxins are all factors that may have an effect on male fertility [1,3]. One of the main factors producing infertility in men is oxidative stress.

1.1 Oxidative stress and male fertility

The concept of oxidative stress (OS) was firstly defined in 1985 by Helmut Sies and defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and/or molecular damage" [18]. Oxidants include reactive oxygen species (ROS), among ROS highly reactive free radicals such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), peroxyl (•ROO), and hydroxyl (•OH) are responsible for creating an imbalance [5,11]. A small amount of ROS is necessary for the physiological function of sperm, including capacitation, hyperactivation, acrosomal reaction, and sperm-oocyte fusion [3,6,7]. However, when ROS levels increase above a certain threshold it may affect the quality of the male germ cells, resulting in defective spermatozoa. High levels of ROS neutralize antioxidants in the seminal plasma and cause oxidative stress (OS), which is the main cause of infertility in 30-80% of infertile men [5]. OS in spermatozoa can produce lipid peroxidation leading to sperm apoptosis, also DNA fragmentation in the nucleus and mitochondria are the result of high ROS levels. Spermatogenic cells or germ cells undergo division at a high rate and contain plenty of unsaturated fatty acids, which makes them highly susceptible to OS. Throughout the spermatogenesis process, ROS affect different epigenetic mechanisms, which can ultimately have a deleterious effect on spermatozoa but also a negative effect on the next generation.

1.2 Oxidative stress and spermatogenesis

Spermatogenesis is a process that takes approximately 74 days in humans and 35 days in mice. As mentioned before impaired spermatogenesis is the main contributor to male infertility. To get a better understanding of why this is the case a clear overview is needed of the entire process. Spermatogenesis starts with spermatogonial stem cells (SSCs) and ends with spermatozoa [8]. Spermatogenesis includes three different functional phases that take place inside testicular seminiferous tubules. These phases are mitosis, meiosis, and spermiogenesis. In the testicular tissue, Sertoli cells, germ cells, epithelial tubular cells, and the blood-testis barrier establish a controlled environment to guarantee that a highly complex series of molecular events are carried out, resulting in successful spermatogenesis. [8,9,10]. The mitotic phase includes undifferentiated spermatogonia that have the ability of self-renewal by mitosis and differentiating spermatogonia that are committed to enter the meiosis phase. Following the spermatogenesis process, differentiating spermatogonia become preleptotene spermatocytes. These cells require close contact with Sertoli cells and need to be transported across the blood-testis barrier. After crossing the barrier, preleptotenes differentiate into leptotene, then zygotene, pachytene, and finally diplotene spermatocytes, which are the four cell types characteristic of the long meiotic prophase I [8]. Diplotene spermatocytes undergo the first meiotic division to produce secondary spermatocytes. Secondary spermatocytes undergo the second meiotic division without previous replication of the DNA, resulting in haploid round spermatids [10]. After this, spermiogenesis starts, the process in which round spermatids will transform into spermatozoa. This is the final cell type of the spermatogenesis process. Spermatozoa are highly specialized cells capable of movement but still require a maturation process in the epididymis after being released inside the lumen of seminiferous tubules.

2. SPERMATOGENESIS AND SPERMIOGENESIS

The process of spermatogenesis occurs inside seminiferous tubules that are located in the testicular capsule. These tubules have a diameter of 150-200 um and show a uniform tissue organization [30]. This organization is predominantly based on Sertoli cells, which are highly specialized somatic cells with basal and luminal regions delimited by tight junctions. These junctions prevent the passage of proteins between the two

compartments and thus create the blood-testis barrier. The Sertoli cells form a sheetlike structure that nourishes all the stages of spermatogenic cells, this is called the seminiferous epithelium. Within this seminiferous epithelium the process of spermatogenesis takes place [31]. SSCs are located in the basal compartment of the seminiferous epithelium, this is the part that is located between the basement membrane and the junctional complex [8,31]. After going through the different phases of spermatogenesis, SSCs finally become immature spermatozoa that are released in the luminal region of the epithelium (Figure 1)[8,9,10].

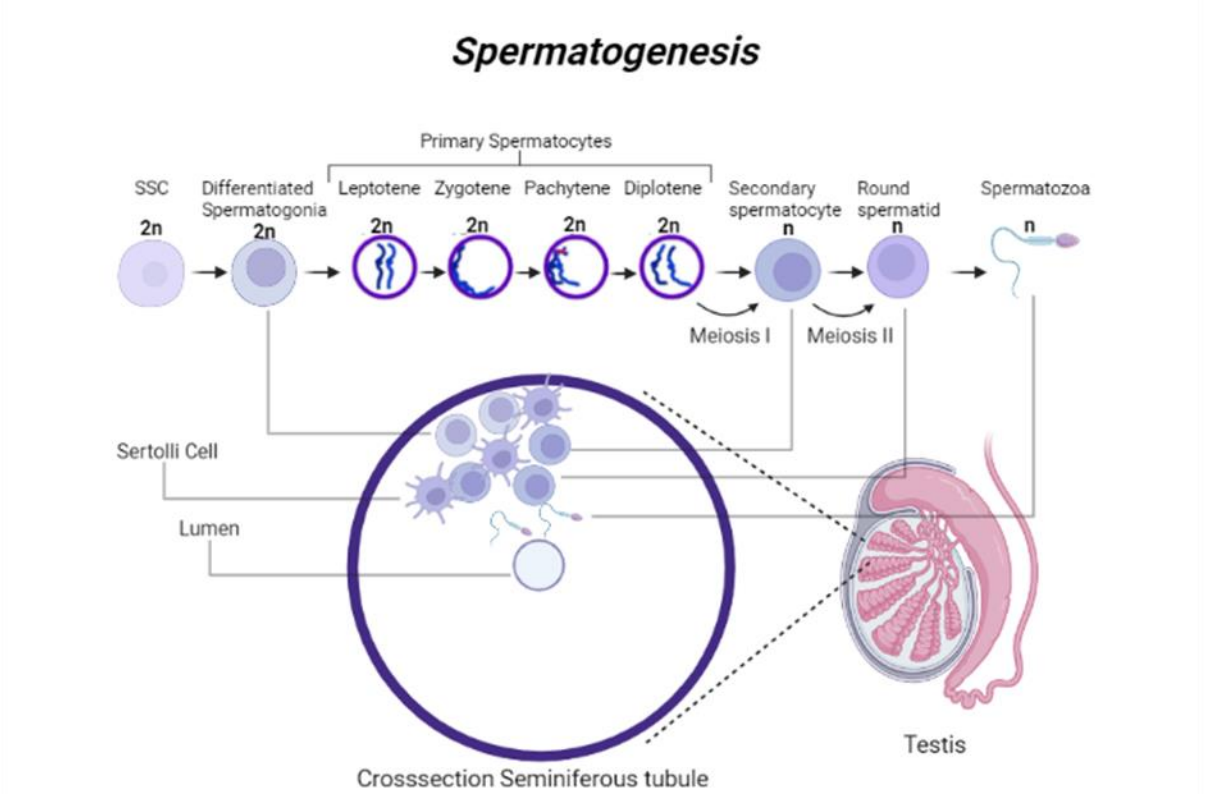

Figure 1. Overview of the different spermatogenesis stages and their organization within the seminiferous tubules.

At the beginning of spermatogenesis, there are two different ligands that either promote differentiation or self-renewal of SSC. The self-renewal promoting ligands are GDNF (Glial Cell-Derived Neurotrophic Factor) and FGF (Fibroblasts Growth Factors). These ligands are scattered over a wide area where SSCs and their differentiating progenies are located. The ligands that promote differentiation are Wnt (Wingless/Integrated) and RA (Retinoic Acid). An interesting phenomenon is that the levels of these ligands fluctuate with different stages of spermatogenesis [33].

The next step of spermatogenesis is the first meiotic division, in order to enter prophase I spermatogonia must duplicate their genetic material. This meiotic division, in the end, gives rise to secondary spermatocytes. Preleptotene spermatocytes are cells entering meiosis, these cells require close contact with Sertoli cells and the integrity of the epithelium to be transported across the blood-testis-barrier to differentiate into leptotene, zygotene, and pachytene spermatocytes.[8] The first step of meiosis is prophase I. During prophase I, primary spermatocytes can be divided into four different stages. At the beginning of prophase I, double-strand breaks occur to provide later the spots for genetic exchange between parental chromosomes. This leads to the first primary spermatocyte stage, the leptotene. After this, the pairing of homolog chromosomes occurs leading to the second primary spermatocyte stage, the zygotene. The third stage is the pachytene, where genetic recombination occurs by the exchange of genetic material between homologs. The final stage of prophase I is the result of homologous chromosome separation except at chiasmata, resulting in the diplotenes. Diplotenes then undergo the first meiotic division to produce diploid secondary spermatocytes. Secondary spermatocytes undergo the second meiotic division to produce haploid round spermatids since this division occurs without previous duplication of the DNA [10].

The final phase of spermatogenesis is spermiogenesis, during this phase round spermatids differentiate into spermatozoa. This phase involves important processes to produce cells that are able to pass genetic information to the next generation. These processes include acrosome and midpiece formation, DNA packaging with protamines, and flagella organization [8]. No further cell division takes place when spermatids undergo their complex cytodifferentiation, which results in elongated spermatids. Finally, elongated spermatids will be released from the seminiferous epithelium as immature spermatozoa through a process called spermiation [34]. The spermiogenesis process can be divided into different phases that are distinguished by the morphology of the developing acrosome and the shape of the nucleus. At the beginning of spermiogenesis, round spermatids have a spherical, central nucleus. Also the formation of the acrosome and the axoneme begins; these are necessary structures for fertilization and motility, respectively [34]. In the second phase, the nucleus and acrosome polarize to one side of the cell, which triggers the elongation phase. When this happens, the spermatids form an intercellular junction with the supporting Sertoli cells. Then the nucleus starts changing its shape and becomes more compact, showing the characteristic hook-shaped head that can be observed in mouse sperms (Figure 2).

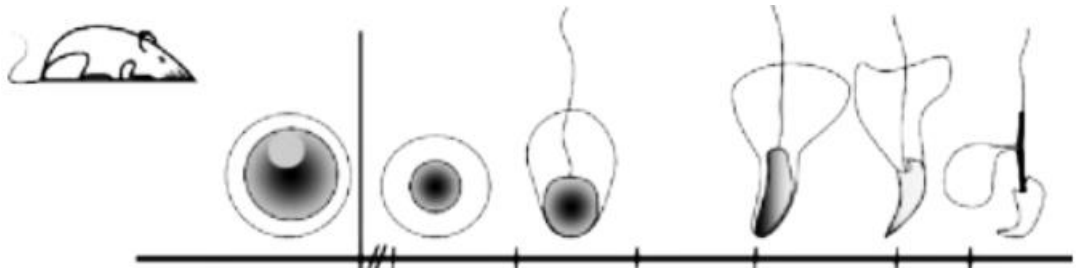

Figure 2. Chromatin condensation in mouse spermiogenesis [52].

During spermiogenesis, several processes take place, including nuclear compaction due to chromatin condensation [34]. In the course of nucleus compaction, histones in the nucleosomal chromatin are replaced with transition proteins, followed by protamine replacement, which results in the compaction of chromatin fibers. Another important event during spermiogenesis is the assembly of the sperm flagellum. This process begins right after the completion of meiosis with the formation of the axoneme, the central part of the flagellum. During spermatid elongation, the additional structures that are needed for proper flagella function are assembled around the central axoneme [34]. As it was mentioned before, the final stage of spermiogenesis is spermiation, consisting of the release of immature spermatozoa capable of active movement after “training” in the epididymis. The epididymal maturation process is needed for spermatozoa's primary goal: to reach the oocyte in the female genital tract [8].

3. EPIGENETICS IN SPERMATOGENESIS

The genome can be defined as the complete set of genes or genetic material present in a cell or organism. This material consists of nucleotide sequences in the DNA. All of the modifications that regulate the gene expression/activity of the genome is known as the epigenome. The modifications that affect gene expression include reversible alterations in DNA and histone structure. Interesting to see is that the DNA sequence of the genome and the epigenome are the same, only the gene expression is altered [9]. The effects produced by epigenetic modifications can have short- or long-term consequences on cell functioning. On top of this, certain modifications can be inherited transgenerationally, which means that gene expression can be influenced at early stages in the offspring [35].

The three most common processes that are responsible for changes in gene expression are DNA methylation, post-translational histone modifications, and chromatin remodeling (Figure 3). The structure of the DNA plays an important role in determining the epigenome. The basic subunits of chromatin are nucleosomes. Nucleosomes consist of two molecules of each of the histones H2A, H2B, H3, and H4, resulting in an octamer that forms the core particle of the nucleosome. How the DNA is wrapped around the histones determines the gene expression. The DNA can either be tightly or loosely wrapped around the histones. Tightly wrapped regions are transcriptionally inactive and are called heterochromatin. Loosely wrapped regions on the other hand are transcriptionally active and are called euchromatin. The difference in compaction between regions in the DNA is

determined by epigenetic modifications that influence gene expression. Noticeably, in contrast to the genome sequence that remains stable throughout the cell cycle, the epigenome is dynamic and can vary between tissues giving cells with the same genetic sequences the ability to perform different specialized functions [36].

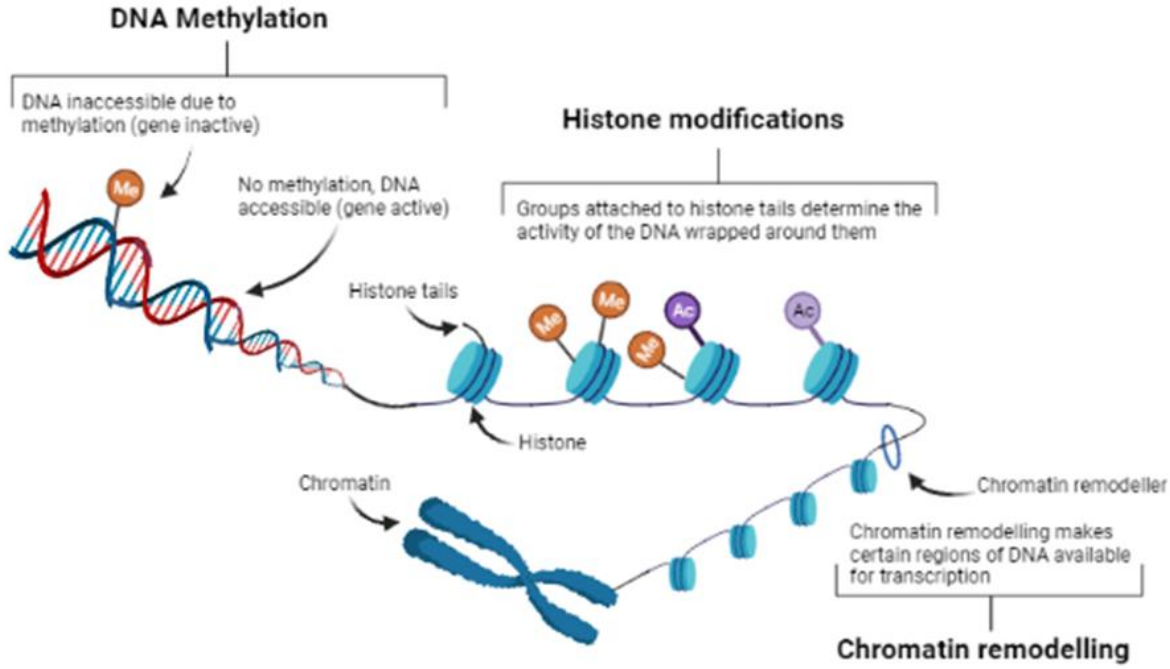

Figure 3. Overview of epigenetic mechanisms that influence gene expression.

3.1 DNA methylation

The methylation site in mammalian DNA is the position 5 of cytosines, primarily within CpG dinucleotides. About 60-80% of the total CpGs within the genome are methylated. DNA methylation is catalyzed by DNA (cytosine-5) -methyltransferases[10]. DNA methylation is mostly related to transcriptional repression, which happens when the CpGs within the promoter regions of certain genes acquire this modification. The process of acquiring new methylated CpG regions is called *de novo* methylation. An essential attribute of DNA methylation is that it is maintained during DNA replication by specific methyltransferases that copy the methylation pattern from the mother DNA molecules to the daughter ones. In the male germline, the methylation is acquired before birth in gonocytes, which are the precursor germ cells responsible for the production of SSCs. The level of DNA methylation is complete when the pachytene phase of meiosis is reached [37]. Spermatogonia that undergo mitotic divisions to preserve their number in the testicles must keep the DNA methylation patterns acquired during the prenatal period. This ensures that every cell at the beginning of spermatogenesis starts with the same methylation pattern [37]. Interestingly, DNA methylation seems to influence the genome beyond gene expression. Some studies have proposed that it also plays a role to maintain a special chromatin state required for germ cell-specific processes such as meiosis and spermiogenesis [38].

3.2 Histone modifications

There are several processes responsible for histone modifications. These include methylation, acetylation, phosphorylation, sumoylation, and ubiquitination, which are all posttranslational histone modifications on the N- and C-terminal of histone tails [39]. These posttranslational modifications alter the structure and charge of the histone tails, as a result the binding to the DNA changes, hence chromatin compaction, and subsequently gene expression. Thus far histone modifications are essential for appropriate cell function [9,39].

The most common histone posttranslational modification is methylation. It works by adding methyl groups to lysine residues, usually in histones H3 and H4. This is performed by histone methyltransferases (HMTases) [9,39]. There are various types of methylation, including mono-, di-, and trimethylation, which determines whether the gene is activated or repressed. An example of this is the methylation of H3K9, often found in silenced promoters [40]. The second most common histone modification is acetylation which is modulated by the balance between the activity of histone deacetylases (HDACs) and histone acetyltransferases (HATs). HDACs usually inhibit gene expression, whereas HATs promote it [41]. Lysine residues that are acetylated have a reduced positive charge, as a result, the binding between negatively charged DNA and the histone tails decreases [39].

During spermiogenesis, it is important to reduce the chromatin volume to give rise to spermatozoa with a highly compact nucleus. The high level of compaction in chromatin is achieved by the replacement of histones with protamines. Protamines are small proteins with several positive charges; they neutralize the negative charges in the DNA, making it possible to tightly compact the chromatin. Before histones can be replaced by protamines in haploid spermatids, histone variants must be initially incorporated, i.e. transition proteins, and histone H4 must be hyperacetylated. As mentioned before, acetylation decreases the binding between the DNA and the histone tails, producing a much looser nucleosome structure, which allows the replacement of the histones by protamines [9]. Following hyperacetylation, protamines replace most histones, ensuring chromatin compaction. In humans, there are two classes of protamines, Prm1 and Prm2. Both of these protamines are required for chromatin condensation.

3.3 Chromatin remodeling

Chromatin remodeling makes certain regions of DNA accessible for transcription. It is a process that changes the structure of chromatin [39]. Chromatin regions can change from a loose to a condensed state and *vice versa*. In loose regions, the DNA is accessible to transcription factors and RNA polymerases, which are the proteins that control gene expression. In contrast, in the condensed regions, it is hard for those proteins to interact with the DNA, which hinders the transcription process [9,39]. Instead of relying on covalent interactions like DNA methylation and histone modifications, chromatin remodeling is carried out by chromatin remodeling complexes that use the energy from ATP hydrolysis to break the binding between DNA and histones. Chromatin remodeling complexes primarily act by sliding histones along the DNA, however, they can also twist and bulge the DNA [43].

4. REACTIVE OXYGEN SPECIES SOURCES (ROS)

Due to the fact that ROS is the main contributing factor in impaired spermatogenesis it is important to understand where ROS come from and how they are able to influence this process. As mentioned before reactive oxygen species are oxidant molecules such as free radicals that contain oxygen in their structure. The imbalance between oxidants and antioxidants in favor of the oxidants is called oxidative stress and can result from various factors. Medication, age, alcohol and drug consumption, genetic problems, systemic diseases, and environmental toxins are all examples of factors that can lead to oxidative stress. In general, all these factors can be classified into two main groups: endogenous and exogenous sources [1,3,5].

4.1 Endogenous sources

Aerobic metabolism goes hand in hand with redox reactions and these reactions produce ROS as byproducts [3]. In living organisms, ROS are produced in several cellular systems located in the cytosol, peroxisomes, as well as in the mitochondrial membrane, and endoplasmic reticulum [19]. ROS production in the cytosol originates from compounds that undergo redox reactions, for example, thiols, catecholamines, and flavins. In addition to this, a number of cytosolic enzymes produce ROS throughout their catalytic activity [19]. Apart from the cytosol, ROS is also generated in peroxisomes. Peroxisomes are organelles involved in H2O2 metabolism and in multiple metabolic pathways. Key functions that are carried out by peroxisomes comprise of fatty acid β- and α-oxidation, amino acid and glyoxylate metabolism, and synthesis of lipidic compounds. During the enzymatic catalyzation of these processes, ROS is generated [19]. The third location where ROS are produced is the mitochondria. Mitochondria are multifunctional organelles in eukaryotic cells that provide adenosine triphosphate (ATP) as a bioenergetic molecule through oxidative phosphorylation, which generates ROS as a

byproduct [11]. This happens mostly in Complex I and Complex III of the electron transport chain (ETC), during the conversion of Coenzyme Q10 (CoQ10) into ubiquinol [17,19]. The last location where ROS is produced is in the endoplasmic reticulum. This network of membranes is involved in several functions, including synthesis of lipids and proteins, protein folding, and transport of Golgi, lysosomal, secretory, and cell-surface proteins [20], calcium storage [21], lipid metabolism, and, in some cell types drug detoxification [22]. The smooth endoplasmic reticulum has an electron transport chain consisting of two systems dedicated to xenobiotic metabolism and the introduction of double bonds in fatty acids, also capable of generating ROS. Xenobiotic metabolism is the process where xenobiotics are detoxified and converted into species that are more water-soluble and easier to excrete in urine or can be coupled with substances that make their urinary or biliary excretion further easier [19].

4.2 Exogenous sources

Exogenous sources are related to lifestyle and environmental exposure that can directly promote ROS generation or suppress the levels of intrinsic antioxidant factors [23]. The most obvious exogenous source is probably smoking which is the most important factor affecting male infertility [24]. Chemicals in cigarettes, for example, nicotine, affect hormone levels in semen [25]. In addition, smoking can cause DNA damage in sperm, this is due to an increase in ROS production in the testicle. Cigarette smoke consists of numerous carcinogens, toxins, and mutagenic substances as well as unstable free radicals and ROS in its particles [5]. Cigarette smoke also enhances the production of superoxide anions, resulting in damage to lipid membranes of cells, proteins, and DNA. This eventually leads to infertility. Another effect of cigarette smoke is the reduction of creatine kinase in sperm, which acts as an energy reservoir for rapid buffering and rebuilding ATP. On top of this, mitochondrial DNA is damaged, leading to reduced ATP production and impaired energy supply. ATP is needed for sperm motility, hence its deficiency affects the fertilization capacity of males gametes [3,5]. Another exogenous source that causes oxidative stress is obesity. People with obesity usually have a general pro-inflammatory state that is linked with systemic oxidative stress, antioxidant reduction, and oxidative sperm DNA damage [26]. Additionally, excessive alcohol intake has a negative effect on the quantity and quality of sperm. It is associated with a reduction of sperm motility and concentration, and it also reduces the percentage of sperm with normal morphology [27]. Alcohol causes spermatic chromatin disorders that affect sperm motility, nuclear maturation, and DNA integrity [28]. In addition, excessive intake of alcohol results in increased production of acetaldehyde, which promotes the generation of ROS due to its interactions with proteins and lipids [29]. On top of this, ethanol causes changes in the structure and function of mitochondria, reducing oxidative phosphorylation and ATP levels and increasing ROS generation through its metabolism and breakdown in the liver [5]. In general, an unhealthy lifestyle seems to increase the level of oxidative stress in the whole body and male germ cells are especially sensitive to this.

5. INFLUENCE OF OXIDATIVE STRESS ON MALE GAMETES

Oxidative stress is defined as the imbalance between the production of reactive oxygen species and the scavenging capacity of available antioxidants [12]. Reactive oxygen species are produced during the intermediate steps of cellular metabolism. Under normal circumstances, a certain amount of ROS is needed to create an environment suitable for cellular metabolism. These include sperm maturation, capacitation, acrosome reaction, hyperactivation, and sperm-oocyte fusion [3,6,7]. However, when the concentration of ROS is too high the neutralizing capacity of antioxidants can be transcended [13]. Research has shown that this could have serious effects on the viability and function of the male germ cells [14]. Under oxidative stress, modifications of major cellular macromolecules occur, such as DNA and RNA. These molecules are particularly sensitive to oxidative stress due to the reactivity between oxygen and nitrogen on the nucleobase. DNA fragmentation due to oxidative stress involves the breaking of DNA strands, base modifications, and chromatin cross-linking [13,15]. On top of this, also proteins and lipids are affected by oxidative stress [13]. Spermatozoa are specifically vulnerable to oxidative damage due to the presence of polyunsaturated fatty acids in their plasma membrane. In addition to this, their cytoplasm rate of antioxidant enzymes is extremely low [5,3].

5.1 Natural defense mechanisms

Spermatozoa are protected by antioxidants present in the semen since spermatozoa themselves do not have many antioxidant enzymes in their cytoplasm [3,5]. Antioxidants in the semen can be divided into two different categories, enzymatic and non-enzymatic. The enzymatic category consists of catalase, superoxide

dismutase, and peroxidase. These enzymatic antioxidants remove ROS by means of catalytic activity. The non-enzymatic antioxidants remove ROS by means of metabolic activity. Some of the non-enzymatic antioxidants are uric acid, glutathione, and coenzyme Q10. This group can be further divided into hydrophobic and hydrophilic antioxidants. Hydrophobic antioxidants protect membranes from lipid peroxidation whereas hydrophilic antioxidants are predominantly found in the seminal plasma [45]. The latter is the main defense against oxidative stress in spermatozoa [46].

DNA damage is the second type of damage caused by oxidative stress. To cope with this there are five repair mechanisms present that together maintain genomic integrity: base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), double-strand break (DSB) repair, and post-replication repair [55]. BER removes incorrect bases in the DNA and polymerase recorrects the base, NER replaces mismatched bases in the double helix, MMR removes incorrect base pairs during replication, DSB repair has two major pathways for repairing double-strand breaks: homologous recombination and nonhomologous DNA end joining. Post-replication repair is the repair of DNA damage taking place after replication. Studies have suggested that alterations in DNA damage repair systems can be linked to infertility [55].

5.2 DNA damage

The most common DNA organic base that is exposed to oxidative stress is the guanine base (G). This exposure may lead to the conversion of guanine (G) to 8-hydroxyguanine (8-OHG). It is shown that 8-OHG is 100 times more present in the sperm of infertile males compared to fertile males [16]. One of the natural defense mechanisms to prevent the accumulation of 8-OHG in the sperm DNA is the so-called base excision repair (BER). This is a cellular mechanism that corrects DNA damage from oxidation, deamination, and alkylation. Spermatozoa lack apurinic endonuclease 1 (APE1) and XRCC1, which are downstream factors of the BER pathway. As a result of this, the DNA repair capability is incomplete [5]. Due to this impairment, abasic sites arise in locations where 8OHdG was before BER. This causes the DNA strands to become unstable, resulting in DNA fragmentation [3].

Spermatids are highly sensitive to DNAse, DNAse is an enzyme responsible for hydrolitic cleavage of phosphodiester bonds in the backbone of DNA. The result of this is a high amount of DNA damage in the form of DNA fragmentation. Especially during the stage where elongating spermatids transform into spermatozoa, there is DNA breakage occurring [53]. This is under normal circumstances needed for chromatin compaction in the late stages of spermiogenesis.

5.3 Lipid peroxidation

The plasma membrane of spermatozoa is rich in unsaturated fatty acids, which have an essential role in several processes, including membrane fusion events such as acrosome reaction and sperm-oocyte fusion. However, these fatty acids make them vulnerable to free radical attacks and lipid peroxidation [5,13]. Lipid peroxidation of the sperm membrane is the second major result of oxidative stress in male gametes. The main cause of lipid peroxidation in the sperm membrane is a high concentration of hydrogen peroxide [3]. The lipid peroxidation cascade itself also contributes to the production of free radicals. The result of this is reduced membrane fluidity and a reduction in sperm motility [11]. High levels of ROS can also affect the membranes of mitochondria, eventually resulting in cytochrome c being released, which activates apoptotic cascades via caspase enzymes[5].

5.4 Apoptosis

Apoptosis plays a major role in controlling spermatogenesis, through apoptosis the number of spermatogonia that will reach the spermatozoa stage is decreased by 75% [47]. In spermatids however, apoptosis is rarely the case [47]. High levels of ROS can promote apoptosis. Apoptosis is the process where programmed cell death occurs [47]. The apoptotic pathway is regulated by two main modulators, the intrinsic mitochondrial pathway, and the extrinsic Fas receptor complex pathway [45]. The first pathway starts with ROS damage to the mitochondrial membrane, when this happens cytochrome C is released [11]. This activates the apoptotic caspases 3 and 9, resulting in apoptosis [47]. The extrinsic pathway is activated through the Fas death receptors by ROS. Sertoli cells are known to regulate the number of germ cells through this pathway. The protein FasL, which is expressed by Sertoli cells, interacts with the Fas receptor in germ cells and induces apoptosis [47].

6. MEASURING OXIDATIVE STRESS IN THE GERM LINE

The most common method used for measuring oxidative stress in sperms is the direct measurement of lipid peroxides in seminal plasma [23]. The marker used for this measurement is malondialdehyde (MDA), which gives an index of lipid peroxidation [48]. The measurement of MDA in seminal plasma is able to show different parameters such as DNA damage in spermatozoa and ROS generation. These parameters give an indication of the oxidative stress that is present.

A second way of measuring oxidative stress is chemiluminescence. Chemiluminescence methods measure the production of electromagnetic radiation observed when a chemical reaction yields an electronically excited intermediate or product, which either luminesces or donates its energy to another molecule responsible for the emission [49]. For instance, electron reduction of lucigenin is used to generate chemiluminescence. This can be achieved by reductases, using NADH or NADPH as electron donors [23]. Another chemical that can be used for chemiluminescence is luminol. It requires one-electron oxidation that creates the luminol radical essential for chemiluminescence. In spermatozoa, an intracellular peroxidase is responsible for this electron oxidation. The signal however is low due to the limited availability of the peroxidase [23].

Flow cytometry is a third method that can be used to detect differences in ROS generation associated with variations in sperm function. By using this method, a distinction can be made between highly functional spermatozoa and spermatozoa with impaired functionality [23]. This can be done with the use of 3 probes: MitoSox red (mitochondrial ROS generation), dihydroethidium (total intracellular ROS generation), and H2DCFDA (dichlorodihydrofluorescein diacetate targeting intracellular oxidants such as hydrogen peroxide). These probes are able to detect differences in redox activity, which are correlated to impaired sperm function [45].

All methods discussed above have some downfalls. They are direct methods that require compounds to react with the free radicals, most of these compounds are nonspecific and usually react with all kinds of reactive species. The more specific probes used in flow cytometry, for example, have other downfalls. It is only possible to measure the amount of free radicals produced between adding the compound and performing the measurement, which means that these probes inform about the history of the sample but not about the real-time behavior of radical levels. In addition, photobleaching of fluorescent probes is also common, limiting the number of measurements that can be performed on the same sample [50].

Diamond magnetometry is another technique used to measure oxidative stress. This technique uses the properties of nanodiamonds to measure free radicals. Diamond magnetometry is superior to the previously mentioned techniques due to various reasons. First of all, diamond magnetometry can measure free radicals in real-time. Secondly, diamonds can be targeted to specific intracellular locations, offering measurements with subcellular resolution [50]. However, one of the main challenges of this technique is to achieve the incorporation of the diamonds by cells to fully exploit the advantages of a nanoscale measurement.

7. DIAMOND MAGNETOMETRY

Diamond magnetometry uses defects in nanodiamonds to measure free radicals. Some defects, *e.g.* the nitrogen-vacancy (NV) centers, are able to detect magnetic and electric fields [51]. The magnetic surroundings of the diamond determine the diamond's optical properties. The advantage of using an optical signal for measurement is that it gives very high sensitivity, which is particularly useful if measurements in specific cell structures are necessary [51]. A specific type of diamond magnetometry is relaxometry. This method is very sensitive to spin noise, which is the result of free radicals that have a free electron spin. The use of nanodiamonds is particularly useful due to their biocompatibility and the ability to perform real-time free radical measurements [50]. A measurement consists of exciting the diamond with a laser such that the NV centers polarize into the ms = 0 state of the ground state. After polarization measurements are performed after specific dark times to see whether the NV centers are still in this state or have returned to equilibrium. The time that it takes to reach the equilibrium gives a quantitative measurement of the radical concentration surrounding the nanodiamond [54]. To be able to utilize this technique it is essential that the diamonds are close to the cells, preferably taken up by them. To see whether or not this has the potential for measuring oxidative stress in spermatids the diamond uptake mechanisms of the cell have to be taken into account.

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