Oxidative stress in male germ cells, with a focus on pachytene and diplotene spermatocytes

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Contents

Summary

Oxidative stress is believed to play an important role in male infertility. This review goes into the role of oxidative stress on spermatogenesis, with a focus on pachytene and diplotene stages. A general overview of the spermatogenesis is given, with additional attention for the synaptonemal complex. This structure is important for differentiation between the various stages of spermatocytes. Following this, the role of oxidative stress is assessed in these various stages of spermatocytes by assessing various markers for DNA damage, DNA repair and lipid peroxidation. This study showed great heterogeneity in results among various stages, resulting in an overall inconclusive result. Lastly, ways to measure oxidative stress were mentioned including indirect and direct measurements of ROS. Indirect measurements would be: detection of lipid peroxidation, low levels of antioxidants and assessing DNA damage. Direct measurements include: chemiluminescence, flow cytometry and the novel technique of diamond magnetometry. These measuring methods are crucial in making advancements in the field of male infertility and the role of oxidative stress.

1. Introduction

Inability to conceive offspring is a prominent problem amongst couples of reproductive age, 8-12% have been reported to be affected by infertility [1]. Out of these cases, men are the sole reason of infertility in 20% of the cases and are at least partly involved in 30-40% of other cases [2]. Although the causes of these high infertility rates remain elusive, several environmental, nutritional and socioeconomic factors are believed to play an important role [3]. The direct link between infertility and these factors is still poorly understood, but there is evidence demonstrating that oxidative stress (the imbalance of reactive oxygen species and antioxidants) is one of the main factors in high infertility rates [3].

1.1 Oxidative stress

Reactive oxygen species (ROS) are byproducts of normal cellular metabolism. Low to moderate amounts of ROS have a positive influence on physiological processes. However, when the levels of ROS are too high, the homeostasis of the human body is impaired and they become pathological. To prevent elevated levels of ROS, cells have natural antioxidant pathways . These pathways contribute to homeostasis and prevent oxidative tissue damage (see figure 1). Nevertheless, high levels of oxidant species , due to either endogenous or exogenous sources (see figure 1), can lead to an oxidative stress condition [4].

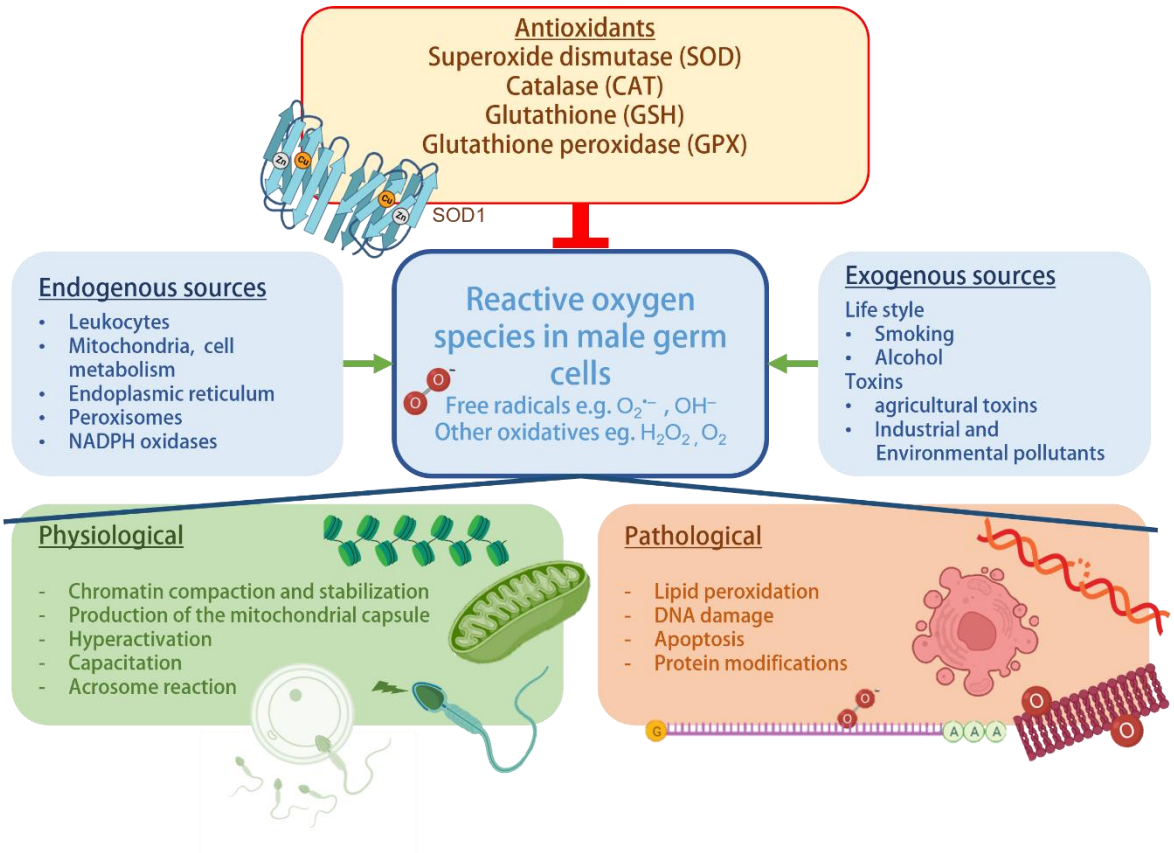

Figure 1. *An overview of the role, sources , and factors influencing oxidative stress.* [5]

1.1.4 Physiological role of ROS

There are several physiological processes in which reactive oxygen species play an important role (see figure 1). For example, during spermatogenesis and epididymal transit, certain levels of ROS are needed for oxidation of cysteine-thiol groups. These are essential for chromatin compaction and stabilisation [3]. Furthermore, ROS play an important role in the production of the mitochondrial capsule, a structural component that is associated with the outer membrane of the mitochondrial membrane in spermatozoa of mammals [6]. Hydrogen peroxide has shown to be of influence in protein thiol oxidation, which is important in forming disulphide bridges. These bridges are of importance for the stability of the mitochondrial capsule [7].

On the other hand, hyperactivation is a motility pattern of sperm to optimise penetration chances. It shows an extremely vigorous whiplash-like flagellar beating [3]. The precise mechanisms of activating the process of hyperactivation are yet to be uncovered. However, it was shown that a low constant presence of O2−• is needed for initiation and preservation of the hyperactivated state [8].

Capacitation is part of the maturation process of sperm and occurs when travelling through the female reproductive tract. ROS are needed as messenger molecules for activation of cascades that mediate significant changes in membrane architecture, which is needed for successful sperm-oocyte interaction and fusion [9].

Acrosome reaction is a process in which acrosomal hydrolytic enzymes are released from the sperm head. Physiological levels of ROS are needed for triggering the activation of tyrosine phosphorylation of proteins on the sperm head region, which are crucial for successful sperm-oocyte fusion [3].

1.1.5 Pathological role of ROS

ROS levels promote pathological conditions when they surpass a certain threshold. In the case of the male germline, pathological levels can have an effect on the membrane, increase DNA damage and apoptosis.

The membrane of sperms contain more polyunsaturated fatty acids (PUFAs) in comparison with other cells. These are important for maintaining membrane fluidity, which is pivotal for membrane fusion events. PUFAs are prone to oxidation due to their highly reactive methylene groups and therefore have enhanced susceptibility to oxidative damage. Lipid radicals can be formed under high levels of ROS, which quickly react with oxygen molecules to form peroxy fatty acids, and finallylipid peroxides. Whenever metal ions are present, lipid peroxides are catalysed to form OH•. This compound reacts with other nearby PUFAs and therefore is able to start the lipid peroxidation chain reaction again [3].

ROS can also cause a significant increase in DNA damage. It has been shown that mainly (NO•, O2−•, and OH• can interact with DNA molecules, causing strand breaks and deletions of nucleotide bases. [10].

Lastly, apoptosis (programmed cell-death) of male germ cells is stimulated by ROS trigger the release of mitochondrial signalling molecules such as cytochrome C. [11]. Of course, apoptosis and its signalling pathways are part of the physiological role of ROS. However, when high levels of ROS are sustained in the male germ line, elevated cell-death is observed, which negatively influences the number of healthy sperms at the end of spermatogenesis.

To answer the question of how oxidative stress influence male fertility, some sub questions need to be addressed. To understand potential causes of male infertility, a general understanding of the spermatogenesis process is required and taking a closer look into the individual stages of this process is crucial to connect gene expression and DNA damage with oxidative stress. Additionally, how oxidative stress effectively measured?

2. Spermatogenesis: general overview

2.1 General outline

Spermatogenesis, the developmental pathway from spermatogonia to spermatozoa, occurs in the seminiferous tubules. The seminiferous tubule is a highly organised structure which contains germ cells in many different stages of maturation [12]. This maturation is facilitated by the Sertoli cells, one of the somatic nurse cells of the testis. They are in direct contact with the germ cells and control the environment within the seminiferous tubules [13].

Spermatogonia as well as the Sertoli cells adhere to the basement membrane of the tubules. Type A spermatogonia will undergo a series of mitotic divisions for self-renewal. Differentiation takes place from A1 to A4 spermatogonia then into intermediate spermatogonia, which will ultimately become type B spermatogonia. Subsequently, primary spermatocytes will be generated from type B cells through a mitotic division [12].

Primary spermatocytes then enter prophase I. In this stage, the cells are diploid and contain two chromatids per chromosome (2n, 2c). This is the only stage in which crossing over (genetic recombination) takes place, causing variation in parental genetic material. The cells go through a series of intricate stages, among which are pachytenes and diplotenes. These stages correspond to the second half of propahse I and are extremely relevant due to the occurrence of genetic recombination in pachytenes and the preparation for the first meiotic division in diplotenes. Secondary spermatocytes, the stage after the first meiotic division, are haploid cells but with two copies of each chromosome (n, 2c). These cells then enter prophase II which prepares them for the second meiotic division. This stage includes four different mechanisms: tight wrapping of DNA into chromosomes, dissolving of nuclear membrane, migration of the centrosomes, and the reformation of the spindle apparatus. Contrary to prophase I, prophase II resembles the simpler process of somatic cell division (mitosis). The number of chromosomes is maintained, whereas the chromatids are separated from each other (n, c). Round spermatids, the result of the second meiotic division, will then proceed to differentiate into sperm cells with the support of the Sertoli cells.

2.2 prophase I

Prophase I can be divided into four stages: Leptonema, Zygonema, Pachynema and Diplonema (see figure 2).

In the leptotene stage, double-strand breaks (DSB) are generated by an enzyme, which produces DNA ends in chromosomes necessary for the later linkage between them (chromosome pairing). This phenomenon results in spatial coalignment of whole homologous chromosomes. In this stage, pairing as well as organisation of the homologous chromosomes takes place [14].

After coalignment, homologous chromosomes become more closely associated. This is also known as synapsis, which is achieved by the formation of the synaptonemal complex (SC). Zygotene stage is characterized by the progress in the formation of the SC. SC formation is mostly initiated at sites of recombinational pairing interactions (DSB hot spots) [14].

After the zygotene stage, the pachytene stage takes place. Pachytenes can be identified based on the moment from which the SC formation in all autosomal chromosomes is completed. During pachytene stage crossing-over (CO) takes place. The sex chromosomes only exchange material in a small area, the so called pseudoautosomal region (PAR). PARs are short regions of homology and behave like autosomes in recombination [15].

After pachytenes, in diplotene stage, the SC disassembles causing the homologous to separate. Only chiasmata, sites at which CO has taken place, remain [14]. This is a logical consequence of the physical link caused by the exchange of genetic material between sister chromatids belonging to different homologous chromosomes.

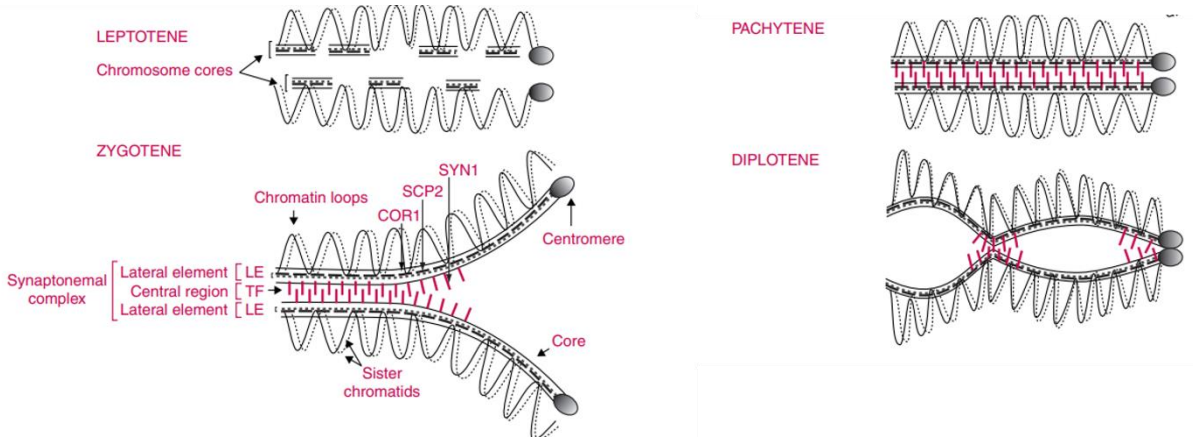

Figure 2. *the four stages of* prophase I. [16] *Leptotene stage: spatial co-alignment of homolog chromosomes. Zygotene stage: synapsis takes place and SC forms. Pachytene stage: crossing over takes place. Diplotene stage: dissociation of SC, chiasmata become visible.*

2.3 Synaptonemal complex

For the identification of the different stages of prophase I, the morphology of the SC is especially relevant. By means of immunology and specific proteins of the complex, the level of progress of its formation informs about prophase I stages. During the leptotene stage, the lateral elements (LEs), composed of SYCP2 and SYCP3, need to be assembled along the homologous chromosomes (figure 3A). This happens in the initial stages of meiotic recombination. Afterwards, the LEs link to the transverse elements (TEs), which primarily consist of SYCP1. Subsequently, a linkage takes place between TEs and the central elements (CEs), these consist of SYCE1, SYCE2, SYCE3 and TEX12, causing the homologous chromosomes to be interconnected [17].

SYCP3 is one of the main components of the LEs and a DNA-binding protein. Along the chromosomes (see figure 3A), SYCP3 polymerizes on the DNA in a zipper-like fashion revealed by immunological staining. As mentioned before, LE formation takes place first in the assembly of the synaptonemal complex. Therefore, using SYCP3 as a marker for immunological staining is effective for differentiating between Leptotene, Zygotene, Pachytene, and Diplotene stages. In the Leptotene stage, expression of the protein is all over the nucleus and not yet organised (figure 3B L). In the Zygotene stage, the zipper-like structure is formed and the transverse elements are partially installed. This is the reason behind the openings at the ends of the synaptonemal complex observed in this stage (figure 3B Z). In the pachytene stage, the zipper structure is fully closed (figure 3B P). However, whether the ends of this zipper structure are yet closed is at times hard to observe. By using γH2AX (a histone variant that is rapidly phosphorylated at sites of double-strand breaks) [18] , the pachytene stage can be more easily distinguished (see figure 3C). For the sex chromosomes the recombination process takes longer, given their drastic difference in length. This is why γH2AX is observed for an extended period of time on sex chromosomes but not on somatic chromosomes. See figure 3C for the differences in γH2AX expression. In the Diplotene stage, the complex starts to disassemble, revealing the chiasmata (see figure 3B D). Finally, the identification of different stages of prophase I is especially relevant for studies addressing alterations in the male germ line that can be associated with infertility, such as the influence of oxidative stress.

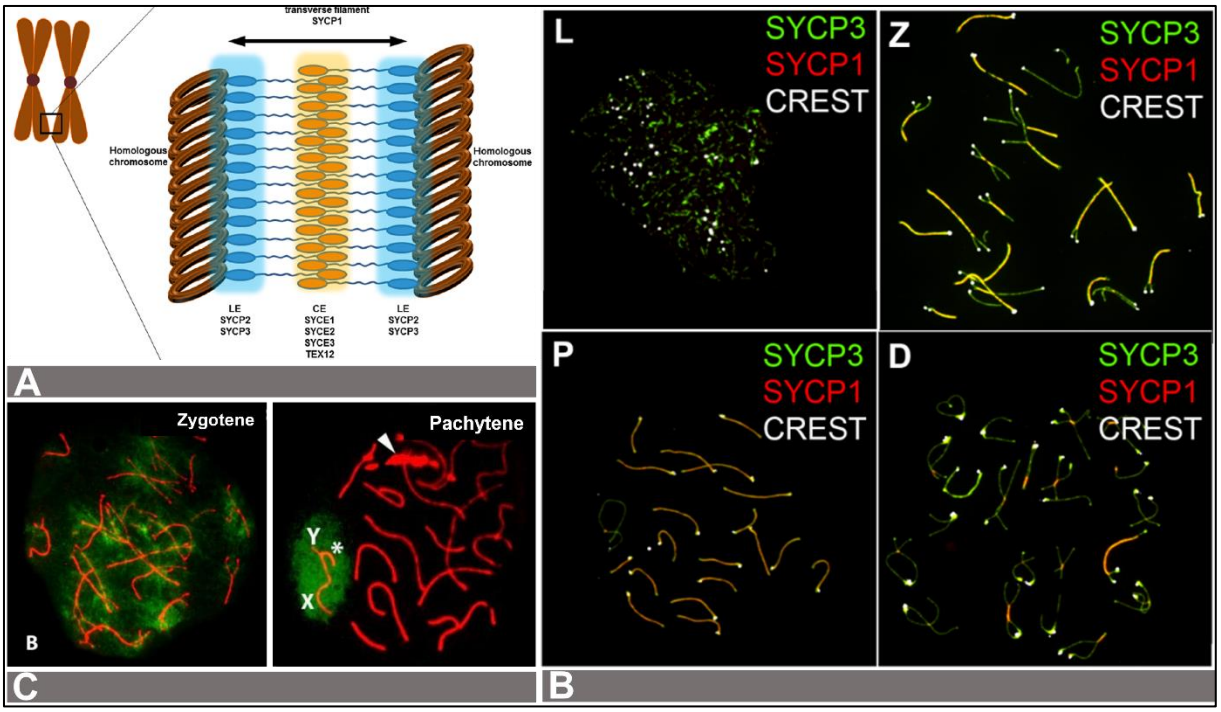

Figure 3. *A. Elements (lateral and central) of the synaptonemal complex* [17]*. B. Immunostaining of leptotene (L), zygotene (Z), pachytene (P), and diplotene (D) stages in mouse?* [18]*. C. Immunostaining of zygotene and pachytene stages in mouse with γH2AX in green showing double-strand break sites and SYCP3 in red showing the synaptonemal complex. [Unpublished images, Arturo Elías Lllumbet]*

3. Oxidative stress and its influence on spermatocytes in pachytene and diplotene stages

3.1 Ageing and oxidative stress alter DNA repair mechanisms

In a study performed on mice by {Nguyen-Powanda et al.} [19], the association between ageing and oxidative stress together with accumulation of DNA damage was assessed. Aged as well as young wild type animals and animals with a superoxide dismutase-1 knockout (Sod1−/−) were used to understand the effects of oxidative stress and underlying mechanisms of male reproductive ageing. Gene expression analysis was conducted to study the key factors in the DNA repair pathway in both pachytene spermatocytes and round spermatids. It was hypothesised that DNA damage would increase in (Sod1−/−) mice because of a deterioration in the DNA damage repair pathways and that ageing further aggravates the damage repair response. The results showed that testis weight of (Sod1−/−) mice were significantly decreased. Besides that, for (Sod1−/−) mice of all ages, both epididymal and total sperm count were reduced, with evidence of increased infertility. Furthermore, this study demonstrated that levels of lipid peroxidation as well as DNA damage are higher in Sod1−/− mice and that ageing has an enhancing effect on top of that. A more detailed description of these findings will follow.

Pachytene spermatocytes are divided into several stages: I–IV, V–VII, VIII–IX, and X–XII (figure 3A left). FITC-labelled peanut-agglutinin lectin (PNA) antibody was used to stain the acrosome (figure 3B left). In this way the different stages of spermatids could be identified [20]. However, the paper does not mention anything on dividing the pachytene spermatocytes. This is crucial information and should have been mentioned.

3.1.2 Lipid peroxidation

4-hydroxynonenal (4HNE) was used as a marker for lipid peroxidation due to oxidative stress in pachytene spermatocytes. In stages I–IV, there were no significant changes in 4HNE intensity between all animals. However, in stages V–VII, 4HNE staining intensity was higher for the aged WT mice group compared to the Young WT animals. Furthermore, spermatocytes of aged Sod1−/− mice were found to have higher levels of lipid peroxidation in comparison to those of aged WT animals in stages VIII–IX. Finally, also in spermatocytes from young Sod1−/− mice 4HNE intensity was significantly higher than young WT mice in stages X–XII. (see figure 3 right). Table 1 summarizes the main findings.

Table 1. Lipid peroxidation in spermatocytes

stage	findings
I–IV	• No significant difference between all groups.
V–VII	• Aged WT higher 4HNE staining intensity compared to young WT animals.
VIII–IX	• A ged Sod1−/− higher 4HNE staining intensity compared to aged WT.
X–XII	• S ignificant 4HNE intensity increase in young Sod1−/− compared to young WT animals.

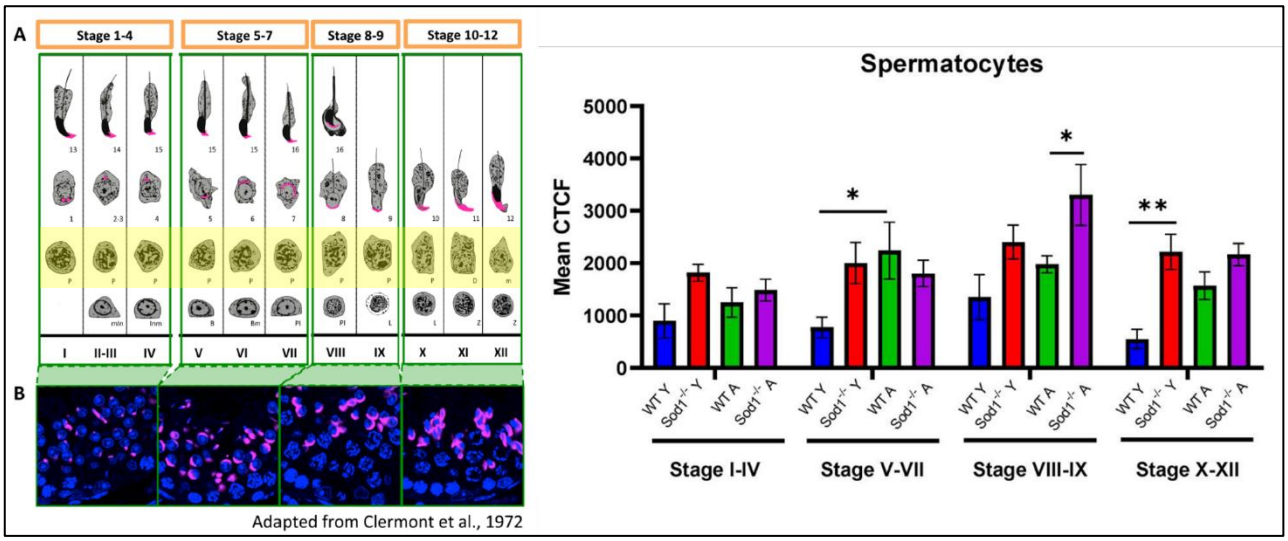

Figure 4 *. Left figure: A. Stage division of pachytene spermatocytes B. Staining of acrosome using* FITC-labelled peanut-agglutinin lectin (pink pseudocolor)[19]. *Right figure: Quantification of the corrected total cell fluorescence (CTCF) of 4HNE showed an increase in 4HNE intensity in spermatocytes of aged WT mice compared to young WT (stage V-VII). Also for aged Sod1−/− compared to aged WT (stage VIII-IX). And lastly, for young Sod1−/− compared to young WT (X-XII).* [19]. *Young wild-type mice (WT Y); aged wild-type mice (WT A); young Sod1−/− mice (Sod1−/− Y); aged Sod1−/− mice (Sod1−/−A). ∗P < 0.05; ∗∗P < 0.01;*

3.1.3 DNA damage

8-hydroxy-2' -deoxyguanosine (8-OHdG) is a common marker for evaluating DNA base damage due to oxidative stress. The use of this marker to evaluate oxidative stress effects on DNA showed a significantly increased damage in only stages I–IV of aged Sod1−/− mice. So both ageing and the lack of SOD contributed to high 8-OHdG levels.

Furthermore, immunological staining was performed using γ-H2AX. With this antibody, detection of double-strand breaks is possible. The results from {Nguyen-Powanda et al.} showed increased values of γ-H2AX staining either for aged, young Sod1−/− mice and aged WT animals compared to young WT, which displays that both the lack of SOD and ageing contribute to DNA damage in the form of double-strand breaks. For the exact findings per stage see table 2.
8-Oxoguanine glycosylase (OGG1) is an enzyme involved in the initiation of the base excision repair pathway (BER) for DNA. It removes 8-oxodG lesions created by high ROS levels. Immunohistochemistry was used for OGG1 detection. For the exact findings per stage see table 2.
DNA ligase IV (LigIV) [21] is another enzyme involved in the nonhomologous end-joining repair pathway. This enzyme repairs DSB by joining DNA strands. A low expression level of LigIV indicates a low need for DSB repair. For the exact findings per stage see table 2.

Table 2. DNA damage in spermatocytes

stage	findings
I–IV	● A ged Sod1−/− mice showed significantly higher 8-OHdG levels compared to aged WT ● No significant differences in γ-H2AX immunostaining ● Significant increase in OGG1 staining intensity in spermatocytes of aged WT mice
V–VII	● No significant differences in γ-H2AX immunostaining ● Significant increase in OGG1 staining intensity in spermatocytes of aged WT mice ● LIGIV staining decreased significantly in young Sod1−/− mice compared to WT
VIII–IX	● Increase in γ-H2AX staining in aged WT mice compared to young WT ● Young Sod1−/− mice showed increased γ-H2AX staining compared to young WT ● Aged Sod1−/− mice showed significantly higher 8-OHdG levels compared to aged WT
X–XII	● Increase in γ-H2AX staining in aged WT mice compared to young WT ● Significant increase in OGG1 staining intensity in spermatocytes of aged WT mice ● LIGIV staining decreased significantly in young Sod1−/− mice compared to WT ● Aged WT showed decrease in LIGIV staining

3.1.3 Altered DNA repair gene expression

A comparison was made between spermatocytes of young and aged Sod1−/− mice and their WT control groups. RT-qPCR was performed with a focus on the following genes: Ogg1, LigIV, Rad51. Their expressions were evaluated in pachytene spermatocytes. For the exact findings per stage see table 3.
Rad51 is an enzyme which is active in homologous recombination. It is responsible for catalysation of the exchange between damaged DNA and the sister chromatid to repair DSBs.

Table 3. Alteration is gene expression in pachytene spermatocytes

stage	findings
Ogg1	• Increased expression in young Sod1−/− mice compared to young WT • No significant changes in Ogg1 expression in aged mice, both WT and aged.
LigIV	• Only aged Sod1−/− mice had a significant lower expression of LigIV compared to aged WT
Rad51	• Sod1−/− mice all showed decreased expression in comparison with their aged matched WT control • Ageing did not contribute in expression

No specific conclusions could be made given the variety between stages of spermatocytes. However, overall findings show that seemingly both aging and a sod1 -/- KO have a significant influence. The following section will go into several of the methods used in this study and will add some valuable other options.

4. Methods to measure oxidative stress

Oxidative stress can be measured directly or indirectly. Direct methods measure reactive oxygen species in real time with the use of certain probes. However, ROS are very reactive and have a short life span. That together with the fact that human gametes produce a very low amount of ROS [22], makes real-time detection challenging. Another possibility is measuring indirectly. Indirect methods detect the damage done by oxidative stress, such as lipid peroxidation, low levels of antioxidants, and DNA or RNA damage. Tables 4 and 5 summarise the indirect and direct measuring methods.

3.1 Indirect methods

3.1.1 Detection of lipid peroxidation

Lipids are an important component of the cell membrane. The membrane of mammalian spermatozoa is distinctly different from the somatic cells in terms of lipid composition. A higher level of PUFAs is observed. These lipids have unconjugated double bonds separated by methylene groups. These groups, located adjacent to the double bond, weaken the methyl carbon-hydrogen bonds. This increases their susceptibility to oxidative damage [23].

Lipid peroxidation (LPO) is a chain reaction from which several subproducts are generated. They can be detected indirectly as markers of oxidative stress. Primary products are unstable hydroperoxides. These molecules decompose to several stable secondary products, including stable aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) [24]. Oxidative stress attributes to the presence of thiobarbituric acid reactive species (TBARS). MDA forms adducts with TBARS, these complexes can be detected by TBAR assays [3]. TBAR assays are a relatively easy method, however a disadvantage is that an TBAR essay is not entirely specific for MDA [25]. Furthermore, it is known that MDA-unrelated species in biological samples can bind to TBARS and thus interfere with the reliability of the measurement. In addition, this type of assay is also not very sensitive [24].

High performance liquid chromatography (HPLC) and Competitive ELISA assays are also feasible methods for detecting MDA [25]. HPLC is said to be an accurate, reliable, and time effective method [24]. Nevertheless, [mention one disadvantage]

HNE, as mentioned before, is another stable secondary product of LPO. Similarly to MDA, methods to detect this compound include ELISA and chromatography combined with mass spectrometry. The

chromatography can be done in both gaseous and liquid state [3]. Nevertheless, [mention one disadvantage]

3.1.2 Antioxidants and redox molecule levels

There are antioxidant pathways in place to naturally combat oxidative stress in cells. Enzymes involved in these pathways are maintained at a certain level under physiological amounts of ROS. A disbalance in these levels indicate for example an elevated ROS presence. See figure 4 for an overview of the antioxidant defence mechanisms.

superoxide radicals are the first target in the antioxidant defence mechanisms and is created through various processes. Firstly, oxygen gets transformed to superoxide radicals (O2-) by means of specialised enzymes such as xanthine and NADPH oxidases. Secondly, other cell processes such as cell metabolism also play an important role in creating superoxide radicals, for example through the mitochondrial electron transport chain [26].

Superoxide dismutase (SOD) also known as CuZnSOD, an antioxidant enzyme, converts superoxide to hydrogen peroxide (H2O2). The enzyme has a catalytic site containing the copper ion, whereas the zinc adds stability [27].

Catalase (CAT) works together with glutathione peroxidase (GPx) to further neutralise H2O2 to water. GPx is a critical factor in the antioxidant cell system because apart from H2O2 it is also able to catalyse HO2• [27]. There are many isoforms of GPXs but GPX4 is the most abundant in the testis and a knockout of this gene is associated with reduced fertility [28]. GPXs use reduced glutathione (GSH) as electron donor. Glutathione reductase reduces the oxidised glutathione (GSSG) back to GSH.

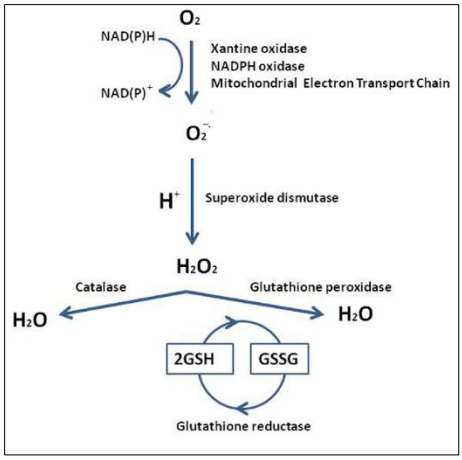

Figure 5. ***Main pathways of the cellular antioxidant defence system:***
O*verproduction of ROS is faced by superoxide dismutase (SOD) and glutathione peroxidase (GPx). The resulting oxidation product is recycled by glutathione reductase (GR) that will transform the oxidised glutathione (GSSG) back to the reduced form of this molecule (GSH)* [26].

3.1.3 DNA damage

Oxidative stress can cause different types of DNA damage. Therefore there are also a lot of options for the detection of DNA damage. The most commonly used marker is 8-hydroxydeoxyguanosine (8-OHdG) (see table 4). (8-OHdG) is an oxidised derivative of deoxyguanosine. This marker is created when a destructive agent interacts with one of the main nucleotides in DNA, guanine [29].

Apart from damage markers, repair markers can be used to assess the level of damaged DNA. This approach measures the activation of the DNA damage response (DDR)system that is triggered after lesion formation in the DNA [29]. After a DSB, phosphorylation of nearby histone variant H2AX occurs. This phosphorylation activates signalling DNA repair machinery [29]. γ-H2AX forms *foci* that can be

microscopically detected as fluorescent spots if immunofluorescence staining is performed. A 1:1 correlation between *foci* and DBS breaks has been confirmed, which makes quantification easier. These *foci* can be analysed through confocal microscopy or epi-fluorescence [29].
Many of these repair markers exist. Some examples are ATM, ATR (protein kinases that phosphorylate substrates to promote effective and accurate DNA repair [30]), but also LIGIV as mentioned before (part of the repair pathway of DSBs by joining the two ends of the DNA)[19].

3.2 Direct methods

3.2.1 Chemiluminescence

Chemiluminescence measures oxidative end products of the reaction of ROS with a certain probe. This reaction creates a certain amount of light that can be detected using a luminometer containing a photomultiplier tube. Chemiluminescence is a direct method of measuring ROS since it detects these molecules in real time through a light producing reaction, whereas indirect methods measure stable end products created by the presence of ROS at a certain moment in time.
A reagent that is often used for this method is luminol, a very sensitive reactant that reacts with a wide range of reactive oxygen species (see table 4). In addition, the reaction with luminol is fast and has a very short half-life, meaning the time between measurement and detection is very limited. However the main disadvantage of this reagent is its inability to differentiate between different types of ROS and intra- or extracellular ROS [22]. A more specific reagent is lucigenin, which detects mostly superoxide species. This reagent is however membrane impermeable, meaning that it is only able to measure extracellular ROS.
The results of chemiluminescence can be affected by many variables such as concentration of reactants (sample and probe), temperature control, background luminescence, reagent injection, and sample volume [22]. Therefore, to obtain reliable and consistent results, familiarity with these constants is crucial. This factor complicates the reproductions of previous studies performed with this method. On top of that, comparing results between research groups may be complex as well. In order to use this method for ROS measurements, a strict quality control must be performed. [31].
There are also multiple luminometer designs. They mainly differ in the processing of the input signal. One way of processing is counting the individual photons that are detected by the photomultiplier tube. These are called photon-counting luminometers. On the contrary, direct current luminometers measure the electric current that is created by, and is proportional to, the photon flux that passes through the photomultiplier tube. These methods either result in measurements given in relative light units (RLU) or counted photons per minute (cpm) or mV/s [22].

3.2.2 Flow cytometry

Flow cytometry is used for making quantitative measurements on single cells or cell constituents at a very high speed. A single particle crosses the centre region of a laser beam. This laser can excite the particle at a certain wavelength to induce fluorescence, which can be measured together with light scatter properties. Light scatter measurements give information on the size and surface characteristics of the cell [32].
Many fluorescent probes can be used to evaluate ROS concentration due to oxidative stress [33]. One of the most common is 2,7-dichlorofluorescein diacetate (H2DCFDA). This compound is membrane permeable and upon diffusion over the membrane deacetylation by esterases takes place, forming $DCFH_2$. This molecule is impermeable and thus encaptured inside the cell. $DCFH_2$ gets oxidised by ROS to DCF, making it fluorescent. This is measurable in a flow cytometer with a fluorescein (FL1) channel [33].
Superoxide does not react strongly with $DCFH_2$ and therefore another probe is used: DHE. DHE reacts with superoxide to form 2-hydroxyethidium, which is fluorescent. The two probes could be used together to measure a wide range of ROS.

A disadvantage of this method is that the sample needs to be a suspension of single cells. Also, when using multiple fluorophores it is important to consider possible overlap of spectral emissions. An advantage of this method is its fast sorting speed possibility. However, to do high speed pressure sorting, particle concentration needs to be high. This is not always feasible when working with viable cells [32].

3.2.3 Diamond magnetometry

Diamond magnetometry is a novel method that makes use of nanodiamonds for sensing specifically free radicals among all kind of reactive oxygen species. The nanodiamonds used for this method have so-called colour centres. These can be defined as impurities that are formed by one or a few foreign atoms or vacancy in the lattice of the diamond structure [34]. Given that these centres are located within the lattice structure, their optical and physical properties are stable. These centres also introduce extra electronic states in the diamond wide band gap. Transitions between these extra states give rise to absorbance and emittance of visible light spectrum [34].

A negatively charged nitrogen-vacancy (NV) centre refers to a diamond structure with a point defect consisting of a substitutional nitrogen atom and a vacancy in its neighbouring lattice site [35]. It has been shown that NV centres offer excellent magnetic sensitivity. Therefore their application is extremely useful for sensing free radical oxygen and nitrogen species [34] , since the optical properties of these centers change according to the magnetic noise in the surroundings of the diamonds. This technique offers subcellular resolution for real-time measurements. So far, the main disadvantage is that the cells need to uptake the diamonds to perform intracellular measurements.

Table 4. Indirect measuring methods

	marker	formation	Assay/method
Lipid peroxidation	Malondialdehyde (MDA)	peroxidation of PUFAs	TBARS assay, Competitive enzyme-linked immunosorbent assay (ELISA), HPLC, gas chromatography mass spectrometry (GC-MS),
	4-hydroxy-nonenal (HNE) and Isoprostanes (F2-isoprostane, 15-(S)-8-isoprostagladin F2α) [3]	By-product of PUFA peroxidation.	ELISA, (GC-MS), liquid chromatography- mass spectrometry (LC-MS), [3]
Antioxidants/redox molecule levels	Superoxide dismutase (SOD)	Reduced levels point to high levels of ROS. SOD reduces hydrogen peroxide to water.	Plate assay [25]
	Glutathione (GSH)	Reduced levels point to high levels of ROS. GSH reduces hydrogen peroxide to water.	Flow Cytometry [25], Adding 5,5' -dithiobis-2-nitrobenzoic acid (Ellman's reagent), producing a quantifiable yellow colour [3]
	Catalase (CAT)	Reduced levels point to high levels of ROS. CAT reduces hydrogen peroxide to water.	Plate assay [25]
General assays to measure total antioxidant capacity	<u>Trolox equivalent antioxidant capacity assay (TEAC):</u> measures the antioxidant capacity of a given substance, as compared to the standard: Trolox [25]. <u>Oxygen radical antioxidant capacity assay (ORAC)</u> Measuring the ability of antioxidants to reduce the quenching ability, caused by ROS, of a fluorescent dye [25].		
DNA damage	8-hydroxydeoxyguanosine (8-OHdG)	Oxidation of base guanine in DNA	GC-MS HPLC [29]
	γ-H2AX	Histones (variant H2AX) local to DSB are able to get phosphorylated	confocal microscopy, epi-fluorescence [29]
Assay to measure DNA damage	<u>Comet assay:</u> Relies on that electrophoresis of DNA embedded in an agarose gel is increased in the presence of a SSB [29].		

Table 5. Direct measuring methods

method	probe	Membrane-permeable	Detection of	mechanism
Chemiluminescence	luminol	yes	superoxide molecules (O2 -), hydroxyl radicals (OH-), Hydrogen peroxide (H2O2) [22]	Oxidants bind, causing a chemical process to take place. Resulting in emission of light [3].
	lucigenin	no	superoxide molecules (O2 -)	Probe is positively charged thus membrane impermeable. Measures hydroxyl radicals in extracellular space. Mechanism is comparable to luminol [3].
Flow cytometry	2,7-dichlorofluorescein diacetate (H2DCFDA)	yes	Hydrogen peroxide (H2O2), hydroxyl radicals (OH-), peroxyl radicals (ROO-)	Inside cell hydrolysis to DCFH. Which reacts with ROS to create fluorescent DCF (2,7-dichlorofluorescein) [36].
	Dihydroethidium/ hydroethidine (DHE)	yes	superoxide molecules (O2 -)	DHE is directly oxidised to 2-hydroxyethidium, by reacting with superoxide molecules, which fluoresces [36].
Diamond magnetometry	nanodiamonds	no, but they can be uptaken by different cell types	Free radicals	The diamonds have NV centres, these are excited which is the ground state. The electrons in the vacancy have a certain spin that is influenced by spin noise in surroundings. T1 measurements gives you an indication of the surrounding spin noise [34].

6. References

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