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Review

Redox regulation and oxidative stress: the particular case of the stallion spermatozoa

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Abstract

Redox regulation and oxidative stress have become areas of major interest in spermatology. Alteration of redox homeostasis is recognized as a significant cause of male factor infertility and is behind the damage that spermatozoa experience after freezing and thawing or conservation in a liquid state. While for a long time, oxidative stress was just considered an overproduction of ROS, nowadays it is considered as a consequence of redox deregulation. Many essential aspects of spermatozoa functionality are redox regulated, with reversible oxidation of thiols in cysteine residues of key proteins acting as an "on-off" switch controlling spermatic function. However, if deregulation occurs, these residues may experience irreversible oxidation and oxidative stress leading to spermatic malfunction and ultimately death. Stallion spermatozoa are "professional producers" of ROS due to their intense mitochondrial activity, and thus sophisticated systems to control redox homeostasis are also characteristic of this species. As a result, combined with the fact that embryos can easily be collected in this species, horses are a good model for the study of redox biology in the spermatozoa and its impact on the embryo.

Key words: horses, spermatozoa, ROS, oxidative stress, redox regulation, equine

Introduction

The male gamete, the spermatozoon, is generated in the germinal epithelium of the testes in a process called spermatogenesis. This epithelium consists of germ cells in different stages of development, intermingled with Sertoli cells that provide

structural support, nursing, and protecting the germ cells. Spermatogenesis is initiated by the differentiation of spermatogonia from a stem cell pool. These cells initiate a proliferative phase entering a continuous process of mitotic division, dramatically increasing spermatogonial numbers. This process is usually termed spermatocytogenesis. In the next step, cells enter a meiotic phase that includes duplication and exchange of genetic information and two meiotic divisions which reduce the chromosome complement to form round haploid spermatids. During the spermiogenesis phase, round spermatids experience a dramatic transformation that includes compaction and silencing of DNA and elongation of the nucleus, development of specific structures such as the sperm tail and acrosome, relocation of the mitochondria in the midpiece in addition to the loss of other organelles and most of the cytoplasm. Fully developed spermatozoa are released in the lumen of the seminiferous tubules in a process termed spermiation. Recent reviews on this topic can be found elsewhere [1-4]. Chemically, oxidation is the loss of an electron, while reduction is the gain of an electron. This nomenclature reflects the tendency of oxygen, a highly electronegative atom, to partially or fully steal an electron from other molecules. Reactive oxygen species (ROS) [5,6] are atoms or molecules with a single unpaired electron, including, among others, superoxide ($O_2^{\bullet-}$), the hydroxyl radical ($HO^{\bullet}$) and the lipid peroxide radical ($LOO^{\bullet}$). Although hydrogen peroxide (H_2O_2) is not a free radical, it is a precursor of $HO^{\bullet}$. UV radiation and the presence of metal ions (Fe^{2+}, Fe^{3+} or Cu^{+}) generate $HO^{\bullet}$. All aerobic organisms depend on the generation of ATP from electrochemical energy generated in the four electron reduction of molecular oxygen into water. During this process the mitochondrial transport chain may lose electrons, leading to the formation of reactive oxygen species.

Moreover mitochondrial dysfunction may exacerbate the loss of electrons and thus increase the production of reactive oxygen species to toxic levels disrupting redox homeostasis [6]. This particular effect is especially critical in horses. The stallion

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spermatozoon is characterized by an unusually intense mitochondrial activity in comparison with other mammals [7-11].

Spermatozoa were the first cells known to be capable of generating reactive oxygen species (ROS) [12]. This early report demonstrated that bovine spermatozoa produce H_2O_2 as a consequence of cellular respiration. It also showed that the production of H_2O_2 inhibits respiration and concluded that bovine spermatozoa must be equipped with a mechanism for the elimination of H_2O_2 at a low rate, to keep it at physiological levels. For a long time the production of ROS was considered solely as a toxic byproduct of sperm metabolism; however nowadays extensive evidence indicates that crucial functions of the spermatozoa are redox regulated, and redox regulation has become a major area of research in sperm biology [13-20]. Since the discovery of ROS production by the spermatozoa, the concept of oxidative stress has evolved, and enormous research interest in this topic has developed in the last decade. As an example, a recent search in PubMed retrieved 215842 entries using the term oxidative stress, when this term was combined with spermatozoa 2777 entries were obtained (https://www.ncbi.nlm.nih.gov/pubmed/, accessed September, 1 2019). Under aerobic conditions, production of ROS is unavoidable. However organisms have evolved to develop complex mechanisms to maintain the production of ROS at physiological levels (oxidative eustress) and the redox signaling dependent on ROS regulated [21-23]. Interestingly the ability to respond to ROS appeared very early in the course of evolution, well before the increase of atmospheric oxygen, probably in response to low ozone levels, since U.V. radiation splits water into ROS [24].

Sources of ROS in the spermatozoa

In general terms, several pathways lead to the generation of ROS, including the production of $O_2^{-\bullet}$, H_2O_2, reactive nitrogen species (RNS), and $OH^{\bullet}$ [25]. The superoxide anion is generated from the coupling of O_2 with an electron (e-). The

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electron donor is usually NADH or NADPH, and the reaction is catalyzed by various oxidases; NADPH oxidases, xanthine oxidase and complex I/II/III/IV from the mitochondria [25]. The generation of H_2O_2 occurs after the dismutation of $O_2^{-\bullet}$, mostly catalyzed by superoxide dismutases (SODs) although a small percentage occurs spontaneously, some oxidases also have dismutase activity, and may contribute to direct production of peroxide from superoxide. The reaction of $O_2^{-\bullet}$ with reduced transition metals may lead to formation of H_2O_2 [25]. Most of the $OH^{\bullet}$ is generated from H_2O_2 and $O_2^{-\bullet}$ in a reaction catalyzed by a metal ion (iron or cupper). This is known as the Habor-Weiss reaction. This reaction occurs in two steps, in the first step, $O_2^{-\bullet}$ reduces Fe^{3+} to Fe^{2+} (Fe^{3+} $O_2^{-\bullet} \rightarrow Fe^{2+} + O_2$), and the second step is the Fenton reaction where Fe^{2+} reacts with H_2O_2 to generate $OH^{\bullet}$ and OH^- ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$) [25]. Nitric oxide and ONOO- (form by the combination of NO and $O_2^{-\bullet}$) are the most important reactive nitrogen species in spermatozoa [25].

Several potential sources can be responsible for ROS production in the spermatozoa, including the spermatozoa itself and contaminating cells in the ejaculate. Dead spermatozoa are a major source of ROS, frequently overlooked in reproductive technologies[26]. L-amino oxidase (LAAO) is present in stallion spermatozoa, being able to generate significant amounts of reactive oxygen species; aromatic amino acids are substrates for this enzyme, producing substantial amounts of ROS, especially in the presence of dead spermatozoa [26]. Interestingly cryopreservation media contain sufficient amounts of aromatic amino acids to activate this enzyme. Ongoing proteomic studies in our laboratory have also confirmed the presence of this enzyme in stallion spermatozoa. A NADP oxidoreductase system has been detected in the membrane [27], however nowadays it is considered that the main source of reactive oxygen species are electron leakage in the mitochondrial electron transport chain (ETC) [7,8,10,28-31]. In particular, defective mitochondria may represent a hallmark of male infertility. Evidences of mitophagy in human sperm were described in our laboratory, suggesting that

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activation of mitophagy is a mechanism that maintains proper sperm function [32]. The sources of reactive oxygen species in the electron transport chain of the stallion spermatozoa have also recently been investigated in our laboratory [9,10], confirming the role of the ETC as a main source of ROS in stallion spermatozoa.

Redox regulation and signaling

Although initially oxidative stress was defined as a disturbance in the pro-oxidant-antioxidant balance in favor of the former, current knowledge has evolved and oxidative stress is better defined in terms of regulation of redox signaling. Numerous processes are redox regulated in biological systems. Redox regulation is similar to pH regulation, the pH varies in different cellular compartments, also the redox state is not an overall redox state and vary in different compartments of the spermatozoa[33]. Redox reactions consist of the transfer of electrons (e^-) from one molecule (oxidation) to another molecule (reduction). Thus, reduction implies a decrease in overall charge (more e^-) of the molecule, while oxidation implies an increase in overall charge (fewer e^-). Reactive oxygen species, such as the superoxide anion $O_2^{-\bullet}$, are low molecular weight compounds that are chemically unstable, particularly in biological systems [21]. The hydroxyl radical is the most reactive and oxidizes virtually any closer molecule. The reactivity of $HO^\bullet$ is $7x10^9$ L mol^{-1} s^{-1}, while the rate constant for $O_2^{-\bullet}$ is <0.3 and is $2x10^{-2}$ L mol^{-1} s^{-1} for H_2O_2 [33]. Another electronically excited state of interest in spermatology is singlet molecular oxygen, generated by photoexcitation mainly by ultraviolet A and B light rays, but even infrared and visible light may also generate photobiological responses. This is the rationale of the customary procedure of avoiding light exposure during semen processing [33]. Other species include alkoxyl and peroxyl radicals, non-radical species such as hypochlorite, peroxynitrite, singlet oxygen and lipid peroxydes, among others [34]. To understand the basis of redox signaling it is important to bear in mind the characteristics of different ROS. As previously mentioned the $HO^\bullet$ is the

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most reactive, and has the shortest half life (10^{-15} s.)[24]. The $HO^{\bullet}$, is considered to be the most harmful oxidant, with no signaling functions. Although $O_2^{-\bullet}$ may have difficulty diffusing through membranes due to its anionic charge, it may use specific channels in some tissues [35-37]. Hydrogen peroxide is a stable compound and in addition is a nonpolar molecule that can easily diffuse through membranes, and is also transported through aquaporin channels [24,38-40]; all of which make H_2O_2 a suitable molecule for redox signaling. ***The primary target of hydrogen peroxide is the thiol group of the amino acid cysteine, which is oxidized in a reversible fashion***. The presence of glutathione and other thiols in spermatozoa is well known [41,42], also the role of oxidative regulation in significant biological processes occurs in very early stages of development. For example, studies in sea urchin, show an oxidative burst that occurs at the time of fertilization preventing polyspermy through the activation of a dual oxidase (Udx1), that induces cross linking of surface proteins on the egg surface [43,44]. Also oxidation reduction processes of sulfhydryl groups of protamines are critical for chromatin condensation during spermatogenesis [45].

Many cellular processes are redox regulated. In spermatozoa redox regulation has been extensively studied in relation to capacitation [13,15,46-51]. Capacitation is the maturational process that sensitizes spermatozoa to recognize and fertilize the oocyte. Capacitation involves, removal of cholesterol from the plasma membrane, removal of coating materials from the membrane, a rise in intracellular Ca^{2+}, an increase in intracellular cAMP, and a dramatic increase in tyrosine phosphorylation. Also during capacitation the sperm plasma membrane potential (E(m)) hyperpolarizes [50,52,53], and spermatozoa experience alkalinization. Interestingly only a subpopulation of spermatozoa is able to experience capacitation. Tyrosine phosphorylation is a redox regulated process [17,20,48,54-59]. Other functions of the spermatozoa, such as activated motility may also be redox regulated [17,60], in relation to tyrosine phosphatases (PTPs), which are intracellular targets for ROS [61]. The activity of PTPs depends on a conserved Cys residue, where oxidation results

in the inactivation of the enzyme [22,62]. On the other hand, ROS can also activate kinases. In addition to hydrogen peroxide, other species such as GSSG, hydrogen sulphide and lipid peroxides can inactivate PTPs [63]. Reversible oxidation of target cysteine residues in specific proteins modulates its activity [22]. In order to function in a reversible manner oxidized cysteine (Cyss) residues need to be reduced. This reversibility depends on adequate availability of reducing molecules including the peroxiredoxin (PRDX) family of antioxidant enzymes [22]. Peroxirredoxins have been described in spermatozoa [13-15,64] and play a major role in sperm function, stressing the importance of redox signaling in these highly specialized cells. Reversing the oxidized Cys residue in this family of pathways involves thiorredoxin or GSH. Reduction of the higher oxidation state (sulphinic acid SO_2H) may require sulfiredoxin or sestrins [22,65]. This reversible sequential oxidation of PRDXs allows a tight regulation of the function of these proteins in a regulation described as a "floodgate" model [66,67]. Spermatozoa are rich in thiols [41], with the majority of thiol groups associated with proteins, which may suggest that redox regulation is an important regulatory mechanism in these cells. Spermatozoa are transcriptionally silent cells whose regulation depends on post transcriptional modification of proteins. One interesting example, since mitophagy has been recently described in spermatozoa [32], of proteins regulated by reversible oxidation of Cys residues, is the large family of Cys-dependent proteases [22]. In particular the cysteine protease HsAtg4 is a direct target for oxidation by H_20_2, specifically a residue located near the protein's catalytic site [68]. The presence of a similar mechanism in spermatozoa is an intriguing possibility and deserves further research [32]. Other functions in the spermatozoa that are redox regulated, include control of motility [60], and binding to the oviductal epithelium to form the sperm reservoir [69-71].

Modern concept of oxidative stress applied to spermatozoa

Since redox regulation is being unveiled as a major mechanism regulating sperm function, probably at the same level as tyrosine phosphorylation and other post translational modifications of sperm proteins, sophisticated mechanisms must be present to maintain redox status under physiological control. Both seminal plasma and the spermatozoa itself contain enzymatic and non-enzymatic systems that contribute to maintenance of oxidative eustress. Recent research from our laboratory shows that in stallion spermatozoa seminal plasma plays a major role in regulating redox status. The steady state redox potential (E_h) can be estimated using the Nerst Equation: Eh= Eo+ RT/Ln [oxidized molecule/reduced molecule], where Eo is the standard reduction potential, R=gas constant, T is the absolute temperature, n= number of electrons transferred and F is the Faraday constant [23]. Recently, a system to easily measure the steady state in semen has become available and is being introduced into reproductive medicine and clinics. Using this system E_h is provided as the static oxidation reduction potential (sORP) and is expressed as millivolts per million spermatozoa. E_h in raw semen (seminal plasma present) was measured and was found to be 1.62 ± 0.06 mV/10^6 spermatozoa, when seminal plasma was removed, it was 7.9 ± 0.79 mV/10^6 spermatozoa, thus showing a much higher overall oxidation status [72]. This finding suggests that regulation of the extracellular medium may also be of great importance as is the case in other cells [72], from this viewpoint it is well recognized that equine seminal plasma is rich in antioxidants [73-78]. On the other hand is important to consider that once the semen is deposited in the mare's uterus or is processed, the antioxidants in seminal plasma are removed from close contact with the spermatozoa; meaning the importance of intrinsic antioxidant defenses in the spermatozoa become critical [13,15,79,80].

The spermatozoa itself also has antioxidant defenses, including glutathione, and other enzymatic antioxidant defenses such as the paraoxonase [81-85], thioredoxin [15,86-92] and peroxiredoxin [13,14,64,79,80,93-95] families of proteins. Ongoing proteomic studies in our laboratory have identified peroxiredoxins 5 and 6, and

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thioredoxin reductase in stallion spermatozoa. Interestingly, the concentration of intracellular GSH in the horse spermatozoa is higher than in most domestic species. A recent study in our laboratory revealed that the mean concentration of GSH in stallions was 8.2 ± 2.1 μM/10^9 spermatozoa [96], while values reported in other species are in the nanomolar ranges per billion spermatozoa [41]. These high levels of GSH in stallion spermatozoa, may be linked to the intense mitochondrial activity of the spermatozoa in this species. Intense mitochondrial activity causes increased ROS production, and thus sophisticated mechanisms to maintain redox homeostasis may have evolved differently between species with spermatozoa less dependent on oxidative phosphorylation for ATP production. In relation to this, evidence of the presence and activity of the Cystine antiporter SLC7A11 in stallion spermatozoa has been discovered [72].This antiporter exchanges extracellular cystine (oxidized form of cysteine) for intracellular glutamate. Once in the cell, cystine is reduced and used for GSH synthesis. Indirect evidence of the presence of a system exporting glutamate in spermatozoa were reported as early as in 1959 [97]. Evidence of GSH synthesis in stallion spermatozoa [96], include the presence of the enzymes glutathione synthetase (GSS) and gamma glutamylcysteine synthetase (GCLC). In addition, functional studies indicate their activity; the use of the specific inhibitor L-Buthioninine sulfoximide (BSO) reduced GSH synthesis from cysteine. In this particular experiment mass spectrometry (MS) was used to specifically identify GSH and avoid interference with other thiols. Overall these results point to a sophisticated redox regulation in stallion spermatozoa. It is considered that most extracellular cysteine is present in the disulfide form (cystine), thus the presence of the xCT /SLCTA11 antiporter may be a major mechanism of cystine incorporation in the spermatozoa. This antiporter is present and active in stallion spermatozoa [72]. In addition to its role in the incorporation of cysteine for GSH synthesis, a potential role in an active Cys/Cyss redox node in the spermatozoa must be considered. Overall, these recent findings support the hypothesis of a complex redox regulation in the spermatozoa. Oxidative stress is thus better defined as the fail in the regulation

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of redox signaling due either to overproduction of ROS, or exhaustion of regulatory mechanisms. This latter point has recently been addressed, and functionality of the stallion spermatozoa is linked to thiol content. When thiols are exhausted stallion spermatozoa rapidly enters senescence, which is characterized by increased production of lipid peroxides, activation of caspase 3, loss of motility and death [98,99]. The stallion spermatozoa is a paradigm of this sophisticated redox regulation; recent research has shown apparently paradoxical results, in this regard more fertile spermatozoa show increased ROS production [8], further underlining the concept that a tightly controlled redox regulation occurs in stallion spermatozoa.

The mitochondria in redox signaling

Electrons can be prematurely leaked to oxygen in the ETC or associated to catabolism of substrates [100,101]. Depending of the number of electrons being leaked different outcomes are possible. If leaked one by one they generate superoxide radicals, if in pairs they generate hydrogen peroxide. When are properly transferred four at a time, they generate water and drive OXPHOS at complex IV of the ETC. A growing body of scientific evidence is stressing the role of proper mitochondrial function in sperm physiology [7,9-11,28,31,32,102-106]; moreover definition of oxidative stress as the result of mitochondrial malfunction, states that it is the result of *"a dysfunction of electron transfer reactions leading to oxidant/antioxidant imbalance and oxidative damage to macromolecules"*[107]. This theory states that $O_2^{-\bullet}$ does not accidentally leak from the ECT, but instead is a signaling molecule [107]. Recent research in our laboratory with an aryl hydrocarbon receptor deficient ($AhR^{-/-}$) mouse strain showing males of unusually high fertility (also in terms of number of pups born) showed that this strain was characterized by higher mitochondrial activity [108], other reports also link mitochondrial activity with fertility in humans and equines [7,8,28,31,104,109,110]. Interestingly the mitochondria are the more sensitive structure in the spermatozoa to stress induced by different biotechnologies,

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and have been proposed as a sensitive marker of spermatic quality and fertilization ability [108]. Mitochondrial roles in the spermatozoa may include Ca_2^+ storage and signaling, production of ATP, control of spermatic lifespan and activation of a specific form of apoptosis for silent, non-inflammatory elimination of redundant spermatozoa after insemination, and potentially control of redox signaling. Numerous evidence points to mitochondria as the hallmark of fertile spermatozoa. However proper evaluation of mitochondrial function in spermatozoa is still elusive, and rarely performed in clinical settings. Fluorescent probes and flow cytometry represent the method of choice to study mitochondrial function in spermatozoa, with the potential for analysis of thousands of spermatozoa and simultaneous functions in every single spermatozoon, together with the recent development of computational methods [29] to study sperm subpopulations makes this the gold standard. However technical difficulties preclude its wider use in reproductive medicine. These difficulties relate to special characteristics of commonly used probes, such as the JC-1. This dye is difficult to compensate using the 488 nm excitation laser due to the spectral characteristics of the fluorochrome, and the dual excitation depending on the formation of monomers (low mitochondrial membrane potential) of aggregates (high mitochondrial membrane potential). This particular issue can be addressed using dual excitation; monomers with the blue 488 nm laser, and aggregates with the 561nm yellow laser. The application of computational methods to the analysis of data, also improves the identification of specific spermatic subpopulations. The production of hydrogen peroxide in stallion mitochondria have been investigated in our laboratory [10], inhibition of complex I of the ETC increased the production of mitochondrial superoxide and hydrogen peroxide, suggesting that mitochondrial malfunction is a potential source of redox deregulation in stallion spermatozoa, inhibition of complex III also caused increased ROS production. In addition, the above-mentioned study underpinned the importance of cautious selection of probes to assess ROS in spermatozoa. However, mitochondrial dysfunction may lead to either reduced or

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increased production of ROS [100] depending on the cause of the dysfunction and caution interpreting the results of the analysis of ROS production in spermatozoa is always advised. Specific antioxidant defenses in the mitochondria of the stallion spermatozoa include mitochondrial GSH, peroxiredoxin 5 and manganese dependent superoxide dismutase (Mn-SOD). Mitochondrial ROS have been implicated in numerous signaling pathways in somatic cells [100] and is also likely that these species may participate in signaling in spermatozoa. Together with its importance in sperm regulation, the special characteristics of the spermatozoa, a cell devoid of most organelles and a very limited cytoplasm, may also mean this cell is a suitable model for the study of mitochondrial function.

Redox regulation and sperm metabolism

Together with mitochondria, stallion sperm metabolism have been of increased interest for scientists focused in equine reproduction in recent years. Mitochondria play major roles in cellular metabolism, being the energetic power-house of the cell [111]. Oxidative phosphorylation (OXPHOS) and the tricarboxylic acid cycle (TCA cycle) are well known mitochondrial functions. Recent specific research in horses has underlined the importance of mitochondria as a provider of energy in the form of ATP, and the consequences it has for sperm physiology and the functional evaluation of the spermatozoa. Early studies suggested that spermatozoa were glycolytic cells, however the participation of oxidative phosphorylation in production of energy is now acknowledged. Early studies also suggested that ATPs produced by mitochondrial respiration could not reach distal parts of the flagellum. To solve this problem shuttle systems and/or glycolysis ought to be present. Also, species specific strategies occur in the predominance of one energy source. Recent proteomic studies indicate that the spermatozoa can use different substrates for energy, possessing the ability to oxidize fatty acids. The stallion spermatozoa is considered to predominantly use OXPHOS for the generation of energy [7,8,11,105].

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The adenine nucleotide translocator (ANT) catalyzes the transmembrane exchange of ATP, generated by oxidative phosphorylation, for cytosolic ADP [112]. Inhibition of this protein leads to reduced sperm motility suggesting that ATP produced by OXPHOS in the mitochondria plays an important role in spermatic motility in horses. Further studies aimed to clarify the role of mitochondrial ATP in stallion sperm motility. Inhibition of OXPHOS reduced spermatic motility and ATP content in stallion but not in human spermatozoa suggesting species specific differences in energetic metabolism [8]. Moreover, this study showed paradoxical relations between fertility and oxidative stress, fertile stallions were characterized by spermatozoa showing increased levels of 8-hydroxiguanidine and the superoxide anion. These increased levels were attributed to increased mitochondrial activity in the spermatozoa of fertile stallions [8]. The relation between increased mitochondrial activity and ROS production has also been confirmed in independent studies [11]. In addition, and in line with these findings a dramatic decrease in sperm ATP content after mitochondrial uncoupling and inhibition of mitochondrial respiration was reported [9]. Reduction of ATP was accompanied by low motilities and velocities, and interestingly inhibition of mitochondrial respiration at the ATP synthase complex collapsed sperm membranes. This may relate to the high ATP consumption necessary to maintain the activity of the Na^+-K^+ ATPase pump in the spermatozoa [113]. The relation between ROS production and mitochondrial activity was also confirmed. Despite the predominance of OXPHOS, glycolysis and other sources of energy are also present in the spermatozoa. OXPHOS takes place in the mitochondria located in the sperm midpiece, while glycolysis occurs mainly in the flagellum in which the fibrous sheath is rich in glycolytic enzymes where they are anchored [114-116]. The substrate for glycolysis is glucose, which is incorporated into the spermatozoa through diverse glucose transporters (GLUTs) [117]. Oxidative phosphorylation uses diverse sources of substrates derived from the metabolism of carbohydrates, lipids and amino acids. While for a long time a debate has existed among spermatologists regarding the main source of energy in spermatozoa, the

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existence of different bioenergetic strategies in different species is now becoming clear [118], and thanks to the introduction of the omics technologies into spermatology, the spermatozoa is being unveiled as a cell with much higher bioenergetic plasticity that previously assumed [119,120]. In this regard, recent proteomic studies in horses and humans reveal that beta oxidation of fatty acids plays an important role in providing energy for the spermatozoa [120,121]. The pentose phosphate cycle pathway (PPP) is also present in spermatozoa [118,122-127]. NADPH produced by the PPP is important for the re-activation of 2-CysPRDXS.[79] In human spermatozoa the pentose phosphate pathway can respond dynamically to oxidative stress [128] and the inhibition glutathione reductase impairs the ability of sperm to resist oxidative stress and lipid peroxidation [126]. Also, NADPH may play a role in relation to the activity of an NADPH oxidase which plays a role in capacitation [123]. The glutathione peroxidase-glutathione reductase-pentose phosphate pathway system is functional and provides an effective antioxidant defense in normal human spermatozoa [126,129]. Overall current knowledge on sperm metabolism, suggests species specific differences and a great metabolic plasticity in the spermatozoa, which are able to adapt their metabolism to the changing environments that they are exposed to on their travels to fertilize the oocyte. Recent research using the strategy of intervention on the metabolic flexibility of stallion spermatozoa seem promising [7,11,26,105,130], both in the development of new extenders for long time liquid storage, and as an intervention for the development of thawing extenders. In this particular aspect, current extenders in use for stallion spermatozoa contain high concentrations of glucose, around 270- 300 mM, these concentrations are far from being physiological, and may preclude long term preservation of liquid semen. It is well know that supraphysiological concentrations of glucose may lead to cell death [131] due to accumulation of advanced glycation end products (AGEs) [132-135]. The discovery of endocrine features in the spermatozoa, also underlines the complex metabolism of these cells that represent an area of great interest for research in the coming decade [124,136].

Finally, amino-acid metabolism ought to be considered, this has been reported in fish spermatozoa and anecdotal reports in mammals using amino-acids as semen additives support this possibility [137,138]. Additionally, indirect evidence of the role of the amino acid glutamine in stallion spermatozoa has been recently reported by our laboratory. Inhibition of the xCT antiporter, and thus increased intracellular glutamate improved sperm function in fresh extended stallion spermatozoa, but not in frozen thawed samples [72]. The amino-acid glutamine may enter the Krebs cycle and improve mitochondrial function under some circumstances [139]. Glutamine metabolism can provide considerable amounts of NADPH, through the pentose phosphate pathway, and can occur in parallel with aerobic glycolysis depending on glucose-6-phosphate availability [140]. The increase in sperm functionality after using de xCT antiporter inhibitor sulfasalazine can be explained through this mechanism.

Consequences of redox deregulation

In accordance with current biochemical literature, redox regulation is tightly regulated in the spermatozoa, with interactions between spermatic metabolism, mitochondrial production and scavenging of Reactive Oxygen Species. A summary of current knowledge on redox regulation in spermatozoa is presented in figure 1. Many factors can deregulate this complex network in humans and other animals, including aging, exposure to toxins, particularly alcohol and tobacco in humans, poor diet, lack of physical activity and systemic diseases including obesity and diabetes [30,141-144]. Also, current sperm biotechnologies such as cryopreservation cause redox deregulation of spermatozoa, mainly through a severe mitochondrial osmotic stress [99,106,113,145,146]. Deregulation of redox homeostasis has a profound impact on sperm physiology and fertility, all spermatic compartments and function may be affected, moreover impacts on the embryo and the offspring may also occur.

Effects on lipids

Lipid peroxidation is well recognized as a consequence of redox deregulation and loss of redox homeostasis in spermatozoa. In the stallion model, lipid peroxidation occurs as a consequence of aging (figure 2) and sperm biotechnologies such as cryopreservation and chromosomal sex sorting [78,98,99,147-150]. Deregulation of redox regulation and aging and cell senescence is well documented, and aged stallions show increased peroxidation of the lipids in spermatic membranes. Cryopreservation leads to a paradoxical situation, while osmotic induced damage in the mitochondria may lead to reduced production of ROS, lipid peroxidation increases after freezing and thawing. On the other hand spermatozoa that withstands cryopreservation better is also characterized by increased production of ROS [31]. Lipid peroxidation (LPO) occurs after the oxidative attack of lipids, mainly the phospholipids and cholesterol of membranes. Interestingly LPO induces changes in the permeability and fluidity of the membranes that can be easily monitored using probes like YoPro-1 [151,152]. LPO results in the production of lipid hydroperoxides, that are unstable and decompose to more stable and less reactive secondary compounds [153-155]. Lipid peroxidation occurs in three phases, in the *initiation* phase abstraction of $H^{\bullet}$ from a lipid chain (LH) gives a lipid radical ($L^{\bullet}$). Formation of $L^{\bullet}$ is favored in the membrane of the horse spermatozoa due to their abundance in PUFAs [156,157], in this type of lipid the resulting radical is resonance stabilized [153]. Following *initiation* the *propagation* phase continues and the lipid radical reacts with oxygen to generate a lipoperoxyl radical ($LOO^{\bullet}$) , that reacts with a lipid to yield a $L^{\bullet}$ and a lipid hydroperoxyde (LOOH), these are unstable molecules that generate new peroxyl and alkoxyl radicals and decompose to form secondary products [154]. (Figure 3) Finally the reaction ends when it gives a non-radical, or non-propagating species [155]. Among the secondary products formed upon lipid peroxidation of the polyunsaturated fatty acids (PUFAs) of the sperm membranes, aldehydes have received special attention due to their toxicity to

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spermatozoa [98,99,158-165]. Depending on the oxidation of different PUFAs, distinct compounds can originate, malondialdehyde originates from the oxidation of PUFAs containing at least three double bonds, like arachidonic acid, 4 hydroxy-2(E)-nonenal (4-HNE) originates from the oxidation of ω6 fatty acids. The composition of the sperm membrane, suggests that 4-HNE should be the prevalent compound upon LPO, since docosopentanoic acid (C22: 5ω6) is the predominant PUFA in the phospholipids of stallion spermatozoa [156]. Interestingly recently seasonal variation in the lipid composition of the sperm membranes has been reported [166]. It should also be noted that 4-HNE, while triggered by an initial oxidative step, can later continue independent of oxidative stress and continues providing a source of ω-6 fatty acids is available [167]. 4-hydroxynonenal reacts with GSH by Michael addition to form GSH conjugates, and although this reaction can happen spontaneously it occurs much faster in the presence of glutathione-S-transferases. Also the aldehyde function of 4-HNE can be reduced into alcohol or oxidized into acid, with the participation of alcohol dehydrogenase and aldehyde dehydrogenase, forming 1, 4, dihydroxinonene and 4-hydroxynonenoic acid, that can undergo beta oxidation [153]. The role of GSH and aldehyde dehydrogenase has recently been investigated in stallion spermatozoa in relation to oxidative stress [96,98,99,161] suggesting that these mechanisms for 4-HNE detoxification are of pivotal importance for spermatic function. The relation between GSH and 4-HNE in cryopreserved stallion spermatozoa suggest that GSH is effectively a major mechanism for detoxifying 4-HNE [99]. Also, aldehyde dehydrogenase has proven to be a major detoxifying mechanism for 4-HNE in stallion spermatozoa [161]. Lipid peroxidation has been traditionally detected using BODIPY dyes [78,168], however its dual fluorescence and its lipid binding can make this dye difficult to interpret upon flow cytometry analysis. More recently, lipid peroxidation is being detected using antibodies against 4-hydroxynonenal (4-HNE) [99,161,169]. The availability of secondary antibodies marked with different probes makes this technique suitable for multicolor panels, and to study the relation between increased levels of 4-HNE and sperm functionality

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using multiparametric analysis. Mass spectrometry is also a suitable tool for the study of lipid peroxidation induced changes in the spermatozoa and has recently been used in our laboratory to monitor GSH [96].

Effects on proteins

Oxidative modifications of structural and functional proteins are one of the major factors involved in protein dysfunction. Protein carbonyl content is a commonly used biomarker of oxidative damage of proteins. Toxic adducts derived from LPO can diffuse through membranes allowing the reactive aldehydes to covalently modify proteins [159,160,170,171]. In addition to advanced lipid peroxidation end products (ALEs), products derived from the glycoxidation of carbohydrates, that will form advanced glycation end products (AGEs) can also induce protein carbonylation [155]. There is an excellent recent review of this particular topic focused on the spermatozoa [93] and the reader is referred to it for complete details.

Oxidative DNA damage

Spermatozoa harbor the haploid paternal genome and also important epigenetic information with regulatory roles of early embryo development [172]. Recently, it has been reported that biotechnologies such as cryopreservation damage sperm genes with important roles in fertilization and early embryo development, even in the absence of detectable DNA fragmentation [173,174]. Cryopreservation can also damage the sperm epigenome [175]. Many assays have been developed to investigate DNA integrity in the spermatozoa [176,177]. It is considered that most of the DNA damage is caused by an oxidative mechanism. Oxidation of nucleotides can cause abasic pairs in DNA, increasing the risk of replication errors. Loss of a base in DNA, i.e., creation of an abasic site leaving a deoxyribose residue in the strand, is a frequent lesion that may occur spontaneously, or under the action of radiation or

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alkylating agents, or enzymatically as an intermediate in the repair of modified or abnormal bases. The abasic site lesion is mutagenic or lethal if not repaired. From a chemical view point, the abasic site is an alkali-labile residue that leads to strand breakage through beta- and delta- elimination [178,179]. More recently, multiple consequences of the electrophilic nature of abasic lesions have been revealed [180], and oxidized abasic sites are nowadays considered irreparable, leading to the most deleterious form of DNA damage, inter-strand cross links and double strand breaks [181,182]. Detection of oxidized nucleotides in sperm with flow cytometry has been reported using a specific antibody against the oxidative derivative of guanosine, 8-hydroxyguanosine [98,183], and threshold values for fertility have recently been reported in humans [184]. Another newly developed flow cytometry based assay, for evaluation of oxidative stress in sperm DNA, is the γHA2AX assay [185]. Although most histones are replaced by protamines, a small fraction remain in the nucleosome (5-15% in humans). This fraction contains the H2AH histone that is phosphorylated in Ser139 when under oxidative stress. The detection of γHA2AX (the phosphorylated form of the histone) has proven to be more sensitive than the TUNEL assay to detect DNA fragmentation, and also to be better correlated with pregnancy outcome in humans [186].

Impact of Early Embryo Development (EED)

Fecundation of the egg by spermatozoa with compromised redox regulation or experiencing non-lethal oxidative stress has important consequences with regard to embryo viability and the health and well-being of the offspring [187]. Assisted reproductive technologies such as in vitro fertilization and ICSI are associated with an increased incidence of birth defects in offspring [188]. Animal studies indicate that fecundation with spermatozoa experiencing oxidative stress may cause embryonic death [189], an effect that has been linked to oxidative damage in the spermatozoa [190]. Recent research from our laboratory has compared the effect of

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cryopreservation on the transcriptome of early equine embryos [191]. Using the same ejaculate, half processed as fresh sperm and the other half frozen and thawed we obtained embryos from the same mare and stallion after artificial insemination with the aliquot of fresh sperm and in the mare's next cycle using the frozen thawed semen aliquot. The transcriptional profile of embryos obtained with frozen thawed spermatozoa differed significantly from that of embryos obtained with the fresh sperm aliquot of the same ejaculate. Significant downregulation of genes involved in biological pathways related to *oxidative phosphorylation, DNA binding, DNA replication, and immune response*. Interestingly many genes with reduced expression were orthologs of genes in which knockouts are embryonic lethal in mice [191]. While the exact mechanism behind these changes remains to be elucidated, redox deregulation and oxidative stress in the spermatozoa seem to be an important factor. The spermatozoa is known to carry proteins [187], and numerous ncRNAs [192] to the oocyte, with important functions in early embryogenesis. However, it has recently been reported that caput epidydimal mouse sperm, which has not yet incorporated RNAs, can support full development [193]. The impact of redox deregulation on sperm proteins is well recognised and has recently been reviewed [93,194], so it is not unlikely that oxidized proteins can be incorporated by the embryo impacting its development. Recently, preimplantation proteins in the human embryo with potential sperm origin have been identified [187]. In particular, 93 different proteins have been proposed as related to zygote and early embryo development before implantation in humans, moreover up to 560 sperm proteins with known roles in the regulation of gene expression in other cells or tissues have also been identified [187]. Even though further investigation is needed in this field, oxidative damage to sperm proteins with important functions during early embryo development may occur. Further supporting this hypothesis is the fact that biological processes such as *DNA binding and replication,* and *Histone Acetylation* were downregulated in embryos obtained with cryopreserved spermatozoa [191], and many of the proteins mentioned above have roles in these processes [187].

Concluding remarks

Redox regulation plays a major role in controlling spermatic functionality, recent research is unveiling the existence of sophisticated redox regulation systems that may constitute targets for the treatment of the male factor subfertility. In addition, the interaction between metabolism and redox regulation may offer alternatives to traditional methods of sperm conservation. The increasing use of proteomic techniques in research in spermatology will provide significant advances in the understanding of redox regulation in the spermatozoa in coming years

References

1. Staub, C.; Johnson, L. Review: Spermatogenesis in the bull. *Animal* **2018**, *12*, s27-s35, doi:10.1017/S1751731118000435.
2. Bose, R.; Sheng, K.; Moawad, A.R.; Manku, G.; O'Flaherty, C.; Taketo, T.; Culty, M.; Fok, K.L.; Wing, S.S. Ubiquitin Ligase Huwe1 Modulates Spermatogenesis by Regulating Spermatogonial Differentiation and Entry into Meiosis. *Sci Rep* **2017**, *7*, 17759, doi:10.1038/s41598-017-17902-0.
3. Gervasi, M.G.; Visconti, P.E. Molecular changes and signaling events occurring in spermatozoa during epididymal maturation. *Andrology* **2017**, *5*, 204-218, doi:10.1111/andr.12320.
4. Shiraishi, K.; Matsuyama, H. Gonadotoropin actions on spermatogenesis and hormonal therapies for spermatogenic disorders [Review]. *Endocr J* **2017**, *64*, 123-131, doi:10.1507/endocrj.EJ17-0001.
5. Kalyanaraman, B.; Cheng, G.; Hardy, M.; Ouari, O.; Bennett, B.; Zielonka, J. Teaching the basics of reactive oxygen species and their relevance to cancer biology: Mitochondrial reactive oxygen species detection, redox signaling,

and targeted therapies. *Redox Biol* **2018**, *15*, 347-362, doi:10.1016/j.redox.2017.12.012.

6. Kalyanaraman, B. Teaching the basics of redox biology to medical and graduate students: Oxidants, antioxidants and disease mechanisms. *Redox Biol* **2013**, *1*, 244-257, doi:10.1016/j.redox.2013.01.014.

7. Swegen, A.; Lambourne, S.R.; Aitken, R.J.; Gibb, Z. Rosiglitazone Improves Stallion Sperm Motility, ATP Content, and Mitochondrial Function. *Biol Reprod* **2016**, *95*, 107, doi:10.1095/biolreprod.116.142687.

8. Gibb, Z.; Lambourne, S.R.; Aitken, R.J. The paradoxical relationship between stallion fertility and oxidative stress. *Biol Reprod* **2014**, *91*, 77, doi:10.1095/biolreprod.114.118539.

9. Davila, M.P.; Munoz, P.M.; Bolanos, J.M.; Stout, T.A.; Gadella, B.M.; Tapia, J.A.; da Silva, C.B.; Ferrusola, C.O.; Pena, F.J. Mitochondrial ATP is required for the maintenance of membrane integrity in stallion spermatozoa, whereas motility requires both glycolysis and oxidative phosphorylation. *Reproduction* **2016**, *152*, 683-694, doi:10.1530/REP-16-0409.

10. Plaza Davila, M.; Martin Munoz, P.; Tapia, J.A.; Ortega Ferrusola, C.; Balao da Silva, C.C.; Pena, F.J. Inhibition of Mitochondrial Complex I Leads to Decreased Motility and Membrane Integrity Related to Increased Hydrogen Peroxide and Reduced ATP Production, while the Inhibition of Glycolysis Has Less Impact on Sperm Motility. *PLoS One* **2015**, *10*, e0138777, doi:10.1371/journal.pone.0138777.

11. Darr, C.R.; Varner, D.D.; Teague, S.; Cortopassi, G.A.; Datta, S.; Meyers, S.A. Lactate and Pyruvate Are Major Sources of Energy for Stallion Sperm with Dose Effects on Mitochondrial Function, Motility, and ROS Production. *Biol Reprod* **2016**, *95*, 34, doi:10.1095/biolreprod.116.140707.

12. Tosic, J.; Walton, A. Formation of hydrogen peroxide by spermatozoa and its inhibitory effect of respiration. *Nature* **1946**, *158*, 485.

13. Lee, D.; Moawad, A.R.; Morielli, T.; Fernandez, M.C.; O'Flaherty, C. Peroxiredoxins prevent oxidative stress during human sperm capacitation. *Mol Hum Reprod* **2017**, *23*, 106-115, doi:10.1093/molehr/gaw081.
14. Liu, Y.; O'Flaherty, C. In vivo oxidative stress alters thiol redox status of peroxiredoxin 1 and 6 and impairs rat sperm quality. *Asian J Androl* **2017**, *19*, 73-79, doi:10.4103/1008-682X.170863.
15. O'Flaherty, C. Redox regulation of mammalian sperm capacitation. *Asian J Androl* **2015**, *17*, 583-590, doi:10.4103/1008-682X.153303.
16. O'Flaherty, C.; de Souza, A.R. Hydrogen peroxide modifies human sperm peroxiredoxins in a dose-dependent manner. *Biol Reprod* **2011**, *84*, 238-247, doi:10.1095/biolreprod.110.085712.
17. de Lamirande, E.; O'Flaherty, C. Sperm activation: role of reactive oxygen species and kinases. *Biochim Biophys Acta* **2008**, *1784*, 106-115, doi:10.1016/j.bbapap.2007.08.024.
18. O'Flaherty, C.; de Lamirande, E.; Gagnon, C. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. *Free Radic Biol Med* **2006**, *41*, 528-540, doi:10.1016/j.freeradbiomed.2006.04.027.
19. O'Flaherty, C.; de Lamirande, E.; Gagnon, C. Reactive oxygen species and protein kinases modulate the level of phospho-MEK-like proteins during human sperm capacitation. *Biol Reprod* **2005**, *73*, 94-105, doi:10.1095/biolreprod.104.038794.
20. O'Flaherty, C.M.; Beorlegui, N.B.; Beconi, M.T. Reactive oxygen species requirements for bovine sperm capacitation and acrosome reaction. *Theriogenology* **1999**, *52*, 289-301, doi:10.1016/S0093-691X(99)00129-6.
21. Fujii, S.; Sawa, T.; Nishida, M.; Ihara, H.; Ida, T.; Motohashi, H.; Akaike, T. Redox signaling regulated by an electrophilic cyclic nucleotide and reactive cysteine persulfides. *Arch Biochem Biophys* **2016**, *595*, 140-146, doi:10.1016/j.abb.2015.11.008.

692 22. Holmstrom, K.M.; Finkel, T. Cellular mechanisms and physiological
693 consequences of redox-dependent signalling. *Nat Rev Mol Cell Biol* **2014**, *15*,
694 411-421, doi:10.1038/nrm3801.
695 23. Go, Y.M.; Jones, D.P. The redox proteome. *J Biol Chem* **2013**, *288*, 26512-
696 26520, doi:10.1074/jbc.R113.464131.
697 24. Briehl, M.M. Oxygen in human health from life to death--An approach to
698 teaching redox biology and signaling to graduate and medical students.
699 *Redox Biol* **2015**, *5*, 124-139, doi:10.1016/j.redox.2015.04.002.
700 25. Zhang, L.; Wang, X.; Cueto, R.; Effi, C.; Zhang, Y.; Tan, H.; Qin, X.; Ji, Y.;
701 Yang, X.; Wang, H. Biochemical basis and metabolic interplay of redox
702 regulation. *Redox Biol* **2019**, *26*, 101284, doi:10.1016/j.redox.2019.101284.
703 26. Aitken, J.B.; Naumovski, N.; Curry, B.; Grupen, C.G.; Gibb, Z.; Aitken, R.J.
704 Characterization of an L-amino acid oxidase in equine spermatozoa. *Biol*
705 *Reprod* **2015**, *92*, 125, doi:10.1095/biolreprod.114.126052.
706 27. Vernet, P.; Fulton, N.; Wallace, C.; Aitken, R.J. Analysis of reactive oxygen
707 species generating systems in rat epididymal spermatozoa. *Biol Reprod* **2001**,
708 *65*, 1102-1113.
709 28. Cueto, R.; Zhang, L.; Shan, H.M.; Huang, X.; Li, X.; Li, Y.F.; Lopez, J.; Yang,
710 W.Y.; Lavallee, M.; Yu, C., et al. Identification of homocysteine-suppressive
711 mitochondrial ETC complex genes and tissue expression profile - Novel
712 hypothesis establishment. *Redox Biol* **2018**, *17*, 70-88,
713 doi:10.1016/j.redox.2018.03.015.
714 29. Ortega-Ferrusola, C.; Anel-Lopez, L.; Martin-Munoz, P.; Ortiz-Rodriguez,
715 J.M.; Gil, M.C.; Alvarez, M.; de Paz, P.; Ezquerra, L.J.; Masot, A.J.; Redondo,
716 E., et al. Computational flow cytometry reveals that cryopreservation
717 induces spermptosis but subpopulations of spermatozoa may experience
718 capacitation-like changes. *Reproduction* **2017**, *153*, 293-304, doi:10.1530/REP-
719 16-0539.

720 30. Aitken, R.J.; Gibb, Z.; Baker, M.A.; Drevet, J.; Gharagozloo, P. Causes and
721 consequences of oxidative stress in spermatozoa. *Reprod Fertil Dev* **2016**, *28*,
722 1-10, doi:10.1071/RD15325.
723 31. Yeste, M.; Estrada, E.; Rocha, L.G.; Marin, H.; Rodriguez-Gil, J.E.; Miro, J.
724 Cryotolerance of stallion spermatozoa is related to ROS production and
725 mitochondrial membrane potential rather than to the integrity of sperm
726 nucleus. *Andrology* **2015**, *3*, 395-407, doi:10.1111/andr.291.
727 32. Aparicio, I.M.; Espino, J.; Bejarano, I.; Gallardo-Soler, A.; Campo, M.L.;
728 Salido, G.M.; Pariente, J.A.; Pena, F.J.; Tapia, J.A. Autophagy-related
729 proteins are functionally active in human spermatozoa and may be involved
730 in the regulation of cell survival and motility. *Sci Rep* **2016**, *6*, 33647,
731 doi:10.1038/srep33647.
732 33. Sies, H.; Berndt, C.; Jones, D.P. Oxidative Stress. *Annu Rev Biochem* **2017**,
733 10.1146/annurev-biochem-061516-045037, doi:10.1146/annurev-biochem-
734 061516-045037.
735 34. Schmidt, H.H.; Stocker, R.; Vollbracht, C.; Paulsen, G.; Riley, D.; Daiber, A.;
736 Cuadrado, A. Antioxidants in Translational Medicine. *Antioxid Redox Signal*
737 **2015**, *23*, 1130-1143, doi:10.1089/ars.2015.6393.
738 35. Han, D.; Antunes, F.; Canali, R.; Rettori, D.; Cadenas, E. Voltage-dependent
739 anion channels control the release of the superoxide anion from
740 mitochondria to cytosol. *J Biol Chem* **2003**, *278*, 5557-5563,
741 doi:10.1074/jbc.M210269200.
742 36. Han, D.; Antunes, F.; Daneri, F.; Cadenas, E. Mitochondrial superoxide
743 anion production and release into intermembrane space. *Methods Enzymol*
744 **2002**, *349*, 271-280.
745 37. Han, D.; Williams, E.; Cadenas, E. Mitochondrial respiratory chain-
746 dependent generation of superoxide anion and its release into the
747 intermembrane space. *Biochem J* **2001**, *353*, 411-416.

748 38. Vieceli Dalla Sega, F.; Zambonin, L.; Fiorentini, D.; Rizzo, B.; Caliceti, C.;
749 Landi, L.; Hrelia, S.; Prata, C. Specific aquaporins facilitate Nox-produced
750 hydrogen peroxide transport through plasma membrane in leukaemia cells.
751 *Biochim Biophys Acta* **2014**, *1843*, 806-814, doi:10.1016/j.bbamcr.2014.01.011.
752 39. Mubarakshina Borisova, M.M.; Kozuleva, M.A.; Rudenko, N.N.; Naydov,
753 I.A.; Klenina, I.B.; Ivanov, B.N. Photosynthetic electron flow to oxygen and
754 diffusion of hydrogen peroxide through the chloroplast envelope via
755 aquaporins. *Biochim Biophys Acta* **2012**, *1817*, 1314-1321,
756 doi:10.1016/j.bbabio.2012.02.036.
757 40. Bienert, G.P.; Moller, A.L.; Kristiansen, K.A.; Schulz, A.; Moller, I.M.;
758 Schjoerring, J.K.; Jahn, T.P. Specific aquaporins facilitate the diffusion of
759 hydrogen peroxide across membranes. *J Biol Chem* **2007**, *282*, 1183-1192,
760 doi:10.1074/jbc.M603761200.
761 41. Li, T.K. The glutathione and thiol content of mammalian spermatozoa and
762 seminal plasma. *Biol Reprod* **1975**, *12*, 641-646.
763 42. Ortega Ferrusola, C.; Martin Munoz, P.; Ortiz-Rodriguez, J.M.; Anel-Lopez,
764 L.; Balao da Silva, C.; Alvarez, M.; de Paz, P.; Tapia, J.A.; Anel, L.; Silva-
765 Rodriguez, A., et al. Depletion of thiols leads to redox deregulation,
766 production of 4-hydroxinonenal and sperm senescence: a possible role for
767 GSH regulation in spermatozoa. *Biol Reprod* **2018**, 10.1093/biolre/ioy241,
768 doi:10.1093/biolre/ioy241.
769 43. Wong, J.L.; Creton, R.; Wessel, G.M. The oxidative burst at fertilization is
770 dependent upon activation of the dual oxidase Udx1. *Dev Cell* **2004**, *7*, 801-
771 814, doi:10.1016/j.devcel.2004.10.014.
772 44. Wong, J.L.; Wessel, G.M. Free-radical crosslinking of specific proteins alters
773 the function of the egg extracellular matrix at fertilization. *Development* **2008**,
774 *135*, 431-440, doi:10.1242/dev.015503.

45. Chapman, J.C.; Michael, S.D. Proposed mechanism for sperm chromatin condensation/decondensation in the male rat. *Reprod Biol Endocrinol* **2003**, *1*, 20.

46. Luque, G.M.; Dalotto-Moreno, T.; Martin-Hidalgo, D.; Ritagliati, C.; Puga Molina, L.C.; Romarowski, A.; Balestrini, P.A.; Schiavi-Ehrenhaus, L.J.; Gilio, N.; Krapf, D., et al. Only a subpopulation of mouse sperm displays a rapid increase in intracellular calcium during capacitation. *J Cell Physiol* **2018**, *233*, 9685-9700, doi:10.1002/jcp.26883.

47. Alvau, A.; Battistone, M.A.; Gervasi, M.G.; Navarrete, F.A.; Xu, X.; Sanchez-Cardenas, C.; De la Vega-Beltran, J.L.; Da Ros, V.G.; Greer, P.A.; Darszon, A., et al. The tyrosine kinase FER is responsible for the capacitation-associated increase in tyrosine phosphorylation in murine sperm. *Development* **2016**, *143*, 2325-2333, doi:10.1242/dev.136499.

48. Stival, C.; Puga Molina Ldel, C.; Paudel, B.; Buffone, M.G.; Visconti, P.E.; Krapf, D. Sperm Capacitation and Acrosome Reaction in Mammalian Sperm. *Adv Anat Embryol Cell Biol* **2016**, *220*, 93-106, doi:10.1007/978-3-319-30567-7_5.

49. Stival, C.; La Spina, F.A.; Baro Graf, C.; Arcelay, E.; Arranz, S.E.; Ferreira, J.J.; Le Grand, S.; Dzikunu, V.A.; Santi, C.M.; Visconti, P.E., et al. Src Kinase Is the Connecting Player between Protein Kinase A (PKA) Activation and Hyperpolarization through SLO3 Potassium Channel Regulation in Mouse Sperm. *J Biol Chem* **2015**, *290*, 18855-18864, doi:10.1074/jbc.M115.640326.

50. Escoffier, J.; Navarrete, F.; Haddad, D.; Santi, C.M.; Darszon, A.; Visconti, P.E. Flow cytometry analysis reveals that only a subpopulation of mouse sperm undergoes hyperpolarization during capacitation. *Biol Reprod* **2015**, *92*, 121, doi:10.1095/biolreprod.114.127266.

51. Visconti, P.E.; Krapf, D.; de la Vega-Beltran, J.L.; Acevedo, J.J.; Darszon, A. Ion channels, phosphorylation and mammalian sperm capacitation. *Asian J Androl* **2011**, *13*, 395-405, doi:10.1038/aja.2010.69.

804 52. Battistone, M.A.; Da Ros, V.G.; Salicioni, A.M.; Navarrete, F.A.; Krapf, D.;
805 Visconti, P.E.; Cuasnicu, P.S. Functional human sperm capacitation requires
806 both bicarbonate-dependent PKA activation and down-regulation of
807 Ser/Thr phosphatases by Src family kinases. *Mol Hum Reprod* **2013**, *19*, 570-
808 580, doi:10.1093/molehr/gat033.
809 53. Chavez, J.C.; Hernandez-Gonzalez, E.O.; Wertheimer, E.; Visconti, P.E.;
810 Darszon, A.; Trevino, C.L. Participation of the Cl-/HCO(3)- exchangers
811 SLC26A3 and SLC26A6, the Cl- channel CFTR, and the regulatory factor
812 SLC9A3R1 in mouse sperm capacitation. *Biol Reprod* **2012**, *86*, 1-14,
813 doi:10.1095/biolreprod.111.094037.
814 54. Salicioni, A.M.; Platt, M.D.; Wertheimer, E.V.; Arcelay, E.; Allaire, A.;
815 Sosnik, J.; Visconti, P.E. Signalling pathways involved in sperm capacitation.
816 *Soc Reprod Fertil Suppl* **2007**, *65*, 245-259.
817 55. Hernandez-Gonzalez, E.O.; Sosnik, J.; Edwards, J.; Acevedo, J.J.; Mendoza-
818 Lujambio, I.; Lopez-Gonzalez, I.; Demarco, I.; Wertheimer, E.; Darszon, A.;
819 Visconti, P.E. Sodium and epithelial sodium channels participate in the
820 regulation of the capacitation-associated hyperpolarization in mouse sperm.
821 *J Biol Chem* **2006**, *281*, 5623-5633, doi:10.1074/jbc.M508172200.
822 56. Lefievre, L.; Jha, K.N.; de Lamirande, E.; Visconti, P.E.; Gagnon, C.
823 Activation of protein kinase A during human sperm capacitation and
824 acrosome reaction. *J Androl* **2002**, *23*, 709-716.
825 57. Visconti, P.E.; Stewart-Savage, J.; Blasco, A.; Battaglia, L.; Miranda, P.; Kopf,
826 G.S.; Tezon, J.G. Roles of bicarbonate, cAMP, and protein tyrosine
827 phosphorylation on capacitation and the spontaneous acrosome reaction of
828 hamster sperm. *Biol Reprod* **1999**, *61*, 76-84.
829 58. O'Flaherty, C.; de Lamirande, E.; Gagnon, C. Phosphorylation of the
830 Arginine-X-X-(Serine/Threonine) motif in human sperm proteins during
831 capacitation: modulation and protein kinase A dependency. *Mol Hum*
832 *Reprod* **2004**, *10*, 355-363, doi:10.1093/molehr/gah046.

59. O'Flaherty, C.; Beorlegui, N.; Beconi, M.T. Participation of superoxide anion in the capacitation of cryopreserved bovine sperm. *Int J Androl* **2003**, *26*, 109-114.

60. Freitas, M.J.; Vijayaraghavan, S.; Fardilha, M. Signaling mechanisms in mammalian sperm motility. *Biol Reprod* **2017**, *96*, 2-12, doi:10.1095/biolreprod.116.144337.

61. Gonzalez-Fernandez, L.; Ortega-Ferrusola, C.; Macias-Garcia, B.; Salido, G.M.; Pena, F.J.; Tapia, J.A. Identification of protein tyrosine phosphatases and dual-specificity phosphatases in mammalian spermatozoa and their role in sperm motility and protein tyrosine phosphorylation. *Biol Reprod* **2009**, *80*, 1239-1252, doi:10.1095/biolreprod.108.073486.

62. Denu, J.M.; Tanner, K.G. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* **1998**, *37*, 5633-5642, doi:10.1021/bi973035t.

63. Frijhoff, J.; Dagnell, M.; Godfrey, R.; Ostman, A. Regulation of protein tyrosine phosphatase oxidation in cell adhesion and migration. *Antioxid Redox Signal* **2014**, *20*, 1994-2010, doi:10.1089/ars.2013.5643.

64. Ozkosem, B.; Feinstein, S.I.; Fisher, A.B.; O'Flaherty, C. Advancing age increases sperm chromatin damage and impairs fertility in peroxiredoxin 6 null mice. *Redox Biol* **2015**, *5*, 15-23, doi:10.1016/j.redox.2015.02.004.

65. Jeong, W.; Bae, S.H.; Toledano, M.B.; Rhee, S.G. Role of sulfiredoxin as a regulator of peroxiredoxin function and regulation of its expression. *Free Radic Biol Med* **2012**, *53*, 447-456, doi:10.1016/j.freeradbiomed.2012.05.020.

66. Wood, Z.A.; Schroder, E.; Robin Harris, J.; Poole, L.B. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* **2003**, *28*, 32-40.

67. Wood, Z.A.; Poole, L.B.; Karplus, P.A. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* **2003**, *300*, 650-653, doi:10.1126/science.1080405.

862 68. Scherz-Shouval, R.; Shvets, E.; Fass, E.; Shorer, H.; Gil, L.; Elazar, Z. Reactive
863 oxygen species are essential for autophagy and specifically regulate the
864 activity of Atg4. *EMBO J* **2007**, *26*, 1749-1760, doi:10.1038/sj.emboj.7601623.
865 69. Gualtieri, R.; Mollo, V.; Duma, G.; Talevi, R. Redox control of surface
866 protein sulphhydryls in bovine spermatozoa reversibly modulates sperm
867 adhesion to the oviductal epithelium and capacitation. *Reproduction* **2009**,
868 *138*, 33-43, doi:10.1530/REP-08-0514.
869 70. Gualtieri, R.; Iaccarino, M.; Mollo, V.; Prisco, M.; Iaccarino, S.; Talevi, R.
870 Slow cooling of human oocytes: ultrastructural injuries and apoptotic status.
871 *Fertil Steril* **2009**, *91*, 1023-1034, doi:10.1016/j.fertnstert.2008.01.076.
872 71. Talevi, R.; Zagami, M.; Castaldo, M.; Gualtieri, R. Redox regulation of sperm
873 surface thiols modulates adhesion to the fallopian tube epithelium. *Biol*
874 *Reprod* **2007**, *76*, 728-735, doi:10.1095/biolreprod.106.056028.
875 72. Ortiz-Rodriguez, J.M.; Martin-Cano, F.E.; Ortega-Ferrusola, C.; Masot, J.;
876 Redondo, E.; Gazquez, A.; Gil, M.C.; Aparicio, I.M.; Rojo-Dominguez, P.;
877 Tapia, J.A., et al. The incorporation of cystine by the soluble carrier family 7
878 member 11 (SLC7A11) is a component of the redox regulatory mechanism in
879 stallion spermatozoa. *Biol Reprod* **2019**, 10.1093/biolre/ioz069,
880 doi:10.1093/biolre/ioz069.
881 73. Ball, B.A.; Gravance, C.G.; Medina, V.; Baumber, J.; Liu, I.K. Catalase
882 activity in equine semen. *Am J Vet Res* **2000**, *61*, 1026-1030.
883 74. Baumber, J.; Ball, B.A. Determination of glutathione peroxidase and
884 superoxide dismutase-like activities in equine spermatozoa, seminal plasma,
885 and reproductive tissues. *Am J Vet Res* **2005**, *66*, 1415-1419.
886 75. Brummer, M.; Hayes, S.; Dawson, K.A.; Lawrence, L.M. Measures of
887 antioxidant status of the horse in response to selenium depletion and
888 repletion. *J Anim Sci* **2013**, *91*, 2158-2168, doi:10.2527/jas.2012-5794.
889 76. Leone, E. Ergothioneine in the equine ampullar secretion. *Nature* **1954**, *174*,
890 404-405, doi:10.1038/174404b0.

77. Mann, T. Biochemistry of stallion semen. *J Reprod Fertil Suppl* **1975**, 47-52.

78. Ortega Ferrusola, C.; Gonzalez Fernandez, L.; Morrell, J.M.; Salazar Sandoval, C.; Macias Garcia, B.; Rodriguez-Martinez, H.; Tapia, J.A.; Pena, F.J. Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. *Reproduction* **2009**, *138*, 55-63, doi:10.1530/REP-08-0484.

79. Fernandez, M.C.; O'Flaherty, C. Peroxiredoxin 6 is the primary antioxidant enzyme for the maintenance of viability and DNA integrity in human spermatozoa. *Hum Reprod* **2018**, 10.1093/humrep/dey221, doi:10.1093/humrep/dey221.

80. Moawad, A.R.; Fernandez, M.C.; Scarlata, E.; Dodia, C.; Feinstein, S.I.; Fisher, A.B.; O'Flaherty, C. Deficiency of peroxiredoxin 6 or inhibition of its phospholipase A2 activity impair the in vitro sperm fertilizing competence in mice. *Sci Rep* **2017**, *7*, 12994, doi:10.1038/s41598-017-13411-2.

81. Efrat, M.; Stein, A.; Pinkas, H.; Breitbart, H.; Unger, R.; Birk, R. Paraoxonase 1 (PON1) attenuates sperm hyperactivity and spontaneous acrosome reaction. *Andrology* **2018**, 10.1111/andr.12552, doi:10.1111/andr.12552.

82. Barranco, I.; Tvarijonaviciute, A.; Perez-Patino, C.; Alkmin, D.V.; Ceron, J.J.; Martinez, E.A.; Rodriguez-Martinez, H.; Roca, J. The activity of paraoxonase type 1 (PON-1) in boar seminal plasma and its relationship with sperm quality, functionality, and in vivo fertility. *Andrology* **2015**, *3*, 315-320, doi:10.1111/andr.309.

83. Barranco, I.; Roca, J.; Tvarijonaviciute, A.; Ruber, M.; Vicente-Carrillo, A.; Atikuzzaman, M.; Ceron, J.J.; Martinez, E.A.; Rodriguez-Martinez, H. Measurement of activity and concentration of paraoxonase 1 (PON-1) in seminal plasma and identification of PON-2 in the sperm of boar ejaculates. *Mol Reprod Dev* **2015**, *82*, 58-65, doi:10.1002/mrd.22444.

84. Lazaros, L.A.; Xita, N.V.; Hatzi, E.G.; Kaponis, A.I.; Stefos, T.J.; Plachouras, N.I.; Makrydimas, G.V.; Sofikitis, N.V.; Zikopoulos, K.A.; Georgiou, I.A. Association of paraoxonase gene polymorphisms with sperm parameters. *J Androl* **2011**, *32*, 394-401, doi:10.2164/jandrol.110.010348.
85. Verit, F.F.; Verit, A.; Ciftci, H.; Erel, O.; Celik, H. Paraoxonase-1 activity in subfertile men and relationship to sperm parameters. *J Androl* **2009**, *30*, 183-189, doi:10.2164/jandrol.108.004929.
86. Moradi, M.N.; Karimi, J.; Khodadadi, I.; Amiri, I.; Karami, M.; Saidijam, M.; Vatannejad, A.; Tavilani, H. Evaluation of the p53 and Thioredoxin reductase in sperm from asthenozoospermic males in comparison to normozoospermic males. *Free Radic Biol Med* **2018**, *116*, 123-128, doi:10.1016/j.freeradbiomed.2017.12.038.
87. Emelyanov, A.V.; Fyodorov, D.V. Thioredoxin-dependent disulfide bond reduction is required for protamine eviction from sperm chromatin. *Genes Dev* **2016**, *30*, 2651-2656, doi:10.1101/gad.290916.116.
88. Tirmarche, S.; Kimura, S.; Dubruille, R.; Horard, B.; Loppin, B. Unlocking sperm chromatin at fertilization requires a dedicated egg thioredoxin in Drosophila. *Nat Commun* **2016**, *7*, 13539, doi:10.1038/ncomms13539.
89. Su, D.; Novoselov, S.V.; Sun, Q.A.; Moustafa, M.E.; Zhou, Y.; Oko, R.; Hatfield, D.L.; Gladyshev, V.N. Mammalian selenoprotein thioredoxin-glutathione reductase. Roles in disulfide bond formation and sperm maturation. *J Biol Chem* **2005**, *280*, 26491-26498, doi:10.1074/jbc.M503638200.
90. Miranda-Vizuete, A.; Tsang, K.; Yu, Y.; Jimenez, A.; Pelto-Huikko, M.; Flickinger, C.J.; Sutovsky, P.; Oko, R. Cloning and developmental analysis of murid spermatid-specific thioredoxin-2 (SPTRX-2), a novel sperm fibrous sheath protein and autoantigen. *J Biol Chem* **2003**, *278*, 44874-44885, doi:10.1074/jbc.M305475200.
91. Yu, Y.; Oko, R.; Miranda-Vizuete, A. Developmental expression of spermatid-specific thioredoxin-1 protein: transient association to the

longitudinal columns of the fibrous sheath during sperm tail formation. *Biol Reprod* **2002**, *67*, 1546-1554.

92. Kuribayashi, Y.; Gagnon, C. Effect of catalase and thioredoxin addition to sperm incubation medium before in vitro fertilization on sperm capacity to support embryo development. *Fertil Steril* **1996**, *66*, 1012-1017.

93. O'Flaherty, C.; Matsushita-Fournier, D. Reactive oxygen species and protein modifications in spermatozoa. *Biol Reprod* **2017**, *97*, 577-585, doi:10.1093/biolre/iox104.

94. Ozkosem, B.; Feinstein, S.I.; Fisher, A.B.; O'Flaherty, C. Absence of Peroxiredoxin 6 Amplifies the Effect of Oxidant Stress on Mobility and SCSA/CMA3 Defined Chromatin Quality and Impairs Fertilizing Ability of Mouse Spermatozoa. *Biol Reprod* **2016**, *94*, 68, doi:10.1095/biolreprod.115.137646.

95. O'Flaherty, C. Peroxiredoxins: hidden players in the antioxidant defence of human spermatozoa. *Basic Clin Androl* **2014**, *24*, 4, doi:10.1186/2051-4190-24-4.

96. Ortega-Ferrusola, C.; Martin Munoz, P.; Ortiz-Rodriguez, J.M.; Anel-Lopez, L.; Balao da Silva, C.; Alvarez, M.; de Paz, P.; Tapia, J.A.; Anel, L.; Silva-Rodriguez, A., et al. Depletion of thiols leads to redox deregulation, production of 4-hydroxinonenal and sperm senescence: a possible role for GSH regulation in spermatozoadagger. *Biol Reprod* **2019**, *100*, 1090-1107, doi:10.1093/biolre/ioy241.

97. Keil, M.; Wetterauer, U.; Heite, H.J. Glutamic acid concentration in human semen--its origin and significance. *Andrologia* **1979**, *11*, 385-391.

98. Munoz, P.M.; Ferrusola, C.O.; Lopez, L.A.; Del Petre, C.; Garcia, M.A.; de Paz Cabello, P.; Anel, L.; Pena, F.J. Caspase 3 Activity and Lipoperoxidative Status in Raw Semen Predict the Outcome of Cryopreservation of Stallion Spermatozoa. *Biol Reprod* **2016**, *95*, 53, doi:10.1095/biolreprod.116.139444.

99. Martin Munoz, P.; Ortega Ferrusola, C.; Vizuete, G.; Plaza Davila, M.; Rodriguez Martinez, H.; Pena, F.J. Depletion of Intracellular Thiols and Increased Production of 4-Hydroxynonenal that Occur During Cryopreservation of Stallion Spermatozoa Lead to Caspase Activation, Loss of Motility, and Cell Death. *Biol Reprod* **2015**, *93*, 143, doi:10.1095/biolreprod.115.132878.

100. Brand, M.D. Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. *Free Radic Biol Med* **2016**, *100*, 14-31, doi:10.1016/j.freeradbiomed.2016.04.001.

101. Goncalves, R.L.; Bunik, V.I.; Brand, M.D. Production of superoxide/hydrogen peroxide by the mitochondrial 2-oxoadipate dehydrogenase complex. *Free Radic Biol Med* **2016**, *91*, 247-255, doi:10.1016/j.freeradbiomed.2015.12.020.

102. Samanta, L.; Agarwal, A.; Swain, N.; Sharma, R.; Gopalan, B.; Esteves, S.C.; Durairajanayagam, D.; Sabanegh, E. Proteomic Signatures of Sperm Mitochondria in Varicocele: Clinical Use as Biomarkers of Varicocele Associated Infertility. *J Urol* **2018**, *200*, 414-422, doi:10.1016/j.juro.2018.03.009.

103. Lu, X.; Zhang, Y.; Bai, H.; Liu, J.; Li, J.; Wu, B. Mitochondria-targeted antioxidant MitoTEMPO improves the post-thaw sperm quality. *Cryobiology* **2018**, *80*, 26-29, doi:10.1016/j.cryobiol.2017.12.009.

104. Amaral, S.; R, S.T.; Baptista, M.; Sousa, M.I.; Silva, A.; Escada-Rebelo, S.; Paiva, C.P.; Ramalho-Santos, J. Mitochondrial Functionality and Chemical Compound Action on Sperm Function. *Curr Med Chem* **2016**, *23*, 3575-3606.

105. Gibb, Z.; Lambourne, S.R.; Quadrelli, J.; Smith, N.D.; Aitken, R.J. L-carnitine and pyruvate are prosurvival factors during the storage of stallion spermatozoa at room temperature. *Biol Reprod* **2015**, *93*, 104, doi:10.1095/biolreprod.115.131326.

1004 106. Pena, F.J.; Plaza Davila, M.; Ball, B.A.; Squires, E.L.; Martin Munoz, P.;
1005 Ortega Ferrusola, C.; Balao da Silva, C. The Impact of Reproductive
1006 Technologies on Stallion Mitochondrial Function. *Reprod Domest Anim* **2015**,
1007 *50*, 529-537, doi:10.1111/rda.12551.
1008 107. Jones, D.P. Disruption of mitochondrial redox circuitry in oxidative stress.
1009 *Chem Biol Interact* **2006**, *163*, 38-53, doi:10.1016/j.cbi.2006.07.008.
1010 108. Rico-Leo, E.M.; Moreno-Marin, N.; Gonzalez-Rico, F.J.; Barrasa, E.; Ortega-
1011 Ferrusola, C.; Martin-Munoz, P.; Sanchez-Guardado, L.O.; Llano, E.;
1012 Alvarez-Barrientos, A.; Infante-Campos, A., et al. piRNA-associated
1013 proteins and retrotransposons are differentially expressed in murine testis
1014 and ovary of aryl hydrocarbon receptor deficient mice. *Open Biol* **2016**, *6*,
1015 doi:10.1098/rsob.160186.
1016 109. Losano, J.D.A.; Angrimani, D.S.R.; Ferreira Leite, R.; Simoes da Silva, B.D.C.;
1017 Barnabe, V.H.; Nichi, M. Spermatic mitochondria: role in oxidative
1018 homeostasis, sperm function and possible tools for their assessment. *Zygote*
1019 **2018**, *26*, 251-260, doi:10.1017/S0967199418000242.
1020 110. Amaral, A.; Lourenco, B.; Marques, M.; Ramalho-Santos, J. Mitochondria
1021 functionality and sperm quality. *Reproduction* **2013**, *146*, R163-174,
1022 doi:10.1530/REP-13-0178.
1023 111. Vakifahmetoglu-Norberg, H.; Ouchida, A.T.; Norberg, E. The role of
1024 mitochondria in metabolism and cell death. *Biochem Biophys Res Commun*
1025 **2017**, *482*, 426-431, doi:10.1016/j.bbrc.2016.11.088.
1026 112. Klingenberg, M. The ADP and ATP transport in mitochondria and its
1027 carrier. *Biochim Biophys Acta* **2008**, *1778*, 1978-2021,
1028 doi:10.1016/j.bbamem.2008.04.011.
1029 113. Ortega Ferrusola, C.; Anel-Lopez, L.; Ortiz-Rodriguez, J.M.; Martin Munoz,
1030 P.; Alvarez, M.; de Paz, P.; Masot, J.; Redondo, E.; Balao da Silva, C.;
1031 Morrell, J.M., et al. Stallion spermatozoa surviving freezing and thawing

experience membrane depolarization and increased intracellular Na(). *Andrology* **2017**, *5*, 1174-1182, doi:10.1111/andr.12419.

114. Moscatelli, N.; Lunetti, P.; Braccia, C.; Armirotti, A.; Pisanello, F.; De Vittorio, M.; Zara, V.; Ferramosca, A. Comparative Proteomic Analysis of Proteins Involved in Bioenergetics Pathways Associated with Human Sperm Motility. *Int J Mol Sci* **2019**, *20*, doi:10.3390/ijms20123000.

115. Ferramosca, A.; Zara, V. Bioenergetics of mammalian sperm capacitation. *Biomed Res Int* **2014**, *2014*, 902953, doi:10.1155/2014/902953.

116. Piomboni, P.; Focarelli, R.; Stendardi, A.; Ferramosca, A.; Zara, V. The role of mitochondria in energy production for human sperm motility. *Int J Androl* **2012**, *35*, 109-124, doi:10.1111/j.1365-2605.2011.01218.x.

117. Bucci, D.; Rodriguez-Gil, J.E.; Vallorani, C.; Spinaci, M.; Galeati, G.; Tamanini, C. GLUTs and mammalian sperm metabolism. *J Androl* **2011**, *32*, 348-355, doi:10.2164/jandrol.110.011197.

118. Marin, S.; Chiang, K.; Bassilian, S.; Lee, W.N.; Boros, L.G.; Fernandez-Novell, J.M.; Centelles, J.J.; Medrano, A.; Rodriguez-Gil, J.E.; Cascante, M. Metabolic strategy of boar spermatozoa revealed by a metabolomic characterization. *FEBS Lett* **2003**, *554*, 342-346, doi:10.1016/s0014-5793(03)01185-2.

119. Asghari, A.; Marashi, S.A.; Ansari-Pour, N. A sperm-specific proteome-scale metabolic network model identifies non-glycolytic genes for energy deficiency in asthenozoospermia. *Syst Biol Reprod Med* **2017**, *63*, 100-112, doi:10.1080/19396368.2016.1263367.

120. Amaral, A.; Castillo, J.; Estanyol, J.M.; Ballesca, J.L.; Ramalho-Santos, J.; Oliva, R. Human sperm tail proteome suggests new endogenous metabolic pathways. *Mol Cell Proteomics* **2013**, *12*, 330-342, doi:10.1074/mcp.M112.020552.

121. Swegen, A.; Curry, B.J.; Gibb, Z.; Lambourne, S.R.; Smith, N.D.; Aitken, R.J. Investigation of the stallion sperm proteome by mass spectrometry. *Reproduction* **2015**, *149*, 235-244, doi:10.1530/REP-14-0500.

122. Qiu, J.H.; Li, Y.W.; Xie, H.L.; Li, Q.; Dong, H.B.; Sun, M.J.; Gao, W.Q.; Tan, J.H. Effects of glucose metabolism pathways on sperm motility and oxidative status during long-term liquid storage of goat semen. *Theriogenology* **2016**, *86*, 839-849, doi:10.1016/j.theriogenology.2016.03.005.

123. Miraglia, E.; Lussiana, C.; Viarisio, D.; Racca, C.; Cipriani, A.; Gazzano, E.; Bosia, A.; Revelli, A.; Ghigo, D. The pentose phosphate pathway plays an essential role in supporting human sperm capacitation. *Fertil Steril* **2010**, *93*, 2437-2440, doi:10.1016/j.fertnstert.2009.09.005.

124. Ando, S.; Aquila, S. Arguments raised by the recent discovery that insulin and leptin are expressed in and secreted by human ejaculated spermatozoa. *Mol Cell Endocrinol* **2005**, *245*, 1-6, doi:10.1016/j.mce.2005.09.011.

125. Urner, F.; Sakkas, D. Involvement of the pentose phosphate pathway and redox regulation in fertilization in the mouse. *Mol Reprod Dev* **2005**, *70*, 494-503, doi:10.1002/mrd.20222.

126. Williams, A.C.; Ford, W.C. Functional significance of the pentose phosphate pathway and glutathione reductase in the antioxidant defenses of human sperm. *Biol Reprod* **2004**, *71*, 1309-1316, doi:10.1095/biolreprod.104.028407.

127. Urner, F.; Sakkas, D. A possible role for the pentose phosphate pathway of spermatozoa in gamete fusion in the mouse. *Biol Reprod* **1999**, *60*, 733-739, doi:10.1095/biolreprod60.3.733.

128. Evdokimov, V.V.; Barinova, K.V.; Turovetskii, V.B.; Muronetz, V.I.; Schmalhausen, E.V. Low Concentrations of Hydrogen Peroxide Activate the Antioxidant Defense System in Human Sperm Cells. *Biochemistry (Mosc)* **2015**, *80*, 1178-1185, doi:10.1134/S0006297915090084.

129. Ford, W.C.; Whittington, K.; Williams, A.C. Reactive oxygen species in human sperm suspensions: production by leukocytes and the generation of

NADPH to protect sperm against their effects. *Int J Androl* **1997**, *20 Suppl 3*, 44-49.

130. Ortiz-Rodriguez, J.M.; Balao da Silva, C.; Masot, J.; Redondo, E.; Gazquez, A.; Tapia, J.A.; Gil, C.; Ortega-Ferrusola, C.; Pena, F.J. Rosiglitazone in the thawing medium improves mitochondrial function in stallion spermatozoa through regulating Akt phosphorylation and reduction of caspase 3. *PLoS One* **2019**, *14*, e0211994, doi:10.1371/journal.pone.0211994.

131. Volpe, C.M.O.; Villar-Delfino, P.H.; Dos Anjos, P.M.F.; Nogueira-Machado, J.A. Cellular death, reactive oxygen species (ROS) and diabetic complications. *Cell Death Dis* **2018**, *9*, 119, doi:10.1038/s41419-017-0135-z.

132. Allaman, I.; Belanger, M.; Magistretti, P.J. Methylglyoxal, the dark side of glycolysis. *Front Neurosci* **2015**, *9*, 23, doi:10.3389/fnins.2015.00023.

133. Nevin, C.; McNeil, L.; Ahmed, N.; Murgatroyd, C.; Brison, D.; Carroll, M. Investigating the Glycating Effects of Glucose, Glyoxal and Methylglyoxal on Human Sperm. *Sci Rep* **2018**, *8*, 9002, doi:10.1038/s41598-018-27108-7.

134. Chen, M.C.; Lin, J.A.; Lin, H.T.; Chen, S.Y.; Yen, G.C. Potential effect of advanced glycation end products (AGEs) on spermatogenesis and sperm quality in rodents. *Food Funct* **2019**, *10*, 3324-3333, doi:10.1039/c9fo00240e.

135. Karimi, J.; Goodarzi, M.T.; Tavilani, H.; Khodadadi, I.; Amiri, I. Relationship between advanced glycation end products and increased lipid peroxidation in semen of diabetic men. *Diabetes Res Clin Pract* **2011**, *91*, 61-66, doi:10.1016/j.diabres.2010.09.024.

136. Aquila, S.; Gentile, M.; Middea, E.; Catalano, S.; Ando, S. Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology* **2005**, *146*, 552-557, doi:10.1210/en.2004-1252.

137. Sangeeta, S.; Arangasamy, A.; Kulkarni, S.; Selvaraju, S. Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. *Anim Reprod Sci* **2015**, *161*, 82-88, doi:10.1016/j.anireprosci.2015.08.008.

138. Lahnsteiner, F. The role of free amino acids in semen of rainbow trout Oncorhynchus mykiss and carp Cyprinus carpio. *J Fish Biol* **2009**, *75*, 816-833, doi:10.1111/j.1095-8649.2009.02317.x.

139. Koppula, P.; Zhang, Y.; Zhuang, L.; Gan, B. Amino acid transporter SLC7A11/xCT at the crossroads of regulating redox homeostasis and nutrient dependency of cancer. *Cancer Commun (Lond)* **2018**, *38*, 12, doi:10.1186/s40880-018-0288-x.

140. Breda, C.N.S.; Davanzo, G.G.; Basso, P.J.; Saraiva Camara, N.O.; Moraes-Vieira, P.M.M. Mitochondria as central hub of the immune system. *Redox Biol* **2019**, *26*, 101255, doi:10.1016/j.redox.2019.101255.

141. Bromfield, E.G.; Aitken, R.J.; Anderson, A.L.; McLaughlin, E.A.; Nixon, B. The impact of oxidative stress on chaperone-mediated human sperm-egg interaction. *Hum Reprod* **2015**, *30*, 2597-2613, doi:10.1093/humrep/dev214.

142. Gharagozloo, P.; Aitken, R.J. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum Reprod* **2011**, *26*, 1628-1640, doi:10.1093/humrep/der132.

143. Aitken, R.J.; De Iuliis, G.N.; Finnie, J.M.; Hedges, A.; McLachlan, R.I. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Hum Reprod* **2010**, *25*, 2415-2426, doi:10.1093/humrep/deq214.

144. Aitken, R.J.; Baker, M.A. Oxidative stress, sperm survival and fertility control. *Mol Cell Endocrinol* **2006**, *250*, 66-69, doi:10.1016/j.mce.2005.12.026.

145. Thomson, L.K.; Fleming, S.D.; Aitken, R.J.; De Iuliis, G.N.; Zieschang, J.A.; Clark, A.M. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod* **2009**, *24*, 2061-2070, doi:10.1093/humrep/dep214.

146. Garcia, B.M.; Moran, A.M.; Fernandez, L.G.; Ferrusola, C.O.; Rodriguez, A.M.; Bolanos, J.M.; da Silva, C.M.; Martinez, H.R.; Tapia, J.A.; Pena, F.J. The mitochondria of stallion spermatozoa are more sensitive than the

plasmalemma to osmotic-induced stress: role of c-Jun N-terminal kinase (JNK) pathway. *J Androl* **2012**, *33*, 105-113, doi:10.2164/jandrol.110.011957.

147. Pena, F.J.; Ball, B.A.; Squires, E.L. A new method for evaluating stallion sperm viability and mitochondrial membrane potential in fixed semen samples. *Cytometry B Clin Cytom* **2016**, 10.1002/cyto.b.21506, doi:10.1002/cyto.b.21506.

148. Balao da Silva, C.M.; Ortega Ferrusola, C.; Morillo Rodriguez, A.; Gallardo Bolanos, J.M.; Plaza Davila, M.; Morrell, J.M.; Rodriguez Martinez, H.; Tapia, J.A.; Aparicio, I.M.; Pena, F.J. Sex sorting increases the permeability of the membrane of stallion spermatozoa. *Anim Reprod Sci* **2013**, *138*, 241-251, doi:10.1016/j.anireprosci.2013.02.021.

149. Rodriguez, A.M.; Ferrusola, C.O.; Garcia, B.M.; Morrell, J.M.; Martinez, H.R.; Tapia, J.A.; Pena, F.J. Freezing stallion semen with the new Caceres extender improves post thaw sperm quality and diminishes stallion-to-stallion variability. *Anim Reprod Sci* **2011**, *127*, 78-83, doi:10.1016/j.anireprosci.2011.07.009.

150. Ortega Ferrusola, C.; Gonzalez Fernandez, L.; Salazar Sandoval, C.; Macias Garcia, B.; Rodriguez Martinez, H.; Tapia, J.A.; Pena, F.J. Inhibition of the mitochondrial permeability transition pore reduces "apoptosis like" changes during cryopreservation of stallion spermatozoa. *Theriogenology* **2010**, *74*, 458-465, doi:10.1016/j.theriogenology.2010.02.029.

151. Ortega-Ferrusola, C.; Gil, M.C.; Rodriguez-Martinez, H.; Anel, L.; Pena, F.J.; Martin-Munoz, P. Flow cytometry in Spermatology: A bright future ahead. *Reprod Domest Anim* **2017**, *52*, 921-931, doi:10.1111/rda.13043.

152. Gallardo Bolanos, J.M.; Miro Moran, A.; Balao da Silva, C.M.; Morillo Rodriguez, A.; Plaza Davila, M.; Aparicio, I.M.; Tapia, J.A.; Ortega Ferrusola, C.; Pena, F.J. Autophagy and apoptosis have a role in the survival or death of stallion spermatozoa during conservation in refrigeration. *PLoS One* **2012**, *7*, e30688, doi:10.1371/journal.pone.0030688.

153. Gueraud, F.; Atalay, M.; Bresgen, N.; Cipak, A.; Eckl, P.M.; Huc, L.; Jouanin, I.; Siems, W.; Uchida, K. Chemistry and biochemistry of lipid peroxidation products. *Free Radic Res* **2010**, *44*, 1098-1124, doi:10.3109/10715762.2010.498477.

154. Uchida, K. Lipid peroxidation and redox-sensitive signaling pathways. *Curr Atheroscler Rep* **2007**, *9*, 216-221.

155. Uchida, K. Cellular response to bioactive lipid peroxidation products. *Free Radic Res* **2000**, *33*, 731-737.

156. Macias Garcia, B.; Gonzalez Fernandez, L.; Ortega Ferrusola, C.; Morillo Rodriguez, A.; Gallardo Bolanos, J.M.; Rodriguez Martinez, H.; Tapia, J.A.; Morcuende, D.; Pena, F.J. Fatty acids and plasmalogens of the phospholipids of the sperm membranes and their relation with the post-thaw quality of stallion spermatozoa. *Theriogenology* **2011**, *75*, 811-818, doi:10.1016/j.theriogenology.2010.10.021.

157. Garcia, B.M.; Fernandez, L.G.; Ferrusola, C.O.; Salazar-Sandoval, C.; Rodriguez, A.M.; Martinez, H.R.; Tapia, J.A.; Morcuende, D.; Pena, F.J. Membrane lipids of the stallion spermatozoon in relation to sperm quality and susceptibility to lipid peroxidation. *Reprod Domest Anim* **2011**, *46*, 141-148, doi:10.1111/j.1439-0531.2010.01609.x.

158. Martin Munoz, P.; Anel-Lopez, L.; Ortiz-Rodriguez, J.M.; Alvarez, M.; de Paz, P.; Balao da Silva, C.; Rodriguez Martinez, H.; Gil, M.C.; Anel, L.; Pena, F.J., et al. Redox cycling induces spermptosis and necrosis in stallion spermatozoa while the hydroxyl radical (OH*) only induces spermptosis. *Reprod Domest Anim* **2018**, *53*, 54-67, doi:10.1111/rda.13052.

159. Hall, S.E.; Aitken, R.J.; Nixon, B.; Smith, N.D.; Gibb, Z. Electrophilic aldehyde products of lipid peroxidation selectively adduct to heat shock protein 90 and arylsulfatase A in stallion spermatozoa. *Biol Reprod* **2017**, *96*, 107-121, doi:10.1095/biolreprod.116.145292.

160. Bromfield, E.G.; Aitken, R.J.; McLaughlin, E.A.; Nixon, B. Proteolytic degradation of heat shock protein A2 occurs in response to oxidative stress in male germ cells of the mouse. *Mol Hum Reprod* **2017**, *23*, 91-105, doi:10.1093/molehr/gaw074.

161. Gibb, Z.; Lambourne, S.R.; Curry, B.J.; Hall, S.E.; Aitken, R.J. Aldehyde Dehydrogenase Plays a Pivotal Role in the Maintenance of Stallion Sperm Motility. *Biol Reprod* **2016**, *94*, 133, doi:10.1095/biolreprod.116.140509.

162. Moazamian, R.; Polhemus, A.; Connaughton, H.; Fraser, B.; Whiting, S.; Gharagozloo, P.; Aitken, R.J. Oxidative stress and human spermatozoa: diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. *Mol Hum Reprod* **2015**, *21*, 502-515, doi:10.1093/molehr/gav014.

163. Baker, M.A.; Weinberg, A.; Hetherington, L.; Villaverde, A.I.; Velkov, T.; Baell, J.; Gordon, C.P. Defining the mechanisms by which the reactive oxygen species by-product, 4-hydroxynonenal, affects human sperm cell function. *Biol Reprod* **2015**, *92*, 108, doi:10.1095/biolreprod.114.126680.

164. Aitken, R.J.; Baker, M.A. Causes and consequences of apoptosis in spermatozoa; contributions to infertility and impacts on development. *Int J Dev Biol* **2013**, *57*, 265-272, doi:10.1387/ijdb.130146ja.

165. Aitken, R.J.; Smith, T.B.; Lord, T.; Kuczera, L.; Koppers, A.J.; Naumovski, N.; Connaughton, H.; Baker, M.A.; De Iuliis, G.N. On methods for the detection of reactive oxygen species generation by human spermatozoa: analysis of the cellular responses to catechol oestrogen, lipid aldehyde, menadione and arachidonic acid. *Andrology* **2013**, *1*, 192-205, doi:10.1111/j.2047-2927.2012.00056.x.

166. Aurich, C.; Ortega Ferrusola, C.; Pena Vega, F.J.; Schrammel, N.; Morcuende, D.; Aurich, J. Seasonal changes in the sperm fatty acid composition of Shetland pony stallions. *Theriogenology* **2018**, *107*, 149-153, doi:10.1016/j.theriogenology.2017.11.004.

1232 167. Zimniak, P. Relationship of electrophilic stress to aging. *Free Radic Biol Med*
1233 **2011**, *51*, 1087-1105, doi:10.1016/j.freeradbiomed.2011.05.039.
1234 168. Martinez-Pastor, F.; Mata-Campuzano, M.; Alvarez-Rodriguez, M.; Alvarez,
1235 M.; Anel, L.; de Paz, P. Probes and techniques for sperm evaluation by flow
1236 cytometry. *Reprod Domest Anim* **2010**, *45 Suppl 2*, 67-78, doi:10.1111/j.1439-
1237 0531.2010.01622.x.
1238 169. Aitken, R.J.; Gibb, Z.; Mitchell, L.A.; Lambourne, S.R.; Connaughton, H.S.;
1239 De Iuliis, G.N. Sperm motility is lost in vitro as a consequence of
1240 mitochondrial free radical production and the generation of electrophilic
1241 aldehydes but can be significantly rescued by the presence of nucleophilic
1242 thiols. *Biol Reprod* **2012**, *87*, 110, doi:10.1095/biolreprod.112.102020.
1243 170. Bromfield, E.G.; McLaughlin, E.A.; Aitken, R.J.; Nixon, B. Heat Shock
1244 Protein member A2 forms a stable complex with angiotensin converting
1245 enzyme and protein disulfide isomerase A6 in human spermatozoa. *Mol*
1246 *Hum Reprod* **2016**, *22*, 93-109, doi:10.1093/molehr/gav073.
1247 171. Aitken, R.J.; Flanagan, H.M.; Connaughton, H.; Whiting, S.; Hedges, A.;
1248 Baker, M.A. Involvement of homocysteine, homocysteine thiolactone, and
1249 paraoxonase type 1 (PON-1) in the etiology of defective human sperm
1250 function. *Andrology* **2016**, *4*, 345-360, doi:10.1111/andr.12157.
1251 172. Teperek, M.; Simeone, A.; Gaggioli, V.; Miyamoto, K.; Allen, G.E.; Erkek, S.;
1252 Kwon, T.; Marcotte, E.M.; Zegerman, P.; Bradshaw, C.R., et al. Sperm is
1253 epigenetically programmed to regulate gene transcription in embryos.
1254 *Genome Res* **2016**, *26*, 1034-1046, doi:10.1101/gr.201541.115.
1255 173. Valcarce, D.G.; Carton-Garcia, F.; Riesco, M.F.; Herraez, M.P.; Robles, V.
1256 Analysis of DNA damage after human sperm cryopreservation in genes
1257 crucial for fertilization and early embryo development. *Andrology* **2013**, *1*,
1258 723-730, doi:10.1111/j.2047-2927.2013.00116.x.
1259 174. Valcarce, D.G.; Carton-Garcia, F.; Herraez, M.P.; Robles, V. Effect of
1260 cryopreservation on human sperm messenger RNAs crucial for fertilization

and early embryo development. *Cryobiology* **2013**, *67*, 84-90, doi:10.1016/j.cryobiol.2013.05.007.

175. Kopeika, J.; Thornhill, A.; Khalaf, Y. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. *Hum Reprod Update* **2015**, *21*, 209-227, doi:10.1093/humupd/dmu063.

176. Rex, A.S.; Aagaard, J.; Fedder, J. DNA fragmentation in spermatozoa: a historical review. *Andrology* **2017**, *5*, 622-630, doi:10.1111/andr.12381.

177. Evenson, D.P.; Kasperson, K.; Wixon, R.L. Analysis of sperm DNA fragmentation using flow cytometry and other techniques. *Soc Reprod Fertil Suppl* **2007**, *65*, 93-113.

178. Lhomme, J.; Constant, J.F.; Demeunynck, M. Abasic DNA structure, reactivity, and recognition. *Biopolymers* **1999**, *52*, 65-83, doi:10.1002/1097-0282(1999)52:2<65::AID-BIP1>3.0.CO;2-U.

179. Belmont, P.; Jourdan, M.; Demeunynck, M.; Constant, J.F.; Garcia, J.; Lhomme, J.; Carez, D.; Croisy, A. Abasic site recognition in DNA as a new strategy to potentiate the action of anticancer alkylating drugs? *J Med Chem* **1999**, *42*, 5153-5159.

180. Greenberg, M.M. Looking beneath the surface to determine what makes DNA damage deleterious. *Curr Opin Chem Biol* **2014**, *21*, 48-55, doi:10.1016/j.cbpa.2014.03.018.

181. San Pedro, J.M.; Greenberg, M.M. 5,6-Dihydropyrimidine peroxyl radical reactivity in DNA. *J Am Chem Soc* **2014**, *136*, 3928-3936, doi:10.1021/ja412562p.

182. Greenberg, M.M. Abasic and oxidized abasic site reactivity in DNA: enzyme inhibition, cross-linking, and nucleosome catalyzed reactions. *Acc Chem Res* **2014**, *47*, 646-655, doi:10.1021/ar400229d.

183. Balao da Silva, C.M.; Ortega-Ferrusola, C.; Morrell, J.M.; Rodriguez Martinez, H.; Pena, F.J. Flow Cytometric Chromosomal Sex Sorting of

Stallion Spermatozoa Induces Oxidative Stress on Mitochondria and Genomic DNA. *Reprod Domest Anim* **2016**, *51*, 18-25, doi:10.1111/rda.12640.

184. Vorilhon, S.; Brugnon, F.; Kocer, A.; Dollet, S.; Bourgne, C.; Berger, M.; Janny, L.; Pereira, B.; Aitken, R.J.; Moazamian, A., et al. Accuracy of human sperm DNA oxidation quantification and threshold determination using an 8-OHdG immuno-detection assay. *Hum Reprod* **2018**, *33*, 553-562, doi:10.1093/humrep/dey038.

185. Li, Z.; Yang, J.; Huang, H. Oxidative stress induces H2AX phosphorylation in human spermatozoa. *FEBS Lett* **2006**, *580*, 6161-6168, doi:10.1016/j.febslet.2006.10.016.

186. Garolla, A.; Cosci, I.; Bertoldo, A.; Sartini, B.; Boudjema, E.; Foresta, C. DNA double strand breaks in human spermatozoa can be predictive for assisted reproductive outcome. *Reprod Biomed Online* **2015**, *31*, 100-107, doi:10.1016/j.rbmo.2015.03.009.

187. Castillo, J.; Jodar, M.; Oliva, R. The contribution of human sperm proteins to the development and epigenome of the preimplantation embryo. *Hum Reprod Update* **2018**, *24*, 535-555, doi:10.1093/humupd/dmy017.

188. Aitken, R.J.; Curry, B.J. Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxid Redox Signal* **2011**, *14*, 367-381, doi:10.1089/ars.2010.3186.

189. Burruel, V.; Klooster, K.L.; Chitwood, J.; Ross, P.J.; Meyers, S.A. Oxidative damage to rhesus macaque spermatozoa results in mitotic arrest and transcript abundance changes in early embryos. *Biol Reprod* **2013**, *89*, 72, doi:10.1095/biolreprod.113.110981.

190. McCarthy, M.J.; Baumber, J.; Kass, P.H.; Meyers, S.A. Osmotic stress induces oxidative cell damage to rhesus macaque spermatozoa. *Biol Reprod* **2010**, *82*, 644-651, doi:10.1095/biolreprod.109.080507.

191. Ortiz-Rodriguez, J.M.; Ortega-Ferrusola, C.; Gil, M.C.; Martin-Cano, F.E.; Gaitskell-Phillips, G.; Rodriguez-Martinez, H.; Hinrichs, K.; Alvarez-Barrientos, A.; Roman, A.; Pena, F.J. Transcriptome analysis reveals that fertilization with cryopreserved sperm downregulates genes relevant for early embryo development in the horse. *PLoS One* **2019**, *14*, e0213420, doi:10.1371/journal.pone.0213420.

192. Jodar, M. Sperm and seminal plasma RNAs: What roles do they play beyond fertilization? *Reproduction* **2019**, 10.1530/REP-18-0639, doi:10.1530/REP-18-0639.

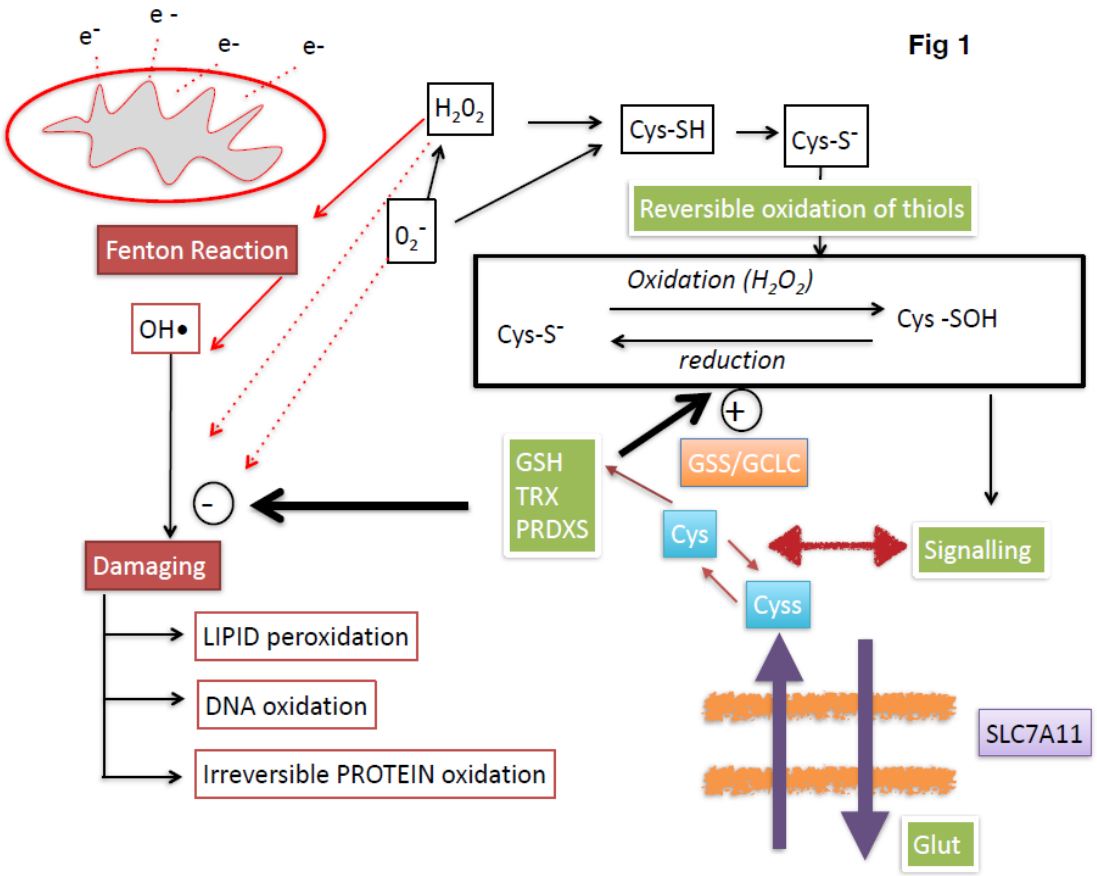
193. Zhou, D.; Suzuki, T.; Asami, M.; Perry, A.C.F. Caput Epididymidal Mouse Sperm Support Full Development. *Dev Cell* **2019**, *50*, 5-6, doi:10.1016/j.devcel.2019.05.012.

194. Morielli, T.; O'Flaherty, C. Oxidative stress impairs function and increases redox protein modifications in human spermatozoa. *Reproduction* **2015**, *149*, 113-123, doi:10.1530/REP-14-0240.

Peer-reviewed version available at Antioxidants 2019, 8, 567; doi:10.3390/antiox8110567

1347 Figure legends

1348



1349

1350 Fig 1.- Redox regulation in stallion spermatozoa. Electron leakage at the
1351 mitochondria is one of the main sources of ROS. Mechanisms to maintain redox
1352 homeostasis include thioredoxin and peroxiredoxin systems and GSH. The stallion
1353 spermatozoa can incorporate cyss to contribute to the intracellular GSH pool.
1354 Controlled levels of ROS regulate sperm functionality through reversible oxidation
1355 of thiols in cysteine containing proteins. If redox regulation is lost, irreversible
1356 oxidation of thiols and oxidative attack to lipids DNA and proteins occurs leading
1357 to sperm malfunction and finally death.

1358

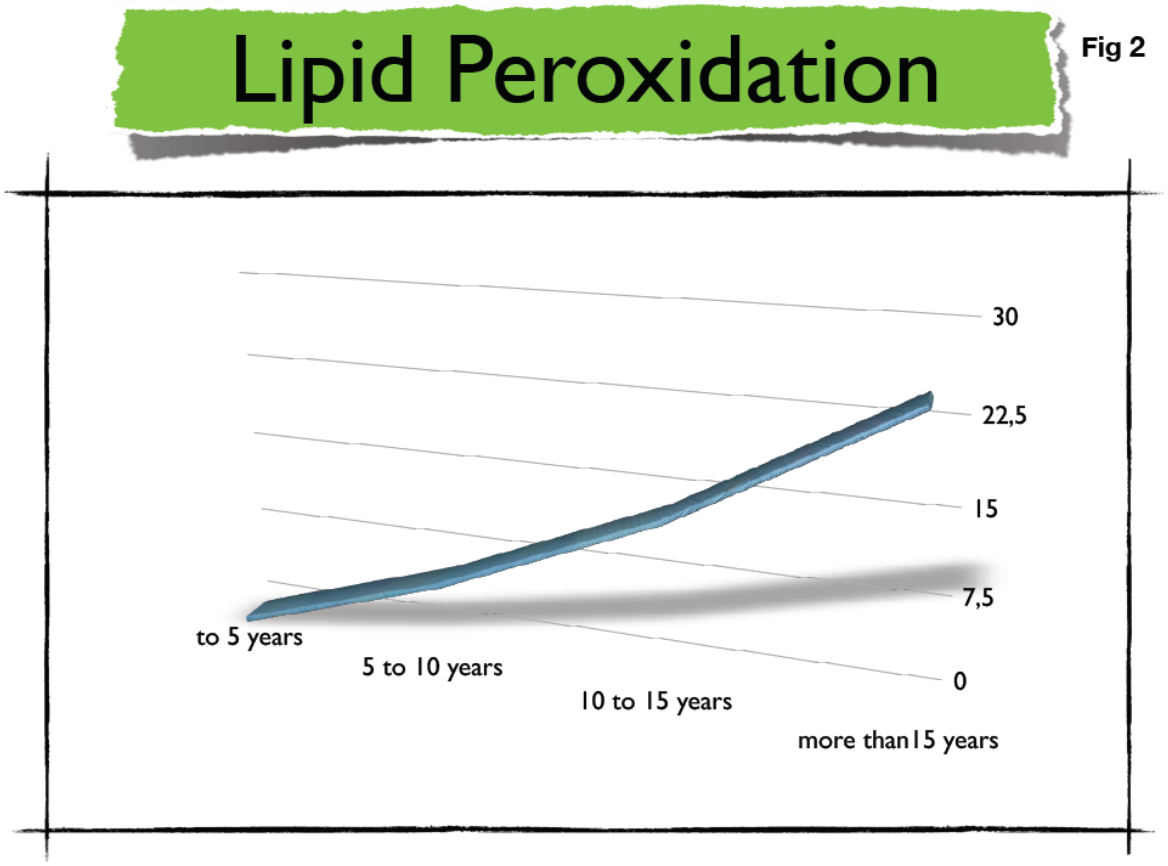


Fig 2.- Effect of stallion age in the peroxidation of sperm membranes, semen was collected from stallions of different ages and lipid peroxidation was assessed flow cytometrically after BODIPY 581/591 C11, as seen in the figure, lipid peroxidation increases with age

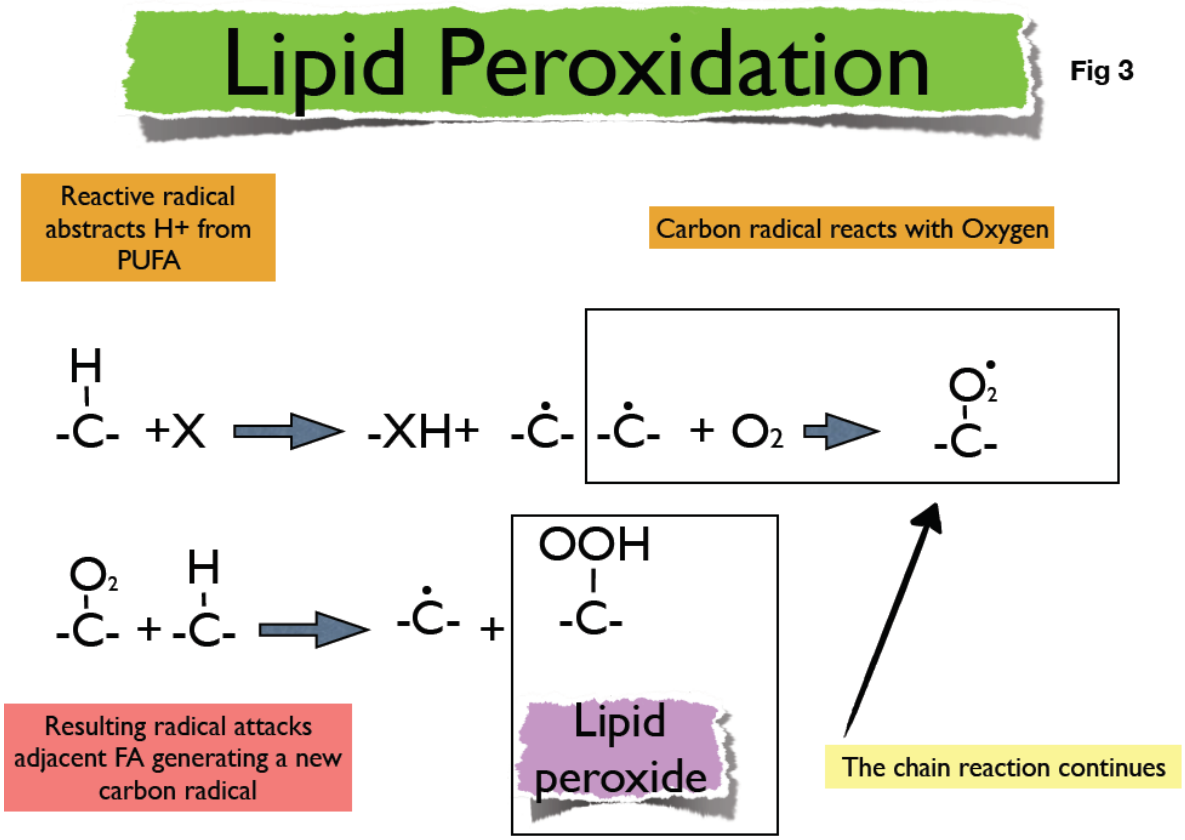


Fig 3.- Schematic overview of lipid peroxidation of the membranes; a lipid radical abstracts and hydrogen from a PUFA, generating a carbon centered radical reacts with and oxygen forming an oxygen centered radical, that abstracts another hydrogen from an adjacent PUFA, forming a lipid peroxide and propagating the cycle