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Effect of Rooibos (*Aspalathus linearis*) Extract on Boar Sperm Samples Submitted to Oxidative Stress

Diploma Thesis

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Declaration

I declare that the Diploma Thesis "Effect of Rooibos (*Aspalathus linearis*) Extract on Boar Sperm Samples Submitted to Oxidative Stress" is my own work and all the sources I cited in it are listed in Bibliography.

Prague, 10/04/2019

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Summary

Rooibos (*Aspalathus linearis*) is a small bush, growing in South Africa. This plant is known especially as rooibos tea which has become popular in the last decades. Rooibos contains numerous substances beneficial to the organism, for instance, antioxidants, comprising flavonoids and phenolic acids. Many studies used rooibos *in vivo* to test its antioxidant effect in different cell lines. In this study, we used rooibos extract *in vitro* in boar spermatozoa submitted to oxidative stress. Sperm samples were incubated in a water bath at 38 °C with a reactive oxygen species (ROS) generating system (Fe^{2+}/ascorbate) and supplemented with three concentrations (0.017 % m/v, 0.006 % m/v and 0.002 % m/v) of fermented and unfermented rooibos extracts. The effects of rooibos extracts on sperm motility, membrane integrity, acrosomal status and lipid peroxidation were evaluated at 3.5 h of sperm incubation. Our results indicated that treatments with rooibos extract showed lower MDA levels than those of control - ox group ($p < 0.05$). The lowest MDA levels were found mainly in the treatments with unfermented rooibos. The extract of *Aspalathus linearis* significantly improved also sperm membrane integrity ($p < 0.05$) and even though significant differences were not found in motility, acrosome integrity and tail membrane integrity, some concentrations (especially the lower ones) have a higher average than the control - ox group. The highest concentrations may have been scavenging higher amounts of ROS, even those that are necessary for the correct sperm functionality. The results of this study provide new insights into the application of rooibos *in vitro* against the negative effects of oxidative stress in boar spermatozoa.

Keywords: acrosome, boar, lipid peroxidation, oxidative stress, rooibos, sperm kinetics, sperm membrane

Účinek extraktu z rooibosu (*Aspalathus linearis*) na vzorky kančích spermií vystavených oxidačnímu stresu

Souhrn

Rooibos (*Aspalathus linearis*) je drobný keř, rostoucí v Jižní Africe. Tato rostlina je známá především jako čaj rooibos, který se stal oblíbeným v posledních desetiletích. Rooibos obsahuje mnoho látek prospěšných pro organismus, například antioxidanty, flavonoidy a fenolické kyseliny. Rooibos byl v mnoha studiích použit *in vivo* k testování jeho antioxidačních účinků v organismu. V této práci jsme použili extrakt z rooibosu *in vitro* a přidali ho ke kančím spermiím, které byly podrobené oxidačnímu stresu. Testované vzorky byly inkubovány ve vodní lázni při teplotě 38 °C se systémem generujícím reaktivní formy kyslíku (ROS) (Fe^{2+}/ askorbát) a byly doplněny třemi koncentracemi fermentovaného a nefermentovaného extraktu z rooibosu (0,017 % m/v; 0,006 % m/v; 0,002 % m/v). Účinky extraktu z roibosu na pohyblivost spermií, celistvost membrán, stav akrozomů a peroxidaci lipidů byly hodnoceny po 3,5 hodinách inkubace daných vzorků. Naše výsledky ukázaly, že vzorky s rooibosem vykazovaly nižší hladiny MDA než u kontrolních skupin ($p < 0,05$). Nejnižší hladiny MDA byly zjištěny hlavně u vzorků s nefermentovaným rooibosem. Extrakt z *Aspalathus linearis* významně zlepšil také celistvost membrán spermií ($p < 0,05$), a i když nebyly zjištěny významné rozdíly v motilitě, celistvosti akrozomů a celistvosti ocasní membrány spermií, některé koncentrace (zejména nižší) mají vyšší hodnoty než kontrolní skupina podrobená oxidačnímu stresu. Nejvyšší koncentrace rooibosu ve vzorcích mohly odstranit vyšší množství volných radikálů, dokonce i těch, které jsou nezbytné pro správnou funkci spermií. Výsledky této studie poskytují nové poznatky o aplikaci rooibosu *in vitro* proti negativním účinkům oxidačního stresu u kančích spermiích.

Klíčová slova: akrosom, kanec, peroxidace lipidů, oxidační stres, rooibos, kinetika spermií, membrána spermií

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1 Introduction

Improving management in livestock artificial insemination (AI) and increasing the use of sires with valuable genomes are high on the list of precedence for livestock breeders worldwide. Significant progress in breeding has been reached in the optimization of female reproductive performance, for instance, good oestrous detection and management of female reproductive functions. Management of reproduction of sires and its improvement is also important for quality and successful AI. Progress can be achieved through the improvement of semen collection, analysis, and cryopreservation (Petruska et al. 2014).

Semen processing (e.g., excessive centrifugation, sperm preservation, etc.), together with the low antioxidant levels in the seminal plasma, leads to higher production of reactive oxygen species (ROS), thus arising an imbalance between free radicals and the antioxidants of extended semen, called oxidative stress (OS) (Sikka 2004). The physiological concentration of ROS has a positive effect on cells, for instance, on the intracellular signalling, differentiation, migration, it also participates in sperm capacitation, hyperactivation, and sperm - oocyte fusion (Saleh & Agarwal 2002). Low levels of ROS have been shown to be necessary for fertilization, acrosome reaction and sperm motility (Sikka 1996; Griveau et al. 1997). Nevertheless, immoderate production of ROS may have a toxic effect on sperm. The high production of free radicals influences functional characteristics of the spermatozoa, such as reducing sperm motility (Sikka 1996), damaging the acrosomal membranes and inactivating glycolytic enzymes (Alvarez & Storey 1984). These changes may lead to loss of fertility of spermatozoa.

Boar spermatozoa have high polyunsaturated fatty acid content and low antioxidant capacity of seminal plasma, so they are very susceptible to lipid peroxidation (Awda et al. 2009). The lipid peroxidation process is incurred by ROS such as H_2O_2 and it is the limiting factor of the lifespan of mammalian spermatozoa. The impacts of lipid peroxidation include irreversible loss of motility, inhibition of respiration, leakage of intracellular enzymes and damage to sperm DNA (White 1993). Recent experiments show that antioxidants, which can improve semen quality, will undoubtedly lead to innovation in AI instrumentation and supplies. The antioxidants against the effect of ROS could be provided in feed, added directly to semen extender, or added to insemination catheters (Petruska et al. 2014). Lots of plants contain these important antioxidants, one of them is *Aspalathus linearis*.

Rooibos (*Aspalathus linearis*) is a small bush, growing in Cederberg Mountains of the Western Cape of South Africa. Rooibos contains a high number of substances beneficial to the

organism. Rooibos tea includes antioxidants, comprising flavonoids and phenolic acid that are potent free radical scavengers (Erickson 2008).

Many studies have tested the effects of rooibos under several pathological conditions with positive results. For example, on diseases including antispasmodic effects, immune system modulation, antimicrobial, antiviral and antiaging properties (Ichiyama et al. 2000; Marnewick et al. 2000; Scheepers 2001). Rooibos tea improved rat's sperm function due to its high level of antioxidants (Opuwari & Monsees 2014). The main goal of the present thesis is to test if Rooibos (*Aspalathus linearis*) extract can protect boar spermatozoa against the negative effects of oxidative stress and keep their quality.

2 Scientific Hypothesis and Objectives of the Work

The hypothesis of the present work was that rooibos (*Aspalathus linearis*) extract can protect boar spermatozoa against the deleterious effects of oxidative stress due to its antioxidant properties. The objective of this thesis was to test the effect of rooibos extract on semen samples submitted to oxidative stress. For this purpose, several sperm parameters (kinetics, membrane and acrosome integrity, and lipid peroxidation) were evaluated after incubation of the semen samples at 38 ºC for 3.5 hours.

3 Literature Overview

3.1 Artificial insemination

Artificial insemination (AI) plays an important role in pig reproduction and it offers several advantages. AI supports an economical use of sires, it also makes the organization of reproduction easier and decreases infectious diseases in the herd (Bajena et al. 2016). The main criterion in the selection of boars for AI is the breeding value, and the high-quality ejaculates play an important role as well (Schulze et al. 2014). These are the two fundamental reasons why the insemination centres concentrate on using the semen of boars providing high-quality ejaculates with respect to the physical parameters (Bajena et al. 2016). There are several parameters, which influence the quality and quantity of ejaculates, such as genetic and environmental factors, the breed, the age of the boar, the size of testes, the season of the year, the microclimate conditions and the dietary regime (Bajena et al. 2016).

The evaluation of male fertility is usually based on semen assessment using basic parameters as sperm motility, semen density and the incidence of sperm abnormalities (Gordon 2017). Diluents and conservation of the collected ejaculate also influence the fertility of the semen (Schulze et al. 2014).

The boar semen is stored at 15 - 20 °C for 1 to 5 days or is used immediately after the collection. This is called short - term conservation and more than 99 % of worldwide inseminations use this method (Johnson et al. 2000). Long-term conservation is not so frequently used for the boar semen, because of the sensitivity of a boar sperm membrane. The boar spermatozoa are known to be very sensitive to cold shock (Gordon 2017). This cold shock causes that the function of the sperm membrane becomes changed by lateral movements of membrane phospholipids which are reduced, thus the separation of the lipid phases arises there (Gadea 2003). Moreover, the boar sperm membrane is defined for the high content in polyunsaturated fatty acids, which are as a good substrate for reactive oxygen species, thus the oxidative stress becomes during the long semen liquid - storage (Brouwers et al. 2005; Awda et al. 2009).

Since 1975, frozen semen has been commercially available (Johnson et al. 2000), even though the use of frozen semen is not recommended for the successful insemination. The major reason for the limited commercial application of frozen semen is the shortened lifespan of frozen-thawed boar sperm in the uterus (Pursel et al. 1978). Successful cryopreservation of boar semen is necessary for international sales (Bailey et al. 2008). Nowadays, the frozen-thawed

boar semen is used for transportation from one country to another in less than 1 % of all inseminations, the cryoconservation of boar semen is still in the progress (Gordon 2017).

3.2 Characteristics of boar ejaculate

Boar ejaculate consists of spermatozoa and seminal plasma (SP) (Carrillo 2016). It is a mix of caudal spermatozoa which are released during ejaculation and secretions of the various accessory sex glands. The ejaculation is an action when the semen is released by the male (Marberger 1974). This process is controlled by the central nervous system and it consists of two phases (deGroat & Booth 1980). One of them is the emission which is a mixture of the caudal spermatozoa and the seminal plasma in the urethra and the other is the ejection via the penile urethra (Marberger 1974; Carrillo 2016).

The boar ejaculation normally takes 5 - 10 minutes. The ejaculate has got 50 to 500ml of semen where the expected volume for insemination is around 250ml (McGlone & Pond 2003; Jackson & Cockcroft 2007), it includes 120×10^9 of total spermatozoa which should have progressive motility (>70 %) and happens in waves of ejaculation fractions (Carrillo 2016). Ejaculation jets are formed of three parts: the pre-sperm-rich fraction (pre-SRF), the sperm-rich fraction (SRF) and the post-sperm-rich fraction (post-SRF) (Rodríguez-Martínez et al. 2009).

The first fraction, the pre-SRF (10 - 15 ml), contains only a few spermatozoa. In this fraction, there is mainly secretion from the accessory sex glands (the seminal vesicles, the bulbourethral glands and the prostate) but also some gel, urine and smegma. In the second fraction, the SRF (50 ml) has the bulk of spermatozoa, primarily in the beginning, first 10 ml (Rodríguez-Martínez et al. 2005; Peña et al. 2006). The rest of the SRF follows, having high numbers of spermatozoa mixed with a richer protein secretion from the vesicular glands (Siqueira et al. 2011). The last, post-sperm-rich fraction (150 - 400 ml) has gradually decreasing sperm numbers and increased secretions from the seminal vesicles, the bulbourethral glands and the prostate (Lavon & Boursnell 1975; Rodríguez-Martínez et al. 2009).

The seminal plasma of boars is a heterogeneous blend including a few organic and inorganic solutes (Lavon & Boursnell 1975). It consists of many components that influence, inhibit or stimulate, a multitude of sperm functions, the female genital tract during sperm transport and events preceding fertilization (Siqueira et al. 2011). The main organic compounds in the SP are proteins such as spermadhesins, antioxidant enzymes and cytokines. The main carbohydrate in the SP is represented by the glucose (Mann & Lutwak-Mann 1981). Spermadhesins are involved in sperm-interaction with the female genital tract, antioxidant enzymes are very important in the reduction of reactive oxygen species (ROS) concentration

and their oxidative damage to spermatozoa and cytokines have got a signalling role towards the female reproductive tract (Töpfer-Petersen et al. 2008). Ca^{2+} and bicarbonate belong to the most relevant inorganic compounds (Carrillo 2016).

3.2.1 Boar spermatozoa

The spermatozoon arises as a final product in the process called spermatogenesis, this process is situated in the testis (Carrillo 2016). Then, testicular spermatozoa mature in the epididymis and in the process, they acquire the capacity to move and fertilize. Mature spermatozoa are stored at the cauda epididymis in a quiescent status until ejaculation occurs (Eddy 2006).

Spermatogenesis is divided into three specific steps: spermatocytogenesis (mitotic phase), meiosis and spermiogenesis (Carrillo 2016). During the mitotic phase, some spermatogonia enter the spermatogenesis to generate primary spermatocytes, while others are maintained as stem cells. Primary spermatocytes go through prolonged prophase with duplication of the genetic material, the crossing over of genomic material between chromatids, to finally divided into two recombined secondary spermatocytes. These spermatocytes enter the meiosis II and create two haploid cells, the spermatids. The last step is the transformation of the spermatids to spermatozoa during the spermiogenesis (Neill 2005; O'Donnell et al. 2011).

The spermiogenesis is also called spermiohistogenesis and has got four phases: the Golgi phase, the cap phase, the formation of the tail and the maturation stage. The Golgi phase is about the formation of the acrosomic vesicle from the Golgi apparatus, also the proximal centriole gives rise to the attachment point of the tail and the distal centriole give rise to the developing axoneme. In the cap phase, the acrosomic vesicle spreads over the nucleus of the round spermatid and the flagellum starts to form. The acrosomal phase is characterized by the elongation of the spermatid nucleus and cytoplasm, in this phase, the acrosome covers most of the anterior nucleus. In the last phase, the mitochondria are assembled around the flagellum and the flagellum is completely formed (Panawala 2017).

Boar spermatogenesis lasts 38 - 41 days from the moment the spermatogonia enter the ad-luminal compartment until eight immature testicular spermatozoa are shed to the lumen to exit the seminiferous tubules towards the epididymis (Carrillo 2016).

3.2.1.1 The general structure of boar spermatozoon

Sperm morphology is a very significant assessment of sperm fertilizing ability because the structure of spermatozoa is responsible for fundamental functions such as reaching and fertilizing the oocyte (Briz & Fabrega 2013). The ejaculated boar spermatozoon measures 53 - 55 µm in length and it can be separated into two main parts: the head which has got "tennis racket"-shaped or bilaterally flatted and oval shaped head and a cylindrical tail. These two compartments are connected by the connecting piece, neck (Fig. 1) (Carrilo 2016). The spermatozoon is a highly compartmentalized cell with specific areas and a plasma membrane, all are important for sperm interactions with the surroundings (Briz & Fabrega 2013).

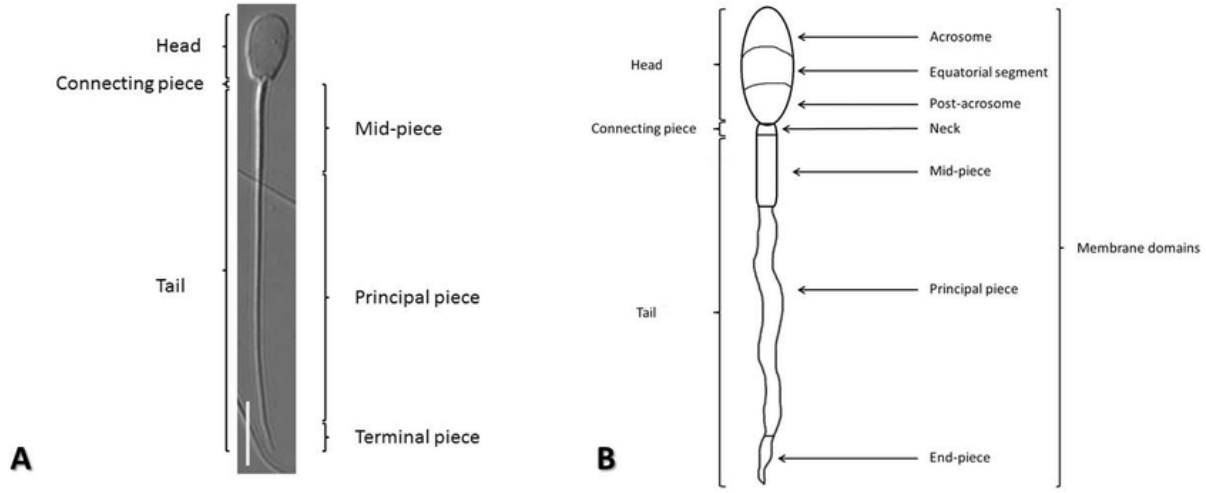

Figure 1: Basic morphological structure and membrane domains of the boar sperm (Carrillo 2016)

3.2.1.1.1 The sperm head

The small and compact boar sperm head is 7 - 9 µm long, 3.7 - 4.2 µm wide and 0.4 µm thick (Thurston et al. 2001). It is formed of the acrosome, the nucleus, cytoskeletal structures and cytoplasm. The other structures in the head post acrosomal dense lamina, the sub acrosomal space and the perinuclear fibrous material (Briz & Fabrega 2013). The sperm nucleus contains very condensed chromatin, its volume is considerably lower than the nuclear volume in a somatic cell (~ 5 %) (Neill 2005). The two meiotic divisions that occur during spermatogenesis result in the sperm containing only one copy of each chromosome, it means that the sperm nucleus is in a haploid version including either an X- or Y- sex chromosome, thus it represents the main genetic material (Nicander & Bane 1962). The sperm nucleus is covered in a nuclear envelope which does not have nuclear pores and the inner and outer membranes are 7 - 10 nm apart. In most other cells the nuclear envelope contains the pores and the two nuclear

membranes are 40 - 60 nm apart. The major nucleus proteins are protamines (Neill 2005; Briz & Fabrega 2013).

The acrosome covers around 80 % of the nucleus. It is a unique sperm organelle which contains hydrolytic enzymes necessary for the sperm to penetrate the zona pellucida during the fertilization. The acrosome is a membrane-enclosed vesicle over the nucleus in the anterior part of the sperm head. It consists of two membranes, with a narrow space inside, the acrosomal matrix, filled with amorphous material distributing hydrolytic enzymes (Neill 2005). The acrosome is formed of three parts: the apical segment, the principal segment and the equatorial segment. The acrosome is very sensitive to osmotic pressure. The post acrosomal dense lamina is from a homogeneous layer of fibrous and electrodense material. This part is in the post acrosomal region of the sperm head and it adheres to the plasmalemma (Briz & Fabrega 2013).

3.2.1.1.2 The flagellum

The flagellum of the spermatozoon consists of four distinct segments: the connecting piece, the middle piece, the principal piece and the end piece (Neill 2005). The connecting piece or the neck is a part connecting the sperm head with the tail. The neck has got a trapezoidal shape and it is 0.7 μm long and 0.5 μm thick (Nicander & Bane 1962). The striated ring separates the connecting piece from the sperm head (Toshimori 1998). The main components of the neck are the basal plate, the laminar bodies, the capitulum, the segmented columns, the basal body and the axoneme. The basal plate is composed of almost the same electrodense material as the post acrosomal dense lamina. The capitulum is a protein structure which is situated from one side to the basal plate to the implantation fossa and from the other to the nine segmented columns, cylinder-shaped. These columns fuse with the outer dense fibers, surrounding the axoneme of the flagellum (Briz & Fabrega 2013).

The mid-piece is 9 μm long and 0.7 μm wide and it is separated from the principal piece by the annulus (Jansen's ring) which has got an electrodense character (Neill 2005). The main parts of the midpiece are the axoneme, the mitochondrial sheath, the outer dense fiber and the peripheral granules (Nicander & Bane 1962). The axoneme is found in the central axis of the midpiece with the classic 9 + 2 microtubular pattern. This complex of microtubules extends the full length of the flagellum and they are situated in the circle as 1 - 9 doublets in the clockwise direction. The axoneme is composed of α- and β- tubulin, in association with dyneins which provide motility to the sperm tail (Briz & Fabrega 2013). The mitochondrial sheath surrounds the outer dense fibers in the middle piece, it is a series of 75 - 100 mitochondria form as a spiral around the axoneme (Eddy 2006).

The principal piece is around 26.2 µm long and 0.4 µm wide and it is the longest segment of the spermatozoon tail (Briz & Fabrega 2013). It is formed by the fibrous sheath, the outer dense fibers, the axoneme and the Jensen's ring. The fibrous sheath surrounds the outer dense fibers which replace the mitochondrial sheath of the midpiece (Nicander & Bane 1962). Two longitudinal axes form the fibrous sheath. It is also formed by basic A-Kinase anchor proteins (AKAPs), mainly AKAP-3 and it contains sperm-specific components of the glycolytic pathway which has a role in the energy production for hyperactivation (Kerr et al. 2006).

The end piece or terminal piece is 2.2 µm long and 0.2 µm wide, and it is the last part of the flagellum. It does not contain any accessory cytoskeletal structures, just the axoneme enclosed by the plasmalemma is present. The axoneme steadily disappears along with the end piece towards the distal part of the spermatozoon (Neill 2005; Briz & Fabrega 2013).

3.2.2 The sperm plasma membrane

The sperm plasma membrane or also the sperm plasmalemma is a significant component of the spermatozoa, which contains an important substance for different processes, such as capacitation, hyperactivation and acrosome reaction (Briz & Fabrega 2013). It ensures homeostasis and controls interaction with various environments. The plasmalemma is built of a typical mosaic fluid model as any other somatic cell membrane is but the lipid and protein domains are different from those somatic cells (Singer & Nicolson 1972). The membrane is organized as a bilayer of amphipathic phospholipids with the hydrophobic moieties arranged next to each other and the hydrophilic part (Gadella et al. 2008). The sperm plasmalemma can be subdivided into different sharply defined domains which are characterised by unique structures inside the cell and domain-specific membrane-receptors. The following three parts of the boar spermatozoon are included into the membrane domains: a) head: acrosome, equatorial segment and post acrosome; b) connecting piece: neck; c) tail: midpiece, principal piece and end-piece. The plasma membrane is in almost direct contact with some intracellular structures due to a small amount of sperm cytoplasm (Toshimori 1998).

As mentioned above the sperm plasma membrane is made from lipids and proteins. This composition of proteins and lipids are found in similar total amounts which are changed during the spermatozoa epididymal transit and capacitation (Briz & Fabrega 2013). Sperm plasma membrane proteins play the main role in sperm-oocyte interaction. Proteins may be either absorbed to extracellular sites or integrated as transmembrane proteins. Membrane lipids are represented by 70 % phospholipids, 25 % neutral lipids and 5 % glycolipids. The boar sperm plasma membrane includes a low amount of cholesterol compared to other cell membranes

(Gadella et al. 2008) which might facilitate surface heterogeneity (Carillo 2008). Phospholipids of boar sperm plasmalemma are susceptible to free radical's attack (Łapkiewicz et al. 1999). Lipid composition of the membrane contains a high concentration of polyunsaturated fatty acids (PUFAs) which serve as preferred substrates for reactive oxygen species (ROS) and HO^{-} generations in membranes (Awda et al. 2009).

3.3 ROS and oxidative stress

Oxidative stress (OS) is defined as a condition that reflects an imbalance between reactive oxygen species (ROS) and a biological system's ability to readily detoxify (antioxidant defences) the reactive intermediates or to repair the resulting damage (Betteridge 2000; Hampl et al. 2012). In a healthy organism, the pro-oxidants and antioxidants are in balance. Spermatozoa are furnished with antioxidant protective mechanisms against ROS (Henkel 2011).

Physiological roles of ROS in cells, when the function of antioxidant is in balance, are cell signalling, immune response or mitochondrial function (Ray et al. 2012). These activities are important in the processes of capacitation, hyperactivation, acrosomal reaction and sperm-oocyte fusion in order to ensure appropriate fertilization (Saleh & Agarwal 2002).

On the other hand, the uncontrolled production of ROS, under the pathological conditions, oversteps the antioxidant capacity of the seminal plasma and it comes to pass negative oxidative stress (Henkel 2011; Trussell 2013). Spermatozoa cannot repair the damage caused by OS because they miss the necessary cytoplasmic-enzyme repair systems (Saleh & Agarwal 2002). Pathological roles of ROS are lipid peroxidation, DNA damage and apoptosis (Fig. 2) (Agarwal et al. 2014).

The main problem is that sperm cell membranes are rich in polyunsaturated fatty acids (PUFAs), so they are highly susceptible to oxygen-induced damage and hence, lipid peroxidation (LPO) (Bansal & Bilaspuri 2011). The loss of intracellular adenosine triphosphate (ATP) due to LPO causes axonemal damage, declined sperm viability, and raised mid-piece sperm morphological defects. In other words, it leads to disruption of the sperm motility (Bansal & Bilaspuri 2011; Gharagozloo & Aitken 2011).

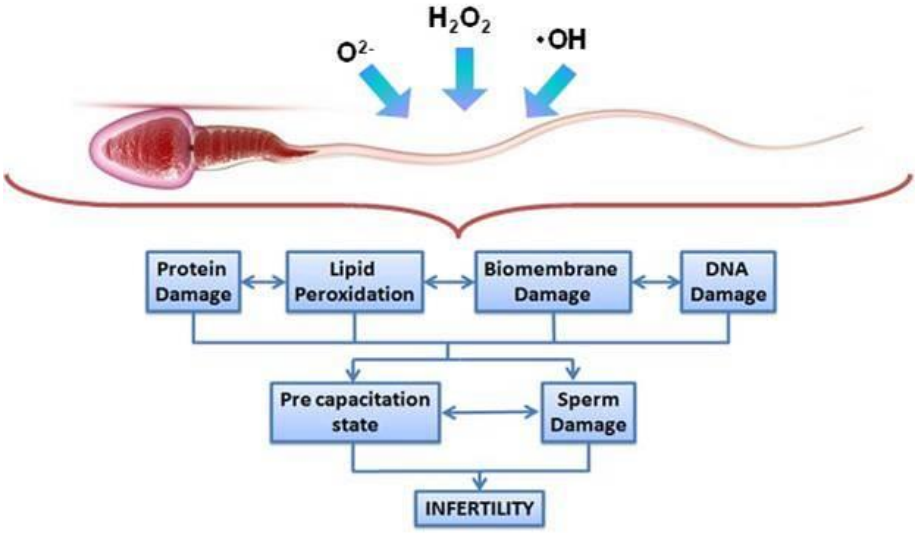

Figure 2: Effect of ROS on sperm physiology (Parodi et al. 2013)

The ability of spermatozoa to generate reactive oxygen species was discovered in 1940. MacLeod (1943) was the first publisher of the article which caused the first sparked interest in oxidative stress and sperm function. MacLeod demonstrated that if spermatozoa were incubated under conditions of high oxygen tension, they quickly lost motility. Thus, MacLeod proved that mammalian spermatozoa have the capacity to generate hydrogen peroxide and that the latter can impair sperm motility. Tosic & Walton (1946) reported the final evidence for the cellular generation of hydrogen peroxide was subsequently secured for bovine spermatozoa. In the same work, the abilities of bull, ram, and boar spermatozoa to generate H_2O_2 were demonstrated and the motility-preserving effects of catalase and peroxidase were confirmed. These conclusions confirm that hydrogen peroxide was particularly cytotoxic for mammalian spermatozoa. The general significance of ROS in the cell biology of mammalian spermatozoa was confirmed in other key papers (Storey et al. 1980; Aitken et al. 2012). Over the past 20 years, this field has moved forward rapidly, for instance, new origins of oxidative stress in the male germ line have been revealed. It has also become evident that ROS can have positive as well as negative impacts on sperm function (Aitken et al. 2012).

ROS, also known as a free radical, have at least one unpaired electron. In generally free radicals there are short lived reactive chemical intermediates which contain one or more the unpaired electrons (Gupta et al. 2005). ROS are oxidizing agents generated as products of the metabolism of oxygen, and because of the unpaired electron, they turn into very reactive molecules which can lead to structural and functional changes and result in cellular damage. ROS represents a broad range of radicals, for instance, hydroxyl ion [OH^-], superoxide ion [O_2^-], nitric oxide [NO], peroxyl [RO_2], lipid peroxyl [LOO], and Thiyl [RS^-] and also non-

radical molecules as singlet oxygen [$^1O_2$], hydrogen peroxide [H_2O_2], hypochloric acid [HOCl], lipid peroxide [LOOH], and ozone [O_3]. The generation of ROS in spermatozoa may occur via two ways, one of them is a hypothetical NADH oxidase at the level of sperm membrane and the other is NADH dependent oxide-reductase reaction at the mitochondrial level (Bansal & Bilaspuri 2011; Agarwal et al. 2014). Leukocytes and immature spermatozoa are the two main sources of ROS. Leukocytes mainly neutrophils and macrophages have been related to excessive ROS production and they ultimately cause sperm dysfunction (Bansal &Bilaspuri 2011).

The absence of endogenous defence mechanism and a second exposure of gametes and embryos to various manipulation techniques as well as environment belong to the major factors conducing to ROS accumulation in vitro. Levels of ROS can seldom change within a fertile individual, but they do not influence sperm concentration and motility. This can happen due to the presence of satisfactory antioxidant defence mechanisms in healthy individuals. The fluctuations in ROS levels may be due to temporary subclinical infection and temporary abnormalities in spermatogenesis, such as retention of cytoplasm or periodic presence of abnormal spermatozoa in semen (Bansal &Bilaspuri 2011).

In other words, endogenous sources of reactive oxygen species are leukocytes, immature spermatozoa and varicocele. Arranged radiation, toxins, smoking and alcohol belong to exogenous sources of ROS (Agarwal et al. 2014). In the case of boar sperms, the main causes are PUFAs (Awda et al. 2009).

3.3.1 Lipid peroxidation

Lipid peroxidation (LPO) cascade arises from the mechanism of ROS - induced damage to spermatozoa and includes an oxidative attack on the sperm membrane lipids. As already mentioned, the boar sperm plasma membrane contains a high concentration of PUFAs which make them delicate to peroxidative damage (Agarwal et al. 2014). These lipids consist of unconjugated double bonds separated by methylene groups. The position of a double bond adjoining to a methylene group debilitates the methyl carbon-hydrogen bond, therefore making hydrogen highly susceptible to abstraction and oxidative damage. Peroxidation of PUFAs in the sperm cell membrane is an autocatalytic, self-propagating reaction (Bansal & Bilaspuri 2011). PUFAs are used as favour substrates for ROS and $HO^{\cdot}$ generation in membranes when there is low antioxidant capacity of boar seminal plasma. Lipid peroxidation also disrupts mitochondrial enzymes and it can interrupt the respiration associated with oxidative phosphorylation (Awda et al. 2009). All things considered, LPO can disrupt membrane

structure and function by disordering membrane phospholipid structure and changing membrane fluidity. This consequently leads to the loss of membrane and morphological integrity, impaired cell functions, along with damaged sperm motility and induction of sperm apoptosis (Bansal & Bilaspuri 2011).

3.4 Antioxidants

Antioxidants are molecules which can safely interact with free radical scavengers that suppress the formation of ROS and oppose their actions (Sikka 2004; Boziaris et al. 2006). In the reproductive system of males, antioxidant systems are contained in spermatozoa and in the seminal plasma. It includes, for instance, glutathione and ergothioneine, capable of counteracting the negative effects of ROS. The antioxidants from seminal plasma replace the deficiency in cytoplasmic enzymes in the spermatozoa (Fraser et al. 2004).

Antioxidants may be divided into two groups according to their action: preventing antioxidants and scavenging antioxidants. The preventing antioxidants are metal chelators or binding proteins, for example, lactoferrin and transferrin which prevent the formation of ROS. To the second group belong, for instance, vitamins C and E as they remove the ROS that are already present (Lampiao 2012). Antioxidants can be also categorized as enzymatic antioxidants and non-enzymatic ones. Non-enzymatic antioxidants are vitamins C, E and B, carotenoids, carnitines, cysteines, pentoxifylline, metals, taurine, hypotaurine and albumin. These antioxidants are gained from vegetables or fruits containing the supplements. Among enzymatic antioxidants there belong glutathione reductase (GSH), superoxide dismutase (SOD) and catalase (Agarwal et al. 2014).

In comparison with other animals' species, boar spermatozoa do not have sufficient protective enzymes against ROS or free radicals. It means that the protection against ROS is secured by low- or high- molecular weight antioxidants of seminal plasma (Łapkiewicz et al. 1999). Boar spermatozoa contain enzymes such as SOD, catalase (CAT) and glutathione peroxidase (GPx). The low cytoplasmic volume in the sperm cell midpiece limits its antioxidant capacity. In addition, the protection of the sperm head and tail membrane lipids against ROS damage is not feasible because antioxidants are in the midpiece (Fraser et al. 2008). Spermatozoa can be also dependent on the attendance of extracellular free radical scavenging systems which interact with biochemical components of the seminal plasma (Fraser et al. 2008). The boar seminal plasma contains numerous non-enzymatic low-molecular weight antioxidants including glutathione, ergothioneine (ERT), ascorbate, tocopherol, urate and several other substances. Their presence and concentration are related (Łapkiewicz et al. 1999). The seminal

plasma is also endowed with an enzymatic defence mechanism to protect spermatozoa against OS. The low volume of GSH and ERT in boar seminal plasma is balanced by the specific antioxidant properties of seminal plasma proteins and the presence of high SOD activity (Strzezek 2002).

Antioxidants seem to work in cooperation, so it is very difficult to measure the specific effects of individual antioxidants separately. The enzyme superoxide dismutase scavenges the superoxide anion and converts it to H_2O_2 and oxygen, then the catalase and peroxidase decompose H_2O_2 in water and oxygen (Bathgate 2011). Glutathione is present in comparatively high concentrations in many cells. It constitutes the main mechanism of protection against ·OH. Glutathione donates an electron and thus stabilizes and after that neutralizes the ROS. Selenium and vitamin E are the co-factors of glutathione. Experiments with these co-factors have been made with a changing success to improve boar sperm quality (Bathgate 2011).

There are two main ways to use antioxidants to improve sperm quality: dietary supplementation and direct addition to the medium used in IVF, for instance, in the cooling media, in the freezing media or in the thawing media, or a combination of them (Bathgate 2011).

3.4.1 Rooibos (*Aspalathus linearis*)

Rooibos (*Aspalathus linearis* (Burm. f.) Dahlgren, *Fabaceae*) is a shrub-like leguminous bush (Fig. 3), indigenous to the Cederberg Mountains of the Western Cape of South Africa, used to make mild-tasting tea, which is caffeine free (Blumberg & McKay 1992; Awoniyi et al. 2012; Opuwari & Monsees 2014). Genus *Aspalathus* (*Fabaceae*, Tribe Crotalarieae) forms more than 270 species most of which are endemic to the Cape Floristic Region (Joubert & Beer 2011). Other names of the rooibos are rooibos tea, rooibosch, rooitea or rooitee. (Blumberg & McKay 1992). *A. linearis* is a polymorphic species which has been described in many wild forms. Some forms are prostrate and remain less than 30 cm tall, compared to other forms, which grow erect and may reach up to 2 m in height. These types of wild rooibos are sometimes called Red, Black, Grey and Red-Brown. For commercial purposes, there are two types: green (natural form, unfermented) and red (fermented). The Red type is also known as the Rocklands type. This type of rooibos grows erect, up to 1.5 m in height. The shrub has got a single basal stem that splits just above the ground surface into multiple thin branches, has bright green thin leaves about 10 - 40 mm in length and small yellow blossoms (Fig. 4). Rooibos can adapt to coarse, nutrient-poor and acidic soil and hot, dry summers (Erickson 2008).

Figure 3: Rooibos bush (Stirton 2012)

Figure 4: Rooibos blossoms (Stirton 2012)

Due to its rich content of different compounds with antioxidant properties, it has recently gained much more attention because of its potential use for clinical purposes (Boor et al. 2006). Rooibos has been consumed as a health beverage for more than a century in South Africa and in Europe. A lot of experiments to prove potential health promoting properties of rooibos have already been made, for instance, on diseases including antispasmodic effects, immune system modulation, liver diseases and it was discovered its antimicrobial, antiviral and antiaging properties (Ichiyama et al. 2000; Marnewick et al. 2000; Scheepers 2001). Many experiments with rooibos in the biomedicine have reached remarkable results (Joubert et al. 2004; Marnewick et al. 2011). Rooibos has been proved as antimutagenic (Marnewick et al. 2004), cancer modulating (Marnewick et al. 2005) and regenerating coenzyme Q10 with the inhibition of lipid peroxidation in rat liver (Kucharska et al. 2004). It has been demonstrated that rooibos also helps adults with cardiovascular diseases as it improves the lipid profile as well as redox status, relevant to heart diseases (Marnewick et al. 2011). Opuwari and Monsees (2014) published in their study that *Aspalathus linearis* improves sperm concentration, viability and motility which may be due to its high level of antioxidants. Recently, it has been reported that rooibos extract enhances sperm velocity and acrosome integrity during boar semen storage (Ros-Santaella & Pintus 2017).

Rooibos had no commercial value at the beginning of the 20th century. Nowadays, it is a well-known herbal drink, which is enjoyed in more than 30 countries. Rooibos has become the second most commonly consumed beverage tea ingredient in the world after the ordinary tea (*Camellia sinensis*) (Joubert & Beer 2011). For the commercial use of rooibos, the needle-like leaves and stems are harvested from January to March, which is the summertime in South Africa (Awoniyi et al. 2012). The harvested rooibos is processed in two different ways to produce two types of tea: green or red tea. The leaves and stems may be bruised and fermented or immediately dried to prevent oxidation. The colour of the unfermented rooibos product is green (Fig. 5), so it is called ‘green’ rooibos while during fermentation the colour changes from green to red (Fig. 6) and thus ‘red’ rooibos, red bush tea is produced (Erickson 2008). The unfermented rooibos contains a higher level of polyphenols than the fermented one. This is because fermented rooibos loses some antioxidants during the fermentation process (Blumberg & McKay 1992).

Rooibos contains a high number of substances beneficial for the organism. Polyphenols are major compounds present in rooibos. Polyphenols are antioxidants with a phenolic ring in their chemical structure. These substances are common in plants and they are divided into two

subgroups: flavonoids and phenolic acids. Rooibos tea includes antioxidants, comprising flavonoids and phenolic acid that are potent free radical scavengers (Erickson 2008).

The following flavonoids can be found in rooibos: aspalathin, nothofagin, quercetin, rutin, isoquercin, orientin, isoorientin, luteolin, vitexin, isovitexin and chrysieriol (Rabe et al. 1994). Currently, rooibos is the only known natural source of aspalathin and one of the two known sources of nothofagin, the other source being heartwood (*Nothofagus fusca* (Hook F.) Oerst, *Nothofagaceae*) (Joubert 1996). Unfermented leaves contain a high level of aspalathin, hot water extracts showed unfermented rooibos to contain higher amounts of flavonoid and total polyphenols than the fermented red tea (Opuwari & Monsees 2014). Rooibos also contains phenolic acids that have been shown to have antioxidant activity, it is, for instance, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (Erickson 2008). Phenolic acids are also found in fruits, vegetables and whole grains (Liu 2013).

Figure 5: Green rooibos (Simpson & Vail 2018)

Figure 6: Red rooibos (Distinctly tea inc. 2017)

4 Material and Methods

4.1 Reagents

Reagents were purchased from Sigma-Aldrich (Prague, Czech Republic), unless otherwise indicated.

4.2 Preparation of plant extract

Fermented and unfermented rooibos (Oxalis, spol. s.r.o., Slušovice, Czech Republic) were used for the preparation of the rooibos extracts. Rooibos leaves and stems (0.2 g) were infused in 100 ml of scalding distilled water (stock solution: 0.2% mass/volume, m/v). After 10 minutes the rooibos extracts passed through a filter paper (Whatman n° 4). Since the filtered extracts had a pH of around 6 at room temperature, the pH of the extracts was adjusted to pH 7 using NaOH 1M as this is the optimal value for boar semen. After this adjustment of the rooibos extract, a semen extender (Solusem®, pH ≈ 7, osmolality ≈ 300 mOsm / kg, AIM Worldwide, Vught, the Netherlands) was prepared using the extract. The stock solution with the semen extender was diluted several times and thus three different concentrations, both for the fermented and unfermented rooibos, were created. The serial dilutions were as follows: 1:6 (0.033 % m/v; R1), 1:18 (0.011 % m/v; R2), and 1:54 (0.004 % m/v; R3).

4.3 Sperm samples collection and processing

Commercial sperm doses from 15 boars of a different breed (i.e., Czech Landrace, Pietrain and Prestice Black-Pied) were used in the experiments. These samples were provided by an animal breeding centre (Chovservis, a.s., Hradec Králové, Czech Republic). The ejaculates were collected once a week by the gloved-hand method and filtered through gauze. Once collected, the samples were diluted with Solusem® extender and then transported to the laboratory at 17 °C.

The motility and abnormalities of the ejaculates were evaluated and only samples with motility of ≥ 75 % and sperm abnormalities of ≤ 25 % were used in the experiments. Then, AI doses from 3 boars were pooled in order to reduce the effect of male variability and centrifuged at 167 g for 3 minutes at 17 °C in order to remove debris and dead sperm cells. The sperm concentration was checked with a Bürker chamber and the pool was adjusted with Solusem® to 40 × 10^6 sperm/ml. Thereafter, the semen pool was split into eight test tubes for each experiment. The control tubes (with or without oxidative stress) were diluted with solusem extender (v/v) while the treatments tubes were diluted with the extender supplemented with the

different rooibos extracts (v/v). All the tubes were supplemented with 0.1 % of bovine serum albumin (BSA).

The final concentrations of the rooibos extracts in the semen samples were 0.017 % m/v (F1; N1), 0.006 % m/v (F2; N2), and 0.002 % m/v (F3; N3). Oxidative stress was induced by a ROS generating system (0.05 mM $FeSO_4$ and 0.5 mM sodium ascorbate (Fe^{2+}/ascorbate)) which is a specific system for inducing lipid peroxidation. All the samples had the final concentration of 20×10^6 sperm/ml. Subsequently, the prepared samples were put in a water bath for incubation at 38 °C and analysed after 20 minutes (control tubes only) and the rest of the samples (control, control - ox and treatments with rooibos) after 3.5 hours. All tubes were analysed for kinetics, lipid peroxidation, acrosomal status and membrane integrity. The experiment was replicated five times with five different pools.

Sperm concentrations were calculated using this formula:

$$\frac{\text{Number of spermatozoa counted} \times \text{Conversion factor to ml}}{(\text{Area of each square} \times \text{Number of squares counted}) \times \text{Depth of the chamber} \times \text{Dilution Factor}}$$

Conversion from µl to ml = 20
Area of each square = 0.02 mm^2
Depth of the chamber = 0.1 mm
Dilution factor = 1:20

4.4 Boar sperm analysis

4.4.1 Motility

The semen samples were incubated at 38 °C for 20 minutes in a water bath. 5 µl of this aliquot was put into a Spermtrack (PROiSER R+D S.L., Spain; chamber depth: 20 µm). Sperm motility was assessed subjectively by estimation of the percentage of motile spermatozoa to the nearest 5 % and the quality of movement (QM) using a scale from 0 (lowest: no motility) to 5 (highest: progressive and vigorous movements). The sperm motility index (SMI) was calculated using the formula: [% individual motility + (QM × 20)] / 2.

Sperm kinetics was evaluated by Computer Assisted Sperm Analysis (CASA; NIS-Elements, Nikon, Tokyo, Japan and Laboratory Imaging, Prague, Czech Republic). CASA is assembled of Eclipse E600 tri-ocular phase contrast microscope (Nikon, Tokyo, Japan) with a warming stage set at 38 °C (Tokai Hit, Shizuoka, Japan) and DMK 23UM021 digital camera (The Imaging Source, Bremen, Germany). The analysis was performed by using a 10× negative phase-contrast objective (Nikon, Tokyo, Japan). It was assessed eight descriptors of sperm kinetics: progressive motility (PM, %), amplitude of lateral head displacement (ALH, µm),

beat-cross frequency (BCF, Hz), linearity (LIN, %), straightness (STR, %), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s) and straight-line velocity (VSL, μm/s).

The standard parameter settings were as follows: frames per second, 60; minimum of frames acquired, 31; VAP ≥ 5 μm/s to classify a spermatozoon as motile, STR ≥ 80 % to classify a spermatozoon as progressive. A minimum of 200 motile sperm cells was analysed per sample.

4.4.2 Lipid peroxidation

For this analysis, there was used the thiobarbituric acid reactive substances (TBARS) assay as previously described (Yagi 1998). The samples were collected and stored at - 80 °C until the test was performed (Pintus et al. 2018). The samples were evaluated by spectrophotometer at 532 nm. A standard curved line was determined by using the known concentrations of 1,1,3,3-tetramethoxypropane (malondialdehyde, MDA). The levels of lipid peroxidation are stated in μmol of MDA per 10^8 spermatozoa. This assay was made two times for each sample.

4.4.3 Acrosomal status

For determining the percentage of sperm cells with a normal apical ridge (NAR; Pursel et al. 1972), the semen samples were fixed in 2% glutaraldehyde solution and the prepared sample was then examined by phase contrast microscopy (40× objective). Two hundred sperm cells were assessed in each sample and the percentage of sperm cells with a normal apical ridge (NAR; Fig. 9) was determined.

4.4.4 Sperm plasma membrane integrity by hypo-osmotic swelling test (HOST)

The HOS test was performed in a hypo-osmotic solution which had been prepared by mixing 7.35 g of sodium citrate with 13.51 g of fructose in 1 L of distilled water (Jeyendran et al. 1992). The final solution was kept in a fridge and then warmed up before use.

150 μl of pre-warmed Host solution (a water bath at 38 °C for 15 minutes) was used for each sample of the experiment. 50 μl of semen sample was added into the tube with the Host solution and mixed with a pipette. Afterwards, the prepared sample was incubated in a water bath at 38°C for 30 minutes. Meanwhile, the slides were pre-warmed (38 °C) on a heating plate. After 30 minutes in the warm bath, 10μl of the prepared sample from each tube was spread on pre-warmed slides and covered with cover slides. The samples were examined by phase contrast microscopy (40× objective). Two hundred sperm cells were assessed in each sample and the

percentage of sperm cells with intact the plasma membrane (swollen or coiled tail; Fig. 7, 8) was determined.

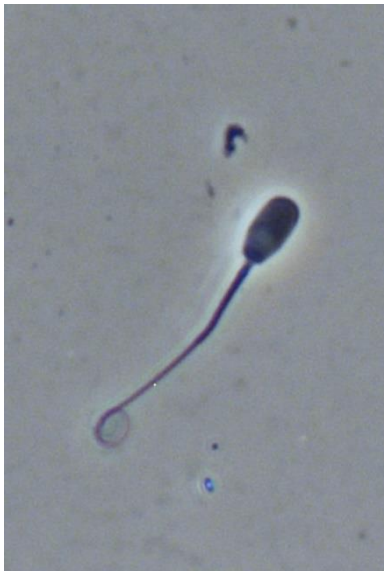

Figure 8: Boar sperm with coiled tail – intact tail integrity (Grieblová et al. 2017)

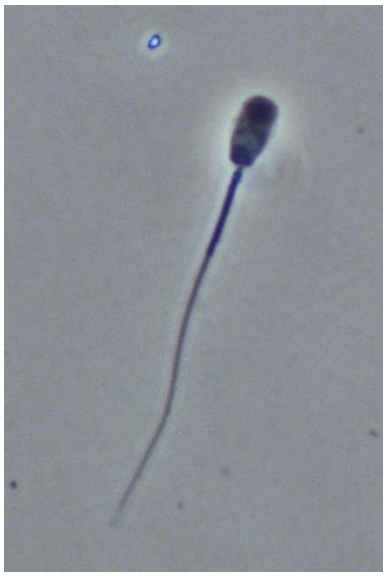

Figure 7: Boar sperm with swollen tail – damaged tail integrity (Grieblová et al. 2017)

4.4.5 Sperm membrane integrity by eosin-nigrosine (E/N)

Eosin-nigrosine solution (Minitube, Tiefenbach, Germany) was used for this analysis. First, eosin-nigrosine stain and the slides were warmed up on a heating plate (38 °C). Afterwards, 10 µl of eosin-nigrosine was used for each semen sample. This drop was put on a pre-warmed slide on the right side, then 10 µl of semen sample was added and mixed up with a tip. Then the smear was made and after drying up, the samples were evaluated under bright field microscopy (40× objective). Two hundred sperm cells were examined in each sample per slide and the percentage of spermatozoa with the intact membrane (E-/N, white head; Fig. 10) was determined.

4.5 Statistical analysis

The SPSS 20.0 statistical software package (IBM Inc, Chicago, IL, USA) was used for statistical analysis. The repeated measures Wilcoxon test was used to check for differences in sperm parameters (control group) during incubation time (0 h and 3.5 h). The generalized linear model (GZLM) was used to check for differences between control groups and treatments. Data are indicated as mean ± standard deviation (SD). Statistical significance was set at $p < 0.05$.

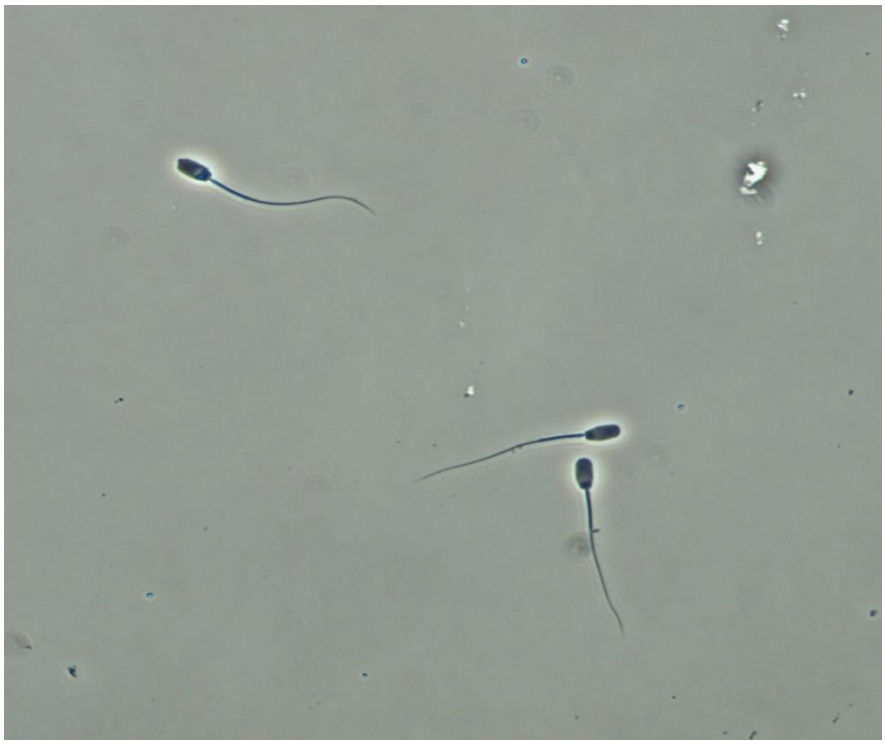

Figure 9: Evaluation of acrosomal status. 2 spermatozoa with the normal apical ridge on the right side and one sperm on a left with damaged acrosome (Ros-Santaella & Pintus 2017).

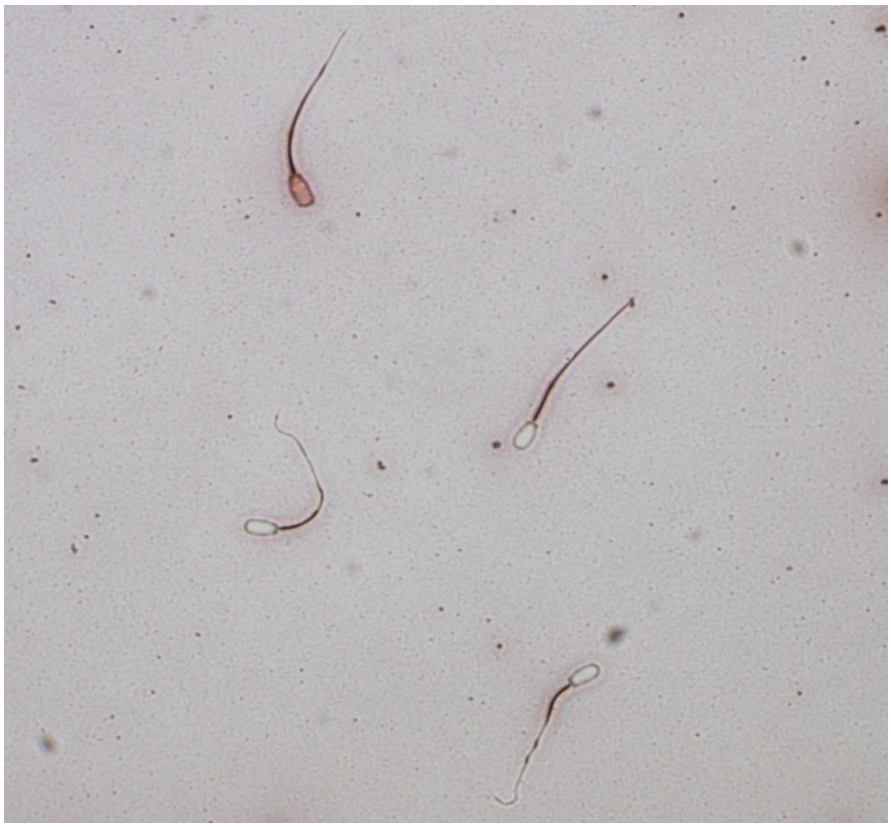

Figure 10: Sperm membrane integrity by eosin-nigrosine (E/N). Spermatozoa with the intact membrane (white head), sperm with damaged head integrity (red head) (Ros-Santaella & Pintus 2017).

5 Results

5.1 Motility and kinetics

During the incubation time (0 h and 3.5 h) there were significant differences ($p < 0.05$) in several parameters. More specifically, the control group at 0 h showed greater TM and VCL, but smaller PM, BCF, LIN and STR than the control group at 3.5 h of incubation (Table 1). Some significant differences were also found between the control group and the control - ox at 3.5 h ($p < 0.05$). For example, the control treatments without oxidative stress had greater TM, VAP, VCL, VSL and ALH than the control treatments with oxidative stress (Table 1).

Conversely, at 3.5 hours of incubation, there were no significant differences ($p > 0.05$) in any sperm kinetic parameter and motility between the rooibos treatments and the control group with oxidative stress (Table 1).

Table 1: Boar sperm kinetics in samples submitted to oxidative stress (CTR except) and supplemented with fermented and unfermented rooibos

Treatment	**Time**	**TM** (%)	**PM** (%)	**VAP** (μm/s)	**VCL** (μm/s)	**VSL** (μm/s)	**ALH** (μm)	**BCF** (Hz)	**LIN** (%)	**STR** (%)
CTR	0	94.21 ± 3.87^{a}	52.92 ± 11.98^{a}	37.81 ± 5.92^{a}	78.00 ± 8.94^{a}	29.55 ± 4.77^{a}	2.63 ± 0.28^{a}	12.82 ± 1.68^{a}	37.14 ± 7.08^{a}	75.58 ± 5.89^{a}
CTR	3.5	89.42 ± 4.10^{b,A}	81.04 ± 17.11^{b,A}	37.34 ± 5.24^{a,A}	60.97 ± 11.37^{b,A}	35.14 ± 5.63^{a,A}	2.35 ± 0.25^{a,A}	15.36 ± 2.01^{b,A}	55.00 ± 11.30^{b,A}	90.34 ± 8.88^{b,A}
CTR – ox	3.5	69.29 ± 9.01^{B}	72.26 ± 4.14^{A}	25.40 ± 6.87^{B}	39.54 ± 11.02^{B}	24.16 ± 6.58^{B}	1.64 ± 0.45^{B}	15.52 ± 1.27^{A}	60.68 ± 5.52^{A}	92.83 ± 2.43^{A}
F1	3.5	63.10 ± 13.55	68.43 ± 8.41	23.18 ± 7.36	36.22 ± 10.28	22.15 ± 7.17	1.45 ± 0.40	15.42 ± 0.78	59.71 ± 1.37	92.72 ± 0.83
F2	3.5	64.27 ± 8.40	68.47 ± 5.94	24.20 ± 4.96	38.33 ± 6.05	23.07 ± 4.94	1.56 ± 0.29	15.03 ± 1.10	58.64 ± 2.92	92.56 ± 0.99
F3	3.5	65.75 ± 11.02	69.97 ± 6.39	25.13 ± 6.62	40.09 ± 9.32	23.80 ± 6.32	1.66 ± 0.44	15.05 ± 0.93	58.16 ± 3.54	92.05 ± 1.98
N1	3.5	68.14 ± 11.21	72.04 ± 8.44	22.39 ± 5.95	33.15 ± 7.51	21.41 ± 5.82	1.44 ± 0.35	15.89 ± 1.40	63.13 ± 6.17	93.20 ± 1.96
N2	3.5	66.66 ± 8.54	69.21 ± 5.70	25.34 ± 7.16	40.22 ± 10.29	24.03 ± 6.89	1.65 ± 0.47	15.26 ± 1.05	59.00 ± 3.92	92.14 ± 1.96
N3	3.5	66.47 ± 7.71	66.90 ± 11.89	26.93 ± 7.01	44.30 ± 12.15	24.80 ± 5.89	1.76 ± 0.45	15.02 ± 1.34	56.15 ± 7.37	90.13 ± 4.99

Different superscript lower-case letters in the same column indicate significant differences ($p < 0.05$) between the control group at 0 h and 3.5 h without induced oxidative stress. Different superscript upper-case letters in the same column indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress at 3.5 h. TM = total motility; PM = progressive motility; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight-line velocity; ALH = amplitude of lateral head displacement; BCF = beat-cross frequency; LIN = linearity; STR = straightness; CTR = control; CTR - ox = control sample with inducted oxidative stress; F= fermented rooibos (F1 = 0.017% m/v; F2 = 0.006% m/v; F3 = 0.002% m/v); N = unfermented rooibos (N1 = 0.017% m/v; N2 = 0.006% m/v, N3 = 0.002% m/v). Data are expressed as mean ± standard deviation of 5 replicates.

5.2 Lipid peroxidation

The oxidative stress induced by Fe^{2+}/ascorbate promoted a significant increase in sperm lipid peroxidation at 3.5 h of semen incubation ($p < 0.05$; Figure 11) compared to the control group. All treatments supplemented with rooibos extract showed lower levels of MDA than those of control - ox group ($p < 0.05$). Significant difference ($p < 0.001$) was detected primarily in treatments with unfermented rooibos (N2, N3). Interestingly, also treatments with a higher concentration of rooibos evinced lower MDA than the control - ox group. Furthermore, there were also significant differences ($p < 0.05$) between the control groups during the incubation time.

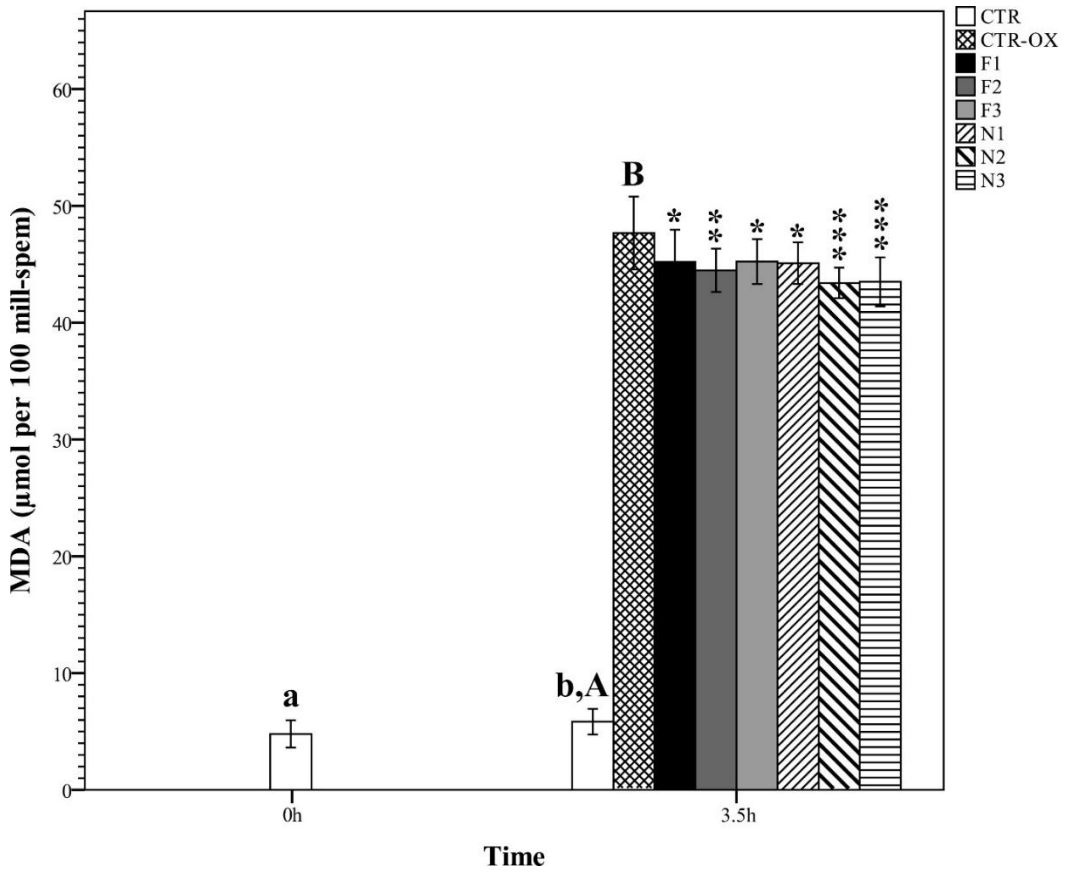

Figure 11: Lipid peroxidation in boar sperm samples submitted to oxidative stress and supplemented with fermented and unfermented rooibos. Different superscript lower-case letters indicate significant differences ($p < 0.05$) between the control group at 0 h and 3.5 h without induced oxidative stress. Different Superscript upper-case letters in the same column indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress at 3.5 h. The asterisks indicate significant differences between the treatments and the control submitted to oxidative stress (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). F= fermented rooibos (F1 = 0.017% m/v; F2 = 0.006% m/v; F3 = 0.002% m/v); N = unfermented rooibos (N1 = 0.017% m/v; N2 = 0.006% m/v, N3 = 0.002% m/v). Data are expressed as mean ± standard deviation of 5 replicates.

5.3 Acrosome integrity

The chart (Fig. 12) shows that the lowest values of acrosome integrity were found in the control - ox samples but had no significant difference ($p > 0.05$) compared to the control groups. The lowest concentrations of rooibos treatments (F3 and N3) reached the highest percentages of undamaged acrosome being similar to control samples at 0 h and 3.5 h. Nevertheless, no significant difference ($p > 0.05$) was found between the control - ox and the treatments in acrosome integrity.

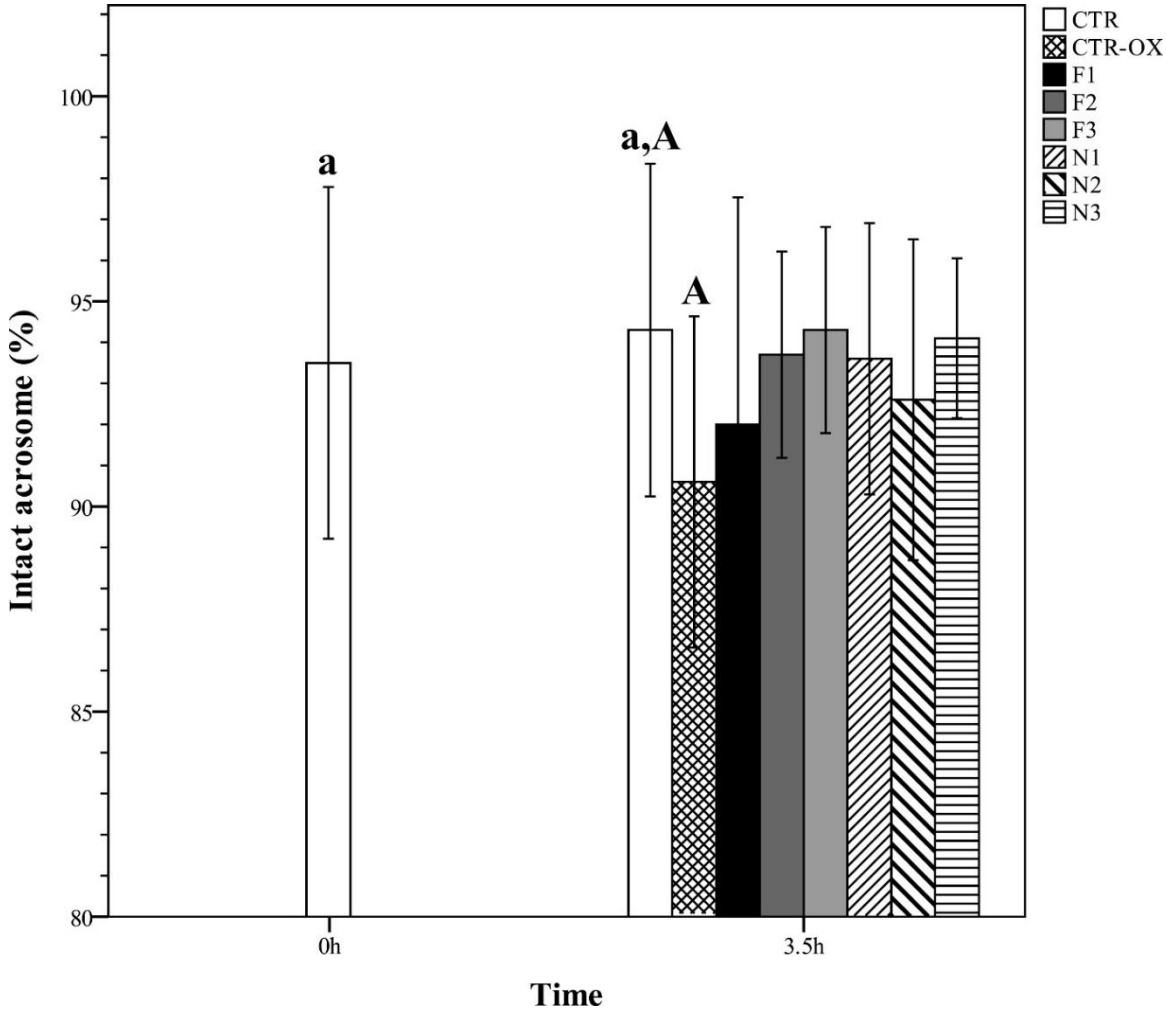

Figure 12: Acrosomal status evaluation through the normal apical ridge. Different superscript lower-case letters indicate significant differences ($p < 0.05$) between the control group in 0 h and 3.5 h without induced oxidative stress. Different superscript upper-case letters in the same column indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress at 3.5 h. F= fermented rooibos (F1 = 0.017% m/v; F2 = 0.006% m/v; F3 = 0.002% m/v); N = unfermented rooibos (N1 = 0.017% m/v; N2 = 0.006% m/v, N3 = 0.002% m/v). Data are expressed as mean ± standard deviation of 5 replicates.

5.4 Tail membrane integrity (HOST)

Following data, presented by Figure 13, did not show any significant differences between control - ox and rooibos treatments ($p > 0.05$). A striking point is that between the control group at 3.5 h and the control - ox was a significant difference ($p < 0.05$). The treatments with unfermented rooibos had almost the same percentage of sperms with undamaged membrane as in the sample with oxidative stress. On the other hand, the treatments with fermented rooibos showed a tendency to be higher than the control - ox group, but without any significant difference ($p > 0.05$).

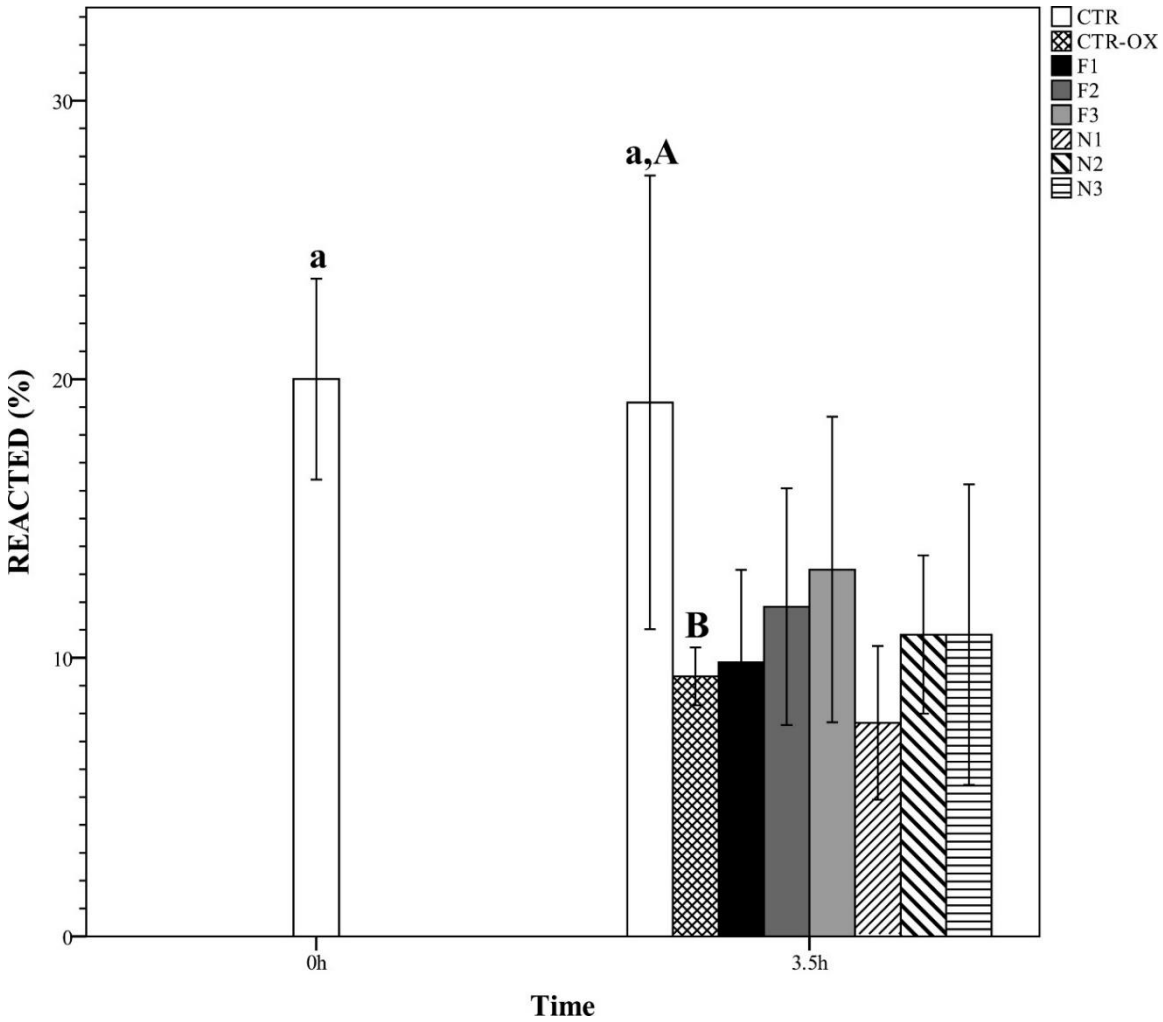

Figure 13: Evaluation of membrane integrity by HOS test. Different superscript lower-case letters indicate significant differences ($p < 0.05$) between the control group in 0 h and 3.5 h without induced oxidative stress. Different superscript upper-case letters in the same column indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress at 3.5 h. F= fermented rooibos (F1 = 0.017% m/v; F2 = 0.006% m/v; F3 = 0.002% m/v); N = unfermented rooibos (N1 = 0.017% m/v; N2 = 0.006% m/v, N3 = 0.002% m/v). Data are expressed as mean ± standard deviation of 5 replicates.

5.5 Head membrane integrity (eosin - nigrosine)

The evaluation of plasma membrane integrity by eosin - nigrosine test provided some significant results ($p < 0.05$; Fig. 14). There were no significant differences between the control and the control - ox samples in head membrane integrity during incubation time ($p > 0.05$). Both fermented and unfermented rooibos treatments showed a higher percentage of sperm with an intact head membrane compared to the control - ox group ($p < 0.05$).

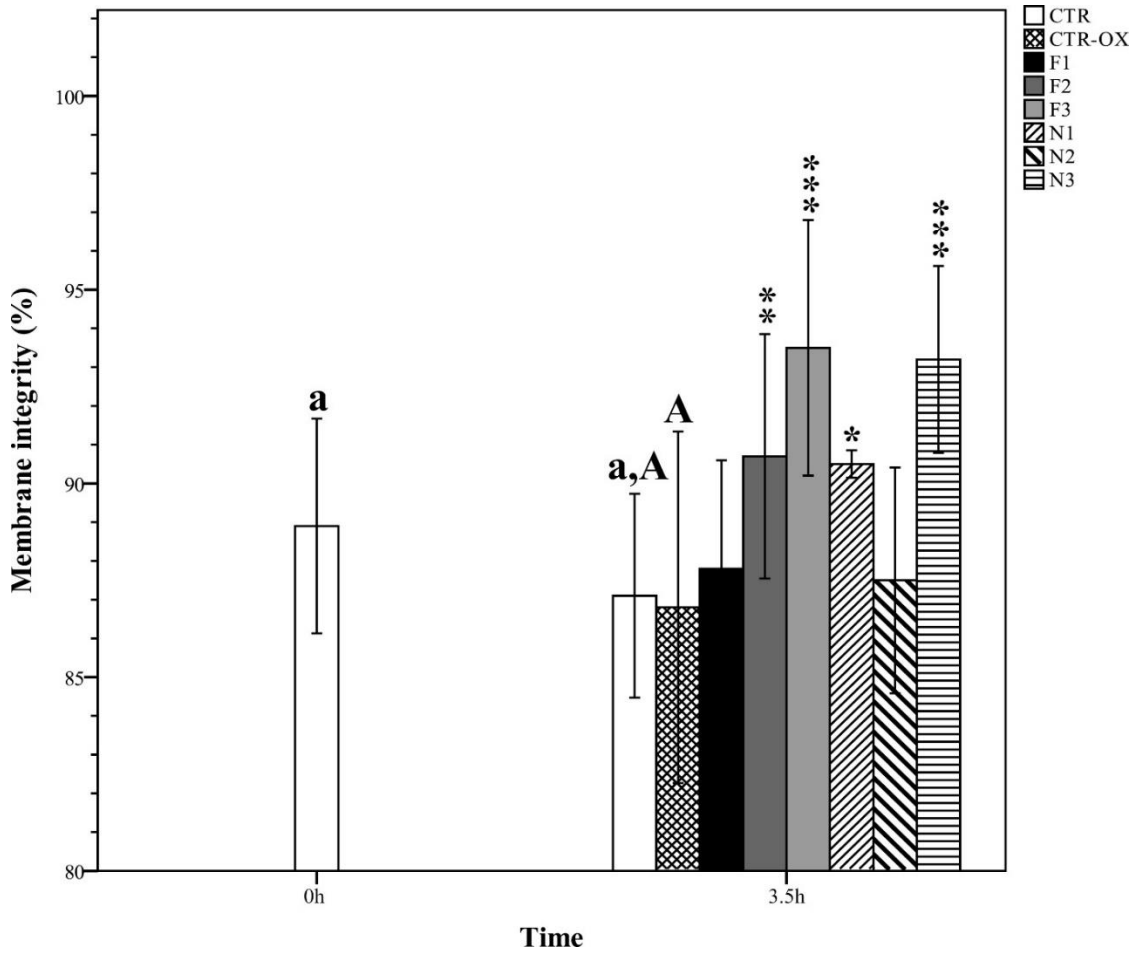

Figure 14: Sperm membrane integrity evaluates by eosin-nigrosine solution. Different superscript lower-case letters indicate significant differences ($p < 0.05$) between the control group in 0 h and 3.5 h without induced oxidative stress. Different superscript upper-case letters in the same column indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress at 3.5 h. The asterisks indicate significant differences between the sample and the control submitted to oxidative stress (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). F= fermented rooibos (F1 = 0.017% m/v; F2 = 0.006% m/v; F3 = 0.002% m/v); N = unfermented rooibos (N1 = 0.017% m/v; N2 = 0.006% m/v, N3 = 0.002% m/v). Data are expressed as mean ± standard deviation of 5 replicates.

6 Discussion

In the present study, it has been found that rooibos extract diminishes the negative effects of oxidative stress in boar spermatozoa. The results clearly show that the extract of rooibos reduces the lipid peroxidation and protects the plasma membrane integrity against oxidative stress. Even though sperm kinetics and acrosome integrity did not show significant differences between the rooibos treatments and the control - ox group, they showed a tendency to be greater at some rooibos concentration.

It was proved that rooibos has a high level of antioxidant capacity (Joubert & De Villiers 1997). The boar sperm membranes are rich in polyunsaturated fatty acids which make them predisposed to oxygen inducted harm promoting oxidative stress and then it leads to lipid peroxidation (Sheweita et al. 2005). Therefore, the antioxidants properties of the rooibos extract added to the semen extender may improve the sperm membrane integrity and reduce lipid peroxidation, as was observed in the present study. Agarwal et al. (2004) found considerable evidence that low amounts of ROS are necessary for spermatozoa to gain fertilizing ability. Nevertheless, the higher production of ROS may have a detrimental effect for spermatozoa due to the production of free radicals that negatively affects the physiology of the spermatozoa, for instance decreasing sperm motility, damaging the spermatozoa membranes, and inactivating glycolytic enzymes (Alvarez & Storey 1984; Sikka 1996). For all these reasons, it is a common practice to add antioxidants to the semen extender and, as an alternative, we used the rooibos extract as a natural source of antioxidants improving the quality of the semen samples under oxidative stress.

In the present study, we tested the motility and kinetic of boar semen submitted to oxidative stress and supplemented with the rooibos extract. We did not find any significant differences between the control group - ox and the treatments with rooibos. However, the lowest concentrations of rooibos extract had a higher average than the control - ox group, so we can suggest that the next time we will test lower rooibos concentration which may show better results. Opuwari and Monsees (2014) used the rooibos extract *in vivo* for experiments on male rat reproductive functions. In this study, Opuwari and Monsees (2014) did not find any significant differences in sperm kinetic parameters. On the other hand, the study by Ros-Santaella and Pintus (2017) brings some significant results with positive effects of rooibos extracts on sperm kinetic. It is important to bear in mind that both studies were not conducted under oxidative stress. In our study, we found a tendency in the kinetic parameters to be higher in the treatments with the lowest concentrations of rooibos in comparison to control - ox group.

For this reason, the highest concentrations maybe are scavenging higher amounts of ROS, even those that are necessary for correct sperm functionality.

In our study, the Fe^{2+}/ascorbate induces a condition of oxidative stress depicted by raised levels of lipid peroxidation. Lipid peroxidation is the pathophysiological process causing many diseases and stress conditions (Aitken et al. 1989). Numerous studies have explored the potential effect of this reaction on the loss of sperm functional characteristics (Geva et al. 1996; Rajesh Kumar et al. 2002). Based on our results, it seems that the antioxidants from rooibos extract protect sperm membrane leading to lower levels of lipid peroxidation. Especially the unfermented rooibos with the lowest concentration in semen samples had a very significant decrease in MDA levels. Other rooibos treatments reached also lower levels of MDA than control - ox samples. Awoniyi et al. (2012) reported that rooibos extracts significantly decreased the lipid peroxidation levels in the testes of rats. Also, many other studies provided the positive effect of rooibos against lipid peroxidation. Ulicna et al. (2006) used rooibos *in vivo* in their study to reduce age-related lipid peroxidation accumulation in brains of rats with successful results. Likewise, Marnewick et al. (2005) proved protective qualities of rooibos *in vivo* against lipid peroxidation in rat livers. It is important to bear on mind that these studies were conducted without oxidative stress.

On the other hand, we did not find any significant results in acrosome integrity and tail membrane integrity. Nevertheless, there was a tendency for both parameters to be higher in the rooibos treatments than in the control - ox, as well as in sperm motility. We found protective effects of rooibos treatments on the head membrane integrity where the treatments with the lowest concentration of rooibos reached a higher percentage of undamaged membrane. This may be also the evidence that the lowest concentrations of rooibos extracts had better effects than high concentrations. Opuwari & Monsees (2014) found a protective effect of rooibos (*in vivo*) on sperm membrane of rats but this study was without inducted oxidative stress. Several studies have shown the importance of an intact membrane on sperm function (Jeyendran et al. 1984; Gadella et al. 1999; Zhu & Liu 2000) highlighting the importance of our results.

Overall, our results clearly show that rooibos extract may be added to the semen extender as a source of natural antioxidants. In the present study, the rooibos signally reduced lipid peroxidation and protected sperm plasma membrane against the deleterious effects of oxidative stress. This study is the first that use rooibos extract in semen samples submitted to oxidative stress.

7 Conclusion

In conclusion, the extract from *Aspalathus linearis* improved the viability and reduced the lipid peroxidation of boar sperm samples submitted to oxidative stress. Although we did not find any significant differences in motility, acrosome integrity and tail membrane integrity, there was a tendency to be higher in the treatments with rooibos extract than in the control - ox group. This study provides evidence that the rooibos extract could replace the commercial antioxidants additives commonly used in the semen extenders. The positive attributes of rooibos are its price and the fact that it is a natural antioxidant which could be used as possible preservation of boar semen.

Overall, we could pronounce that the hypotheses of this study were confirmed due to the fact that rooibos could protect boar spermatozoa against oxidative stress. Next step for a following study should be investigating the effect of rooibos extract in sperm fertilization ability and try to use different concentrations of rooibos extract for better results.

8 Bibliography

Agarwal A, Nallella KP, Allamaneni SS, Said TM. 2004. Role of antioxidants in treatment of male infertility: an overview of the literature. Reproductive Biomedicine Online **8**:616-627.

Agarwal A, Virk G, Ong Ch, Plessis SS. 2014. Effect of Oxidative Stress on Male Reproduction. World J Mens Health **32**:1-17.

Aitken RJ, Clarkson JS, Fishel S. 1989. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biology of Reproduction **41**:183-197.

Aitken RJ, Wingate JK, De Iuliis GN, Koppers AJ, McLaughlin EA. 2006. Cis-unsaturated fatty acids stimulate reactive oxygen species generation and lipid peroxidation in human spermatozoa. The Journal of Clinical Endocrinology & Metabolism **91**:4154-4163.

Aitken RJ, Jones KT, Robertson SA. 2012. Reactive Oxygen Species and Sperm Function – In Sickness and In Health. Journal of Andrology **33**:1096-1106.

Alvarez JG, Storey BT. 1984.Assessment of cell damage caused by spontaneous lipid peroxidation in rabbit spermatozoa. Biology of Reproduction **30**:323-331.

Awda BJ, Mackenzie-Bell M, Buhr MM. 2009. Reactive Oxygen Species and Boar Sperm Function. Biology of Reproduction **81**:553-561.

Awoniyi DO, Aboua YG, Marnewick J, Brooks N. 2012. The Effects of Rooibos (*Aspalathus linearis*), Green Tea (*Camellia sinensis*) and Commercial Rooibos and Green Tea Supplements on Epididymal Sperm in Oxidative Stress-induced Rats. Phytotherapy Research **26**:1231-1239.

Bailey JL, Lessard Ch, Jacques J, Breque Ch, Dobrinski I, Zeng W, Galantino-Homer HL. 2008. Cryopreservation of boar semen and its future importance to the industry. Theriogenology **70**:1251-1259.

Bajena M, Kondracki S, Iwanina M, Wysokińska A, Adamiak A. 2016. Physical characteristics of ejaculates produced by insemination boars depending on the interval between successive ejaculate collections. Journal of Central European Agriculture **17**:260-271.

Bansal AK, Bilaspuri GS. 2011. Impacts of Oxidative Stress and Antioxidants on Semen Functions. Veterinary Medicine International. DOI:10.4061/2011/686137.

Bathgate R. 2011. Antioxidant Mechanism and their Benefit on Post-thaw Boar Sperm Quality. Reproduction in Domestic Animals **46**:23-25.

Betteridge DJ. 2000. What is oxidative stress?. Metabolism **49**:3-8.

Blumberg JB, McKay DL. 2006. A Review of the Bioactivity of South African Herbal Teas: Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia intermedia*). Phytotherapy Research **21**:1-16.

Boor P, Božek P, Čársky J, Greksák M, Nakano M, Šebeková K, Uličná O, Vančová O. 2006. Rooibos Tea (*Aspalathus linearis*) Partially Prevents Oxidative Stress in Streptozotocin-Induced Diabetic Rats. Physiol. Res. **55**:157-164.

Boziaris IS, Kapsokefalou M, Kamaitis M, Proestos Ch. 2006. Natural Antioxidant Constituents from Selected Aromatic Plants and Their Antimicrobial Activity Against Selected Pathogenic Microorganisms. Food Technol. Biotechnol. **46**:151-156.

Briz MD, Fabrega A. 2013. The Boar Spermatozoon. Pages 3-49 in: Bonet S, Casas I, Holt WV, Yeste M, editors. Boar Reproduction: Fundamentals and New Biotechnological Trends. Springer Science & Business, London.

Brouwers JF, Silva PFN, Gadella BM. 2005. New assays for detection and localization of endogenous lipid peroxidation products in living boar sperm after BTS dilution or after freeze-thawing. Theriogenology. **63**:458-469.

Carrillo AV. 2016. Sperm membrane channels, receptors and kinematics. Linköping University Electronic Press, Linköping.

deGroat WC, Booth AM. 1980. Physiology of male sexual function. Annals Internal Medicine **92**:329-331.

Distinctly tea inc. 2017. Rooibos - Steeped® Distinctly me and tea. Available from https://meandtea.ca/rooibos/ (accessed November 2018).

Eddy EM. 2006. The spermatozoon in Knobil and Neill's Physiology of Reproduction, 3rd Edition. Academic Press, USA.

Erickson L. 2008. Rooibos Tea: Research into Antioxidant and Antimutagenic Properties. HerbalGram **59**:34-45.

Fraser L, Dziekońska A, Kukliňska M, Lecewicz M, Strzežek J. 2004. Effects of dietary supplementation with polyunsaturated fatty acids and antioxidants on biochemical characteristics of boar semen. Reproductive biology **4**:271-289.

Fraser L, Kowalowka M, Strzezek J, Wysocki P. 2008. Extracellular Superoxide Dismutase of Boar Seminal Plasma. Reproduction in Domestic Animals **43**:490-496.

Gadea J. 2003. Review: Semen extenders used in the artificial insemination of swine. Spanish Journal of Agricultural Research **1**:17-27.

Gadella BM, Flesh FM, Golde LMG, Colenbrander B. 1999. Dynamics in the membrane organization of the mammalian sperm cell and functionality in fertilization. Veterinary Quarterly **21**:142-146.

Gadella BM, Tsai PS, Boerke A, Brewis A. 2008. Sperm head membrane reorganisation during capacitation. International Journal of Developmental Biology **52**:473-480.

Geva E, Bartoov B, Zabludovsky N, Lessing JB, Lerner-Geva L, Amit A. 1996. The effect of antioxidant treatment on human spermatozoa and fertilization rate in an in vitro fertilization program. Fertility and Sterility **66**:430-434.

Gharagozloo P, Aitken RJ. 2011. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. Human Reproduction **26**:1628-40.

Gordon I. 2017. Reproductive Technologies in Farm Animals, 2nd Edition. CABI, Ireland.

Gupta MD, Tandon VMD, Tandon MBBS. 2005. Free radicals / Reactive oxygen species. JK-Practitioner **12**:143-148.

Grieblová A, Pintus E, Ros-Santaella JL. 2017. Integrity of head and tail plasmalemma is associated with different kinetic variables in boar sperm. Animal Reproduction Science **184**:218-227.

Griveau JF, Le Lannou D. 1997. Reactive oxygen species and human spermatozoa: physiology and pathology. International Journal of Andrology **20**:61-69.

Hampl R, Drábková P, Kanďár R, Stěpán J. 2012. Impact of oxidative stress on male infertility. Česká Gynekologie **77**:241-5.

Henkel RR. 2011. Leukocytes and oxidative stress: dilemma for sperm function and male fertility. Asian Journal of Andrology **13**:43-52.

Ichiyama K, Tai A, Yamamoto I. 2007. Augmentation of antigenspecific antibody production and IL-120 generation with a fraction from rooibos (Aspalathus linearis) tea. Bioscience, Biotechnology, and Biochemistry **71**:589-602.

Jackson PGG, Cockcroft PD. 2007. Handbook of Pig Medicine. Elsevier Health Sciences, USA.
Johnson LA, Weitze KF, Fiser P, Maxwell WMC. 2000. Storage of boar semen. Animal Reproduction Science **62**:143-172.

Jeyendran RS, Ven HHV, Perez-Pelaez M, Crabo BG, Zaneveld LJD. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. Journal of Reproduction & Fertility Ltd **70**:219-228.

Jeyendran RS, Ven HHV, Zaneveld LJD. 1992. The Hypoosmotic Swelling Test: An Update. Archives of Andrology **29**:105-116.

Joubert E. 1996. HPLC quantification of the dihydrochalcones, aspalathin and nothofagin in rooibos tea (*Aspalathus linearis*) as affected by processing. Food Chemistry **55**:403-11.

Joubert E, Ferreira D. 1996. Antioxidants of Rooibos tea – a possible explanation for its health promoting properties?. S Afr J Food Sci Nutr **8**:79-83.

Joubert E, De Villiers OT. 1997. Effect of fermentation and drying conditions on the quality of rooibos tea. International Journal of Food Science & Technology **32**:127-134.

Joubert E, Winterton P, Britz TJ, Ferreira D. 2004. Superoxide anion radical and ,-diphenyl-B-picrylhydrazyl radical scavenging capacity of rooibos (*Aspalathus linearis*) aqueous extracts, crude phenolic fractions, tannin and flavonoids. Food Research International **37**:133-138.

Joubert E, Beer D. 2011. Rooibos (*Aspalathus lineris*) beyond the farm gate: From herbal tea to potential phytopharmaceutical. South African Journal of Botany **77**:869-886.

Kerr JB, Loveland KL, O'Bryan MK, Krestser DM. 2006. Cytology of the testis and intrinsic control mechanisms in Knobil and Neill's Physiology of Reproduction, 3rd Edition. Academic Press, USA.

Kucharska J, Ulicna O, Gvozdjakova A, Sumbalova Z, Vancova O, Bozek P, Nakano M, Greksak M. 2004. Regeneration of coenzyme Q10 redox state and inhibition of oxidative stress by rooibos tea (*Aspalathus linearis*) administration in carbon tetrachloride liver damage. Physiology Research **53**:515-521.

Lampiao F. 2012. Free radicals generation in an in vitro fertilization setting and how to minimize them. World Journal of Obstetrics Gynecology **1**:29-34.

Łapkiewicz S, Lecewicz M, Strrzežek J. 1999. A note on antioxidant capacity of boar seminal plasma. Animal science papers and reports **17**:181-188.

Lavon U, Boursnell JC. 1975. The split ejaculate of the boar: contributions of the epididymides and seminal vesicles. Journal of Reproduction and Fertility **42**:541-552.

Liu RH. 2013. Health-Promoting Components of Fruits and Vegetables in the Diet. American Society for Nutrition **4**:3845-3925.

MacLeod J. 1943. The role of oxygen in the metabolism and motility of human spermatozoa. Am J Physiol **138**:512-518.

Mann T, Lutwak-Mann C. 1981. Male Reproductive Function and Semen. Springer-Verlag Berlin Heidelberg, New York.

Marberger H. 1974. The mechanisms of ejaculation. Basic Life Sciences **4**:99-110.

Marnewick JL, Gelderblom WC, Joubert E. 2000. An investigation on the antimutagenic properties of South African herbal teas. Mutation Research **471**:157-166.

Marnewick JL, Batenburg W, Swart P, Joubert E, Swanevelder S, Gelderblom WCA. 2004. Ex vivo modulation of chemical-induced mutagenesis by subcellular liver fractions of rats treated with rooibos (*Aspalathus linearis*) tea, honeybush (*Cyclopia intermedia*) tea, as well as green and black (*Camellia sinensis*) teas. Mutation Research **558**:145-154.

Marnewick JL, Joubert E, Joseph S, Swanevelder S, Swart P, Gelderblom W. 2005. Inhibition of tumor promotion in mouse skin by extract of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*), unique South African Herbal teas. Cancer Letters **224**:193-202.

Marnewick J, Rautenbach F, Venter I, Neethling H, Blackhurst DM, Wolmarans P, Macharia M. 2011. Effects of rooibos (*Aspalathus linearis*) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease. Journal of Ethnopharmacology **133**:46-52.

McGlone J, Pond WG. 2003. Pig Production: Biological Principles and Applications. Cengage Learning, United States.

Neill JD. 2005. Knobil and Neill's Physiology of Reproduction. Academic Press, USA.
Nicander L, Bane A. 1962. Fine structure of boar spermatozoa. Z Zellforsch Mikrosk Anat **57**:390-405.

O'Donnell L, Nicholls PK, O'Bryan MK, McLachlan RI, Stanton PG. 2011. Spermiation: The process of sperm release. Spermatogenesis **1**:14-35.

Opuwari CS, Monsees TK. 2014. *In vivo* effects of *Aspalathus linearis* (rooibos) on male rat reproductive functions. Journal of Andrology **46**:867-877.

Panawala L. 2017. Difference Between Spermatogenesis and Spermiogenesis. Sydney. Available from http://pediaa.com/difference-between-spermatogenesis-and-apermiogenesis/ (accessed November 2018).

Parodi J, Cueva M, Romero F. 2013. Reactive oxygen species can alter physiological parameters of sperm; the future of macromolecules in boar semen dilution. Porcine Research **3**:40-48.

Peña FJ, Saravia F, Núñez-Martínez I, Johannisson A, Wallgren M, Rodriguez Martinez H. 2006. Do different portions of the boar ejaculate vary in their ability to sustain cryopreservation? Animal Reproduction Science **93**:101-113.

Petruska P, Capcarova M, Sutovsky P. 2014. Antioxidant supplementation and purification of semen for improved artificial insemination in livestock species. Turkish Journal of Veterinary and Animal Sciences **38**:643-652.

Pintus E, Kadlec M, Jovičić M, Sedmíková M, Ros-Santaella JL. 2018. Aminoguanidine Protects Boar Spermatozoa against the Deleterious Effects of Oxidative Stress. Pharmaceutics 10 (212) DOI:10.3390/pharmaceutics10040212.

Pursel VG, Johnson LA, Rampacek GB. 1972. Acrosome morphology of boar spermatozoa incubated before cold shock. Journal of Animal Science **34**:278-283.

Pursel VG, Schulman LL, Johnson LA. 1978. Effect of orvus es paste on acrosome morphology, motility and fertilizing capacity of frozen-thawed boar semen. Journal of Animal Science **471**: 198-202.

Rabe C, Steenkamp JA, Joubert E, Burger JF, Ferreira D. 1994. Phenolic metabolites from rooibos tea (*Aspalathus linearis*). Phytochemistry **35**:1559-1565.

Rajesh Kumar T, Doreswamy K, Shrilatha B, Muralidhara. 2002. Oxidative stress associated DNA damage in testis of mice: induction of abnormal sperms and effects on fertility. Mutation Research **513**:103-111.

Ray PD, Huang BW, Tsuji Y. 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cellular Signalling **24**:981-990.

Rodríguez-Martínez H, Saravia F, Wallgren M, Tienthai P, Johannisson A, Vázquez JM, Martínez E, Roca J, Sanz L, Calvete JJ. 2005. Boar spermatozoa in the oviduct. Theriogenology **63**:514-535.

Rodríguez-Martínez H et al. 2009. The physiological roles of the boar ejaculate. Soc Reprod Fertil Suppl **66**:1-21.

Ros-Santaella JL, Pintus E. 2017. Rooibos (*Aspalathus linearis*) extract enhances boar sperm velocity up to 96 hours of semen storage. PLoS ONE 12(8) (e0183682) DOI: 10.1371/journal.pone.0183682.

Ros-Santaella JL, Pintus E. 2017. Uso del extracto de rooibos (*Aspalathus linearis*) para la conservación del semen porcino. Suis **143**:14-18.

Saleh RA, Agarwal A. 2002. Oxidative stress and male infertility: from research bench to clinical practice. Journal of Andrology **23**:737-52.

Sheweita SA, Tilmisany AM, Al-Sawaf H. 2005. Mechanisms of male infertility: role of antioxidants. Current Drug Metabolism **6**:495-501.

Scheepers S. 2001. Anti-microbial activity of rooibos tea (Aspalathus linearis) on food spoilage organisms and potential pathogens [MSc. Thesis]. Stellenbosch University Stellenbosch, South Africa.

Schulze M, Buder S, Rüdiger K, Beyerbach M. 2014. Influence on semen traits used for selection of young AI boars. Animal Reproduction Science **148**:3-4.

Sikka SC. 1996. Oxidative stress and role of antioxidants in normal and abnormal sperm function. Frontiers in Bioscience **1**:78-86.

Sikka AC. 2004. Role of Oxidative Stress and Antioxidants in Andrology and Assisted Reproductive Technology. Journal of Andrology **25**:5-18.

Singer SJ, Nicolson GL. 1972. The fluid mosaic model of the structure of cell membranes. Science **175**:720-731.

Simpson & Vail. 2018. Organic green tea rooibos herbal tea. Available from https://www.svtea.com/Organic-Green-Rooibos-Herbal-Tea/productinfo/T0807/ (accessed November 2018).

Siqueira AP, Wallgren M, Hossain MS, Johannisson A, Sanz L, Calvete JJ, Rodriguez-Martinez H. 2011. Quality of boar spermatozoa from the sperm-peak portion of the ejaculate after simplified freezing in MiniFlatpacks compared to the remaining spermatozoa of the sperm-rich fraction. Theriogenology **75**:1175-1184.

Stirton Ch. 2012. *Aspalathus linearis*. Available from https://www.ispotnature.org/communities/southernafrica/view/observation/374021/aspalathus-linearis (accessed November 2018).

Strzezek J, Saiz-Cidoncha F, Wysocki P, Tyszkiewicz A, Jastrzebski M. 2002. Seminal plasma proteins as markers of biological value of boar semen. Animal Science Papers and Reports **20**:255-266.

Thurston LM, Watson PF, Mileham AJ, Holt WV. 2001. Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. Journal of Andrology. **22**:382-394.

Toshimori K. 1998. Maturation of mammalian spermatozoa: modifications of the acrosome and plasma membrane leading to fertilization. Cell and Tissue Research **293**:177-187.

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

Töpfer-Petersen E, Ekhlasi-Hundrieser M, Tsolova M. 2008. Glycobiology of fertilization in the pig. The International Journal of Developmental Biology **52**:717-736.

Trussell JC. 2013. Optimal diagnosis and medical treatment of male infertility. Seminars in Reproductive Medicine **31**:235-236.

Ulicna O, Vancova O, Bozek P, Carsky J, Sebkova P, Boor P. 2006. Rooibos (*Aspalathus linearis*) partially prevents oxidative stress in streptozotocin-induced diabetic rats. Physiological Research **55**:157-164.

White IG. 1993. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. Reproduction, Fertility and Development **5**:639-658.

Yagi K. 1998. Free Radical and Antioxidant Protocols. Methods in Molecular Biology **108**:101-106.

Zhu WJ, Liu XG. 2000. Cryodamage to plasma membrane integrity in head and tail regions of human sperm. Asian Journal of Andrology **2**:135-138.

9 List of Abbreviations

AI	artificial insemination
ALH	amplitude of lateral head displacement
AKAPs	A-Kinase anchor proteins
ATP	adenosine tri-phosphate
BCF	beat-cross frequency
BSA	bovine serum albumin
CASA	Computer Assisted Sperm Analysis
CAT	catalase
CTR	control treatment
CTR ox	control treatment with oxidative stress
DNA	deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
E/N	eosin - nigrosine
ERT	ergothioneine
F	fermented rooibos
F1	fermented rooibos 0.017 % m/v
F2	fermented rooibos 0.006 % m/v
F3	fermented rooibos 0.002 % m/v
GPx	glutathione peroxidase
GSH	glutathione reductase
GZLM	generalized linear model
H_2O_2	hydrogen peroxide
HOCl	hypochloric acid
HOST	hypo-osmotic swelling test
IVF	in vitro fertilisation
LIN	linearity
LOO	lipid peroxyl
LOOH	lipid peroxide
LPO	lipid peroxidation
MDA	1,1,3,3-tetramethoxypropane (malondialdehyde)
N	unfermented rooibos
N1	unfermented rooibos 0.017 % m/v

N2	unfermented rooibos 0.006 % m/v
N3	unfermented rooibos 0.002 % m/v
NADH	nicotinamide adenine dinucleotide
NAR	normal apical ridge
NO	nitric oxide
$^1O_2$	singlet oxygen
O_2^-	superoxide ion
O_3	ozone
OH^-	hydroxyl ion
OS	oxidative stress
PM	progressive motility
post-SRF	post-sperm rich fraction
pre-SRF	pre-sperm rich fraction
PUFAs	polyunsaturated fatty acids
QM	quality of movement
R1	rooibos extract 0.033 %
R2	rooibos extract 0.011 %
R3	rooibos extract 0.004 %
RO_2	peroxyl
ROS	reactive oxygen species
RS^-	thiyl
SD	standard deviation
SMI	sperm motility index
SP	seminal plasma
SOD	superoxide dismutase
SRF	sperm rich fraction
STR	straightness
TBARS	thiobarbituric acid reactive substances
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight-line velocity