PhD thesis

"Counteracting oxidative stress improves quality of chilled stallion semen"

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AI	Acrosome intact
AI	Artificial insemination
AIF	Apoptosis inducing factors
ALH	Amplitude of lateral head displacement
AR	Acrosome reacted
ATP	Adenosine triphosphate
AV	Artificial Vagina
BCF	Beat cross frequency
BHA	Butylhydroxyanisole
BHT	Butylhydroxytoluene
C	Control group
CAT	Catalase
CLCs	Cholesterol-loaded cyclodextrins
CRISPs	Cysteine-rich secretory proteins
DFI	DNAfragmentation index
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetra-acetic acid
G6PD	Glucose-6-phosphate dehydrogenase
GPX	Glutathione Peroxidase
GPX/GRD	Glutathione peroxidase/ glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GWS	Glass wool-Sephadex
H_2O	Water
H_2O_2	Hydrogen peroxide
LDL	Low density lipoproteins
LIN	Linearity
LPO	Lipid peroxidation
M	Treatment group (Maca supplementation)
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOX5	NADPH oxidase system
NPPC	Native phosphocaseinate
O_2	Dioxygen molecule
O_2-	Superoxide anion radical
OH	Hydroxyl radical
OS	Oxidative stress
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid

List of abbreviations

PVDF	Polyvinylidene difluoride
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
SCA	Sperm Class Analyzer
SCSA	Sperm chromatin structure assay
SD	Standard deviation
SD	Standard deviation
Se	Selenium
SOD	Superoxide dismutase
STR	Straightness
T	Treated semen
TBARS	Thiobarbituric acid reactive species
TNE	Tris-NaCl-ethylenediaminetetra-acetic acid
TSC	Total sperm count
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUDP nick end-labeling
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
WOB	Wobble

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Abstract

Stallion semen experiences oxidative stress during cooling and transport and is consequently damaged by reactive oxygen species. Two different approaches have been investigated to enhance the intrinsic antioxidant defense mechanisms against oxidative stress in liquid-preserved semen. First, a dietary antioxidant supplementation to improve antioxidant status in tissue, seminal plasma and spermatozoa was tested. Second, the combined addition of three enzymatic antioxidants to semen extender in order to increase the antioxidant status of seminal plasma was evaluated.

In the first experiment, the effect of dietary supplementation with *Lepidium meyenii* (Maca) on fresh and chilled stallion semen characteristics were evaluated. Maca is a traditional Andean crop used as a nutraceutical for its fertility-enhancing properties which is linked with its antioxidant activity. The diet of five stallions was supplemented daily with 20 mg of Maca powder for a total of 60 days. A control group of another five stallions received the same diet without Maca. Semen was collected once before the administration of Maca (D0), twice during the administration at 30 and 60 days (D30-D60), and finally twice at 30 and 60 days after the end of the administration (D90-D120). Ejaculates were processed for cooled shipping at 5°C and evaluated in the laboratory for total and progressive motility, acrosome integrity and lipid peroxidation after collection and after 24 hours, 48 hours, and 72 hours storage, respectively. Dietary supplementation with Maca significantly improved sperm concentration and total sperm ($p<0.05$). The beneficial effects of Maca supplementation on motility and acrosome integrity in the raw semen appeared from the end of treatment with Maca (D60) until the end of the study (D120). Furthermore, total motility, progressive motility, and acrosome integrity decline slower during storage in the Maca-treated group than in the control group. The lipid peroxidation did not change during cooling storage in each group and did not show a significant difference between two groups. The results from this study indicated that the dietary supplementation with Maca was able to increase sperm production and in stabilizing semen quality during storage of chilled semen.

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxide (GPX) constitute the principal enzymatic components of the endogenous antioxidant system of equine spermatozoa and seminal plasma. The objective of the second experiment was to evaluate the effect of adding a combination of SOD, CAT and GPX to a semen extender on the quality of stallion semen stored at 5°Cfor 72 hours. Ejaculates from seven stallions were split in two aliquots and diluted with semen extender without (control)

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or with the addition of 15 IU/mL SOD, CAT, and GPX. Semen analysis was performed within 3 hours after semen collection (T0) and every 24 hours during storage of chilled semen (T24, T48, T72). At each time point, total and progressive motility, kinetic parameters of sperm movement, DNA fragmentation and the levels of activated caspase-3 were evaluated. In the first 24 h of storage, almost no difference between control samples and treated samples was evident. After 48 and 72 hours, beneficial effects of a combination of antioxidants became evident. The antioxidant supplementation significantly inhibited the activation of activated caspase 3 and concomitantly maintained total motility and the percentage of rapid moving sperm cells at a higher level ($p < 0.05$). A storage-dependent increase in DNA damage was alleviated only to a minor extend after prolonged storage time, i.e. 72 hours. The results suggest that the tested combination of SOD, CAT and GPX added to a cooling extender improves viability, motility and kinetic features and reduce DNA fragmentation in semen stored for more than 24 hours.

In conclusion, a dietary supplementation of stallions with an antioxidant or measures to supplement the semen extender with a combination antioxidants are both valid tools to counteract oxidative stress and maintain the quality of chilled equine semen at a high level for a prolonged time.

Over the past decades, artificial insemination (AI) has become a major breeding tool in many horse breeds (Aurich and Aurich, 2006). Especially the use of cooled-transported semen allowed breeders to benefit from the genetics of the best stallions and avoided animal transportation (Aurich C, 2008). The cooling and storage of spermatozoa is associated with a reduction in cell viability, motility and fertilizing capacity (Yoshida, 2000). One of the underlying mechanism is an imbalance in the production and scavenging of reactive oxygen species (ROS; reviewed by Pagl et al., 2006) The presence of ROS in the male genital tract and in spermatozoa is not per se a negative phenomenon. Low concentrations of ROS are indispensable for maturation of spermatozoa in the testis and for important functions of spermatozoa, such as capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion (Pagl et al., 2006). Spermatogenesis and Leydig cell steroidogenesis are sensitive to excessive concentrations of ROS in the testis (Hales et al. 2005). Indeed, oxidative stress in the testis mediates pathological consequences on sperm production which result in the generation of immature and defected spermatozoa (Aziz et al., 2004). During cold storage of equine semen, ROS production increases (Aurich C, 2005), provoking damages to all components of the sperm cell including lipids, protein layers ofthe sperm plasma membrane and to the DNA (Aitken et al., 2010; Ball, 2008). Physiologically, enzymatic and non-enzymatic antioxidants in the gonads, seminal plasma, and the cytoplasm defend spermatozoa from excessive concentration of ROS. An inefficient antioxidant production or an excessive production of ROS during sperm production and storage in the male genital tract or during cooled or frozen storage of semen (Aitken et al., 2012) can lead to oxidative stress, and thereby damage to the sperm cells (Roca et al., 2013).

In order to maintain the quality and fertility of cooled equine semen for prolonged time, the semen processing has been optimized. The supplementation of extenders with antioxidants was proposed with encouraging results (Aurich et al., 1997; Bruemmer et al., 2002; Kankofer et al. 2005; Aurich C, 2008; Cocchia et al., 2011). An emerging alternative strategy aims at preventing oxidative damage to spermatozoa in the testis and epididymis as well as at improving the antioxidative capacity of semen during storage by food supplementation with antioxidant and/or PUFA (Brinsko et al., 2005; Deichsel et al., 2008; Contri et al., 2011; Schmid-Lausigk and Aurich, 2014; Freitas et al., 2016).

In the light of current research about the dietary antioxidant therapies to preserve semen quality, it seems interesting to investigate the effects of

further antioxidants. An intriguing natural antioxidant is *Lepidium meyenii* (maca), an Andean crop which has effects on the fertility of mammals, such as humans and bulls (Clément et al., 2010, Lee et al., 2016). In the first study, the effect of 60-days of dietary supplementation with the maca powder on the quantity of fresh stallion sperm and the quality, i.e. motility, viability, acrosome integrity and lipid peroxidation, of diluted stallion semen during storage at 5°C up to 72 hours was evaluated. In a second study, the potential beneficial effect of a combination of enzymatic ROS scavengers, namely Superoxide dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPX), as supplements to semen extender was tested. Motility parameters and DNA damage in spermatozoa during 72 hours of cooled storage were investigated.

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Chapter 1

Artificial insemination in equine species

1.1 Evolution and current status of AI in Europe

Artificial insemination (AI) is the first important biotechnology devised to improve the breeding and genetics of animals. Artificial insemination was mentioned for the first time in an Arabian legend of 1322. The curious tale tells of a birth of a beautiful and healthy foal obtained by insemination with semen scooped from a recently mated mare's vagina and transported diluted in camel milk in a goat skin bag (Bowen 1969, Pickett 2000). However, this legend does not provide information about the process of dilution or insemination and it has to be classified to the realm of myths.

First documented use of artificial insemination in mammals was reported in 1780 when an Italian physiologist, Spallanzani, obtained three beagle puppies (Spallanzani, 1781). In 1888, the French veterinarian Repiquet proposed to the Société de Médecine Vétérinaire to use AI in order to overcome subfertility in horses and cows, to breed more mares to one stallion, and to produce hybrid animals such as mules (Raillet, 1888). The first controlled experiments about equine artificial insemination were conducted by the Russian scientist Elia Iwanoff in Russia, France, and Germany, respectively (Iwanoff 1903, 1907, 1912). The first methods for equine AI were described in his publications, i.e. the collection of semen with a vaginal sponge and the insemination with a rubber tube inserted transcervically into the uterus. Furthermore, he discussed the various advantages of AI, especially in obtaining more offspring from the same stallion and in the possibility to ship the semen to close-by place. In addition, to genetic progress, AI was purposed as a veterinary tool for breeding of subfertile horses.

Progress in assisted reproduction techniques for equine breeding was biphasic in the 20th century, due to a continuously changing role of the horse in society. After World War I and the subsequent economic crisis, horses lost their importance and the breeding works shifted to other species (Aurich and Aurich, 2012). However, advances in reproductive biotechnology in other species stimulated further research in horses.

Techniques for equine AI were rather advanced in the late 1930s. The major advances of these periods were the development of an Artificial Vagina (AV) and phantoms, allowing the contact of stallion with mare and avoiding the transmission of venereal diseases. The first equine AV (Cambridge model; Walton, 1933) and phantom were made in the early 1930s, followed by modified version in other countries (Götze, 1949). Equine AI programs emerged in different countries such as United States, United Kingdom, Italy,

Germany, Denmark and Bulgaria (Götze, 1949). More than 140,000 mares were inseminated in the Soviet Union in 1938.

Dilution of stallion semen with adequate extenders was also a topic of interest for European scientists. In order to preserve and to store the semen, a large variety of extenders have been tested, such as cow milk, Ringer's solution and Locke's solutions. Different scientist also tried to develop new extenders by combining various components as sugars, electrolytes, egg yolk, milk and milk products (Milowanow, 1940¸ Berliner, 1942, Götze, 1949). Centrifugation and refrigeration (0°C< T<8° C) were recommended to maintain the fertility of stored semen, especially before antibiotics where added to semen extender.

Between 1930 and 1960 equine AI dramatically decreased in Europe (Allen, 2005). The still limited knowledge on reproductive biology, and financial aspects due to the decreasing role of horses in agriculture caused a temporary halt. Independent from those developments, Russian and Chinese scientists continued using AI in horses with fresh semen and testing methods for the preservation of frozen and chilled semen (Allen, 2005). In China, 600,000 mares were inseminated with fresh semen in 1959 (Cheng et al. 1964). In the late 1960s researchers from Colorado University in the United States commenced to extend the physiological knowledge about equine reproduction. Their topics of interest were factors influencing stallion spermatogenesis, the different methods for the collection, dilution, cooling, deep freezing and insemination of semen (Squires et al., 1999; Pickett et al., 2000). These studies were a starting point for the further research in equine AI.

During the last 60 years the role of horses in Europe changed from an agricultural working animal to a partner for equestrian sports and leisure activities. Starting in the 80s, AI programs in horse were established in different countries in Europe, especially in sport horse breeds (Figure 1.1; Ponsart et al., 2004; Aurich, 2012). A significant increase in the horse population and acceptance of the technology by numerous breed registries caused an increasing interest in equine AI (Aurich, 2012).
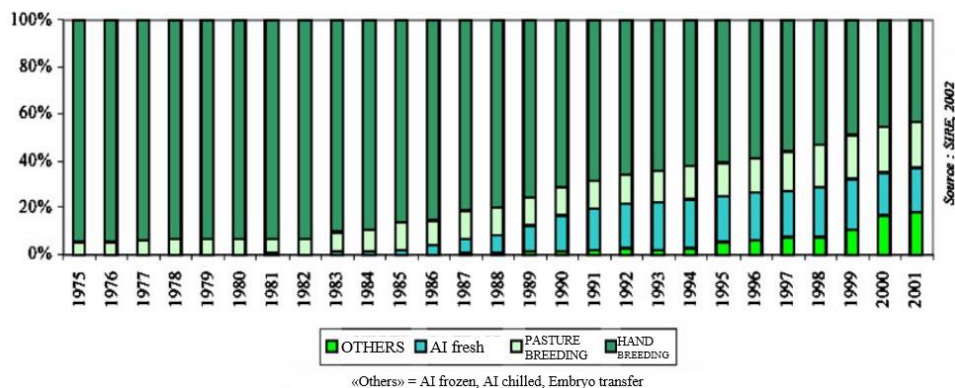
Figure 1.1 *Evolution of equine techniques after 1975 in France (www.haras-nationaux.fr ; source SIRE, 2002).*

In 1985, 98% of German sport mares were still bred by natural cover and only 1,4 % inseminated, but the value for AI increased to 50% and 80%, respectively in 1995 and 2004 (German Equestrian Federation. Jahresbericht 2010/ Annual Report2010). In Germany, equine AI with cooled semen achieved a rate of 90% already more than 10 years ago and this was maintained until now (Figure 1.2A; Dohms, 2002; German Equestrian Federation. Jahresbericht 2010/ Annual Report 2010). In France only 5% of sport mares were artificially inseminated in 1985, almost 50% in 1995 (Figure 1.1) and 56% in 2010, with both cooled (29%) and frozen-thawed (27%) semen (Figure 1.2B; Haras nationaux, Chiffres d'élevage 2010/ Breeding data 2010). In small countries like Austria and the Netherlands approximately 70% of the breeding mares were artificially inseminated (Association of Austrian Horse Breeders 2005, Aurich JE and Aurich C, 2006).

A. *GERMANY*

B. *FRANCE*
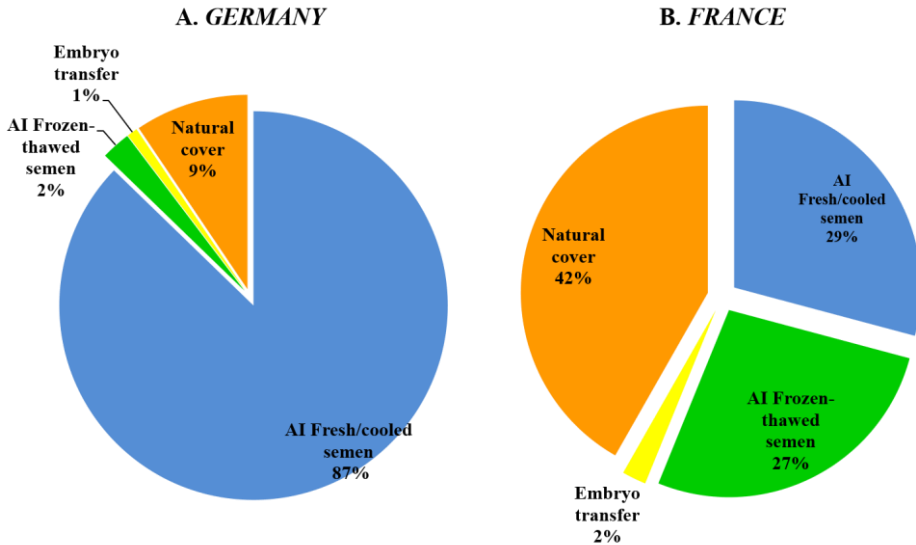
Figure 1.2 *Use of Artificial Insemination in German (A; German Equestrian Federation. Jahresbericht 2010/ Annual Report 2010) and French (B; Haras nationaux, Chiffres d'élevage 2010/ Breeding data 2010) sport horses breeding in 2010 (Aurich JE and Aurich C, 2012)*

Nowadays, AI with mainly fresh or cooled-transported semen has become the major horse breeding tools (Aurich JE and Aurich C, 2006). AI with frozen-thawed semen is less popular. The main causes of this situation are the demanding mare management and the relative lower pregnancy rates when performing AI with frozen-thawed (from 30 to 50%) semen compared to fresh (51%) or cooled (40%) stallion semen (Jasko et al.,1992; Vidament, 1997; Barbacini et al., 1999; Loomis, 2001; Samper et al., 2002). The poor ability of equine spermatozoa to survive the freezing and thawing process is generally accepted (Amann and Pickett, 1987).

In the last three decades, new extenders for cryopreservation and new protocols have been successfully developed to increase the outcomes of the use of frozen semen (Loomis, 2001; Peña et al., 2011). Anyway, recent field data of seasonal pregnancy rates with frozen semen are not readily available.

1.2 Advances in liquid storage of equine stallion semen

Although the use of cooled-shipped semen in the equine industry has become increasingly routine, seasonal pregnancy rates still vary from 60 to 90% (Loomis, 2001). In general, the fertilizing capacity of semen stored at 5°C is maintained similar to that of fresh semen for about 24h and is satisfactory at most until 48h (Jasko et al.,1992). Over time, spermatozoa suffer an irreversible loss in fertilizing ability. Preservation of semen quality during refrigeration depends on individual stallion factors and on semen processing (reviewed by Aurich C, 2008).

1.2.1 Main stallion factors affecting quality of cooled semen

The suitability of semen to be processed for storage at different temperatures depends on the quality of the native semen, on the amount and the composition of the stallion's seminal plasma and on the sensitivity of the sperm's plasma membrane (reviewed by Aurich C, 2005). The capability of preserving the fertility of semen varies between stallions (Squires et al.,1988; Varner et al., 1989). Not all sires are a good candidates for cooled semen processing. Based on the cooling ability of their semen, stallions are classified as "bad" or "good" coolers (Brinsko et al, 2000; Batellier et al., 2001).
Intrinsic properties of stallion spermatozoa are related to their capability to be processed and stored at different temperatures (Aurich, 2005; 2008). Apparently, sperm morphologic (Love et al., 1999) or chromatin abnormalities (Kenney et al., 1995; Love and Kenney, 1998) and plasma membrane sensitivity interfere with its fertilizing ability, especially when exposed to storage conditions. Chromatin quality is related to the extent of disulfide bonding (Love and Kenney, 1999) as well as the ratio of protamine types associated with the DNA (Balhorn et al., 1988). Susceptibility of sperm DNA to acid denaturation and the entity of DNA damage under storage is higher in subfertile stallions than in fertile stallions (Love et al., 2005). Even if, under cooling at 5°C, it seems that semen from all stallions did not show chromatin quality alteration at least until 20h of storage (Love et al., 2005; Ball, 2008). Meanwhile, moderate and high levels of DNA denaturation have been shown at storage temperatures of 20°C and 37° C.

During cooling process, the sperm's sensitivity to lipid peroxidation induced by oxidative stress depends on the plasma membrane composition (Cross, 1998). Especially, the cholesterol content and the cholesterol: phospholipid ratio differ from stallion to stallion and influence the temperature at which the membrane phase transition occurs. Indeed, an experimental study reduced and can even eliminated this phase transition by adding cholesterol-loaded cyclodextrins (CLCs) to stallion spermatozoa (Spizziri et al., 2010). Therefore, changes in lipid composition of essential membrane components should improve membrane stability, thereby reducing as well the vulnerability to oxidative stress during storage. Dietary intake of polyunsaturated fatty acid (PUFA) increased the quality of fresh and cooled stallion semen (Brinsko et al., 2005; Contri et al., 2011) probably due to a change in lipid composition such as observed in other species. (Zaniboni et al., 2006; Cerolini et al., 2006; Mourvaki et al., 2010; Radomil et al., 2011). Furthermore, recent research with the aim to protect stallion spermatozoa against oxidative stress suggested that diet supplementation with natural antioxidants, with fatty acids or a combination of both is a promising strategy (Bruemmer et al., 2002; Contri et al., 2011; Schmid-Lausigk and Aurich, 2014).

Both, beneficial and detrimental effects of seminal plasma on spermatozoa have been described (reviewed by Bergeron and Manjunath, 2006). Seminal plasma is a transport medium for sperm into the female genital tract (Manjunath, 2012) and preserves sperm function, probably by its antioxidant capacity (Aurich C., 2008). However, the detrimental effect of high concentrations of seminal plasma on the sperm plasma membrane (Jasko et al., 1992) and chromatin (Love, 2005) during cooled-storage has been demonstrated. Mechanism behind its detrimental effect are still unclear, but can be related to individual variations in composition of seminal plasma. Thus, some stallions present a seminal plasma that has a toxic effect on motility, especially at cooled conditions (Rigby et al., 2001). Individual plasma enzymes and proteins, such as lipase and cysteine-rich secretory proteins (CRISPs), could be responsible for the sperm damages (Carver and Ball, 2002; Leed et al., 2005). However, several studies asserted that low amounts of seminal plasma (0.6 to 20%) preserve motility and acrosome integrity of stallion spermatozoa (Jasko et al., 1992; Moore et al., 2005; Gibb and Aurich, 2016) and their fertilizing ability (Loomis, 2006). The positive effects are probably due to inhibition of polymorphonuclear neutrophils adhesion to sperm, thereby reducing sperm phagocytosis within the female reproductive tract (Alghamdi et al., 2004). Moreover, an improvement in the

antioxidant property of seminal plasma by interaction with semen extender has been supposed (Kankofer et al., 2005).

1.2.2 Processing of semen for cooled storage

Steps of semen processing, such as addition of extender, centrifugation, dilution, cooling and storage, contribute to a decrease in motility and fertilizing ability (reviewed by Aurich, 2005). Techniques for sperm processing should be adjusted to reduce these losses as much as possible.

1.2.2.1 Extenders

Extenders have been developed firstly to control pH and osmolarity and secondly to reduce the deleterious effect of seminal plasma and of the metabolic products released from active cells, such as reactive oxygen species (ROS). Dilution of cooled semen preserves the viability of spermatozoa and reduces sperm concentration. The optimal sperm concentration for diluted semen is 25 to 50 million sperm/mL (Varner et al., 1987). In case of ejaculates with high volume and low concentration (<100 million sperm/ml) a centrifugation at 300 g to 500 g for 10 to15minutes is recommended before the dilution.

Addition of antimicrobial agents to semen extender is necessary to inhibit bacterial growth during storage. The main antibiotic activity is normally given at a temperature above 15 °C, and thus present during the cooling of semen from collection temperature to storage temperature (Aurich and Spergser, 2007). Antimicrobials regularly included in stallion semen extender are amikacin (1 mg/ml) alone, a combination of amikacin and potassium penicillin G (1000 U/ml), or sodium ticarcillin plus clavulanic acid (1 mg/ml; McCue, 2014). Gentamicin is also recommended by many authors (Jasko et al., 1993; Clément et al., 1995; Varner et al., 1998), even if some negative effects were found on sperm functions, depending on sperm concentration and eh basic semen extender (Jasko et al., 1993; Aurich and Spergser, 2007).

Many extenders are based mainly on egg-yolk or on milk, starting from a recipe published by Kenney et al. back in 1975 (Kenney et al. 1975). Milk extenders are the most wide-spread used extenders, because they are inexpensive, easy to prepare and maintain a good semen quality when compared to purely egg-yolk-based extenders (Malmgren et al., 1994; Aurich C, 2008). Moreover, it has been recently demonstrated that milk-

based extenders could increase the antioxidant capacity of semen (Kankofer et al., 2005). Skim milk-glucose extender supplemented with a high-potassium modified Tyrode's medium improves sperm motility and fertility of cooled storage semen (Padilla and Foote, 1991; Rigby et al., 2001). However, modified Tyrode's medium has negative effect in the presence of seminal plasma (Rigby et al., 2001). Most common extenders such as INRA96 (IMV) and EquiPro (Minitub) provide optimal longevity of spermatozoa at cooled conditions. Their protective effect is supported by purified milk fraction native phosphocaseinate (NPPC) and β-lactoglobuline (Batellier et al., 2001; Pagl et al., 2006). Over the last few years, the idea to develop a chemically defined extender that allows semen preservation at ambient temperature for at least one week has emerged (Gibb and Aitken, 2016). With an ambient temperature storage, the irreversible membrane damage induced by cooling and/or freezing can be avoided. Indeed, semen processing does not require the addition of animal-derived products for membrane stabilization. At the same time, stallion sperm metabolism is not restrained by low temperatures, leading to ROS production and higher ATP consumption. To contrast ROS production and ATP depletion, supplementation of semen extender with osmolytes and various antioxidants has been proposed. For example, L-carnitine has been shown to be a powerful organic osmolyte and it has antioxidant proprieties (Gibb et al., 2015; Lisboa et al., 2007). L- carnitine supplementation in room temperature semen extender improved sperm motility and reduced oxidative DNA damage at 20°C to 25°C; Gibb et al., 2015). Carnitine supplementation was also tested in semen extender during cold storage, resulting in a maintained sperm motility (Lisboa et al., 2007). The addition of various antioxidants to semen extenders in chilled and frozen storage has been tested successfully in canine, boar and other species (Peña et al., 2003; Gadea et al., 2005; Michael et al., 2007 and 2009). Only few studies were performed in stallion (Aurich et al., 1997; Cocchia et al., 2011).

1.2.2.2 Seminal plasma removal/reduction

During cooled-storage, spermatozoa preserve their viability longer after being separated from sources of ROS such as seminal plasma, defected or dying spermatozoa (Morrell et al., 2009). To obtain this separation, centrifugation and partial removal of seminal plasma (5-20% remaining plasma) and of damaged and dead spermatozoa has been suggested (Jasko et al., 2002; reviewed by Loomis, 2006). A typical recommendation for

routine processing of stallion semen for cooled storage is a centrifugation at 400 g to 600g for10 min to 15 min, aspiration of approximately 80% of supernatant followed by resuspension of spermatozoa in milk-extender to prepare insemination doses. Higher centrifugation forces can damage sperm cells and subsequent reduce sperm quality (Macpherson et al., 2003). However, higher centrifugation speed of semen can be used with the "cushion" technique (Waite et al., 2008; Bliss et al., 2012). In order to avoid a packaging of spermatozoa at the bottom of the centrifugation tubes, a solution of high specific density is layered below the diluted semen. The cushion makes it possible that semen can be centrifuged at 1000 g for 20 min and semen still maintains a good motility and velocity (Knop et al., 2005; Ecot et al., 2005).

The main disadvantage of a standard centrifugation is the loss of approximately 25% of spermatozoa when removing the supernatant. The filtration of semen with a glass wool and glass wool-Sephadex (GWS) can be useful to preserve more spermatozoa (Sieme et al., 2003). Column separation using glass beads was also suggested as a method to protect semen quality, especially before freezing of spermatozoa (Klinc et al. 2005). Besides a cushioned centrifugation technique, many new methods in semen processing have been purposed in recent time. Most prominent is the sperm separation by density gradient centrifugation or by a simplified single layer procedure which have been demonstrated to improve the longevity of stored stallion spermatozoa (Macpherson et al. 2002; Morrell et al., 2008, 2009).

1.2.2.3 Cooling rates

There is a critical temperature range from 18°C to 5°C during the cooling processes of extended semen in which a phenomenon called "cold shock" can be induced. Cold shock manifests in a damage of the sperm plasma membrane (Moran et al., 1992). The cold shock damages are produced by cooling rates of more than -0.3°C/min and are due to lipid phase transitions that cause lateral lipid re-arrangement and may even include a loss of lipid fractions from the plasma membrane (Drobnis et al., 1993). The membrane component rearrangement predisposes the cell to membrane lipid peroxidation as a result of formation of ROS and consequently membrane integrity is compromised (Ricker et al., 2006). It has been recommended to use a cooling rate of less than -0.3 °C/minto reduce cold shock injuries in sperm (Amann and Pickett, 1987; Varner et al., 1988). In order to permit a controlled, slow initial rate of cooling (−0.3°C/min) and maintenance of a

final temperature of 4°–6°C a special container were designed (i.e. Equitainers; Douglas-Hamilton et al., 1984). In order to improve sperm tolerance to faster cooling rates, various techniques have been developed. The addition of cholesterol (CLCs) or new developed cryoprotectants can be used to enable faster cooling rates (Squires et al., 2001; Spizziri et al., 2014).

1.2.2.4 Storage temperature

A temperature range between 4°C and 6°C has been defined as the optimal storage temperature for maintenance of sperm motility (Varner et al., 1988 and 1989; Moran et al., 1992) and fertility (Palmer et al., 1984; Squires et al., 1988). Cold temperature arrests microbial growth and reduces the metabolism of sperm cells, thereby prolonging their life span (Yoshida, 2000). In order to maintain a constant temperature during semen shipment a proper packaging should be used (Bradecamp, 2014). Cooled semen may be packaged in plastic bags, heat-sealed, plastic pouches, all-plastic syringes (without rubber plungers), or plastic containers such as conical centrifuge tubes. Then, those packages should be placed into a commercially shipping containers. There are several reusable and single–use containers designed for the shipment of cooled semen.

The existence of "bad cooler" stallions stimulated research on room temperature storage of semen. Research group of Love and Varner in 1989 and in 2002 demonstrated that the semen stored at room temperature (20°C to 22°C) can be used for insemination but not after more than 12 hours after collection. Batellier et al. (2001) demonstrated that semen diluted in INRA96 extender and stored at 15°C can be an alternative to semen diluted in milk-based extenders and stored at 4°C. Recently, the development of a new extender prolonged the preservation of semen at room temperature to one week (Gibb et al., 2015; Gibb and Aitken, 2016).

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Chapter 2

The relationship between
reactive oxygen species and spermatozoa

2.1 Reactive oxygen species

The term *reactive oxygen species* (ROS) represent a broad group of molecules derived from oxygen which are characterized by an intrinsic instability and high reactivity. ROS includes also a subclass of nitrogen-containing compounds collectively known as reactive nitrogen species (RNS), such asthe peroxynitrite anion, the nitroxyl ion, nitrosyl-containing compounds, and nitric oxide (reviewed by Doshi et al., 2012). The phrases *free radicals* and *reactive oxygen species* (ROS)are often used interchangeably in literature. However, ROS is a more general term and includes free oxygen radicals and certain non-radical oxygen derivates that are oxidizing agents or are easily converted into radicals (Table 2.1; Halliwell and Gutteridge, 1989; Halliwell, 2001). ROS contain one or more unpaired electrons in their outer orbit and cause damage to other molecules by extracting electrons from them in order to attain stability. In this process of electron pairing, a former free radical initiates a chain reaction that produce other free radicals (reviewed by Nordberg and Arner, 2001). In biological systems, this chain reaction is mainly started through splitting off a hydrogen atom from compounds such as proteins and lipids (Lushchak, 2014).

Table 2.2 *List of Radical and Non radical reactive oxygen species: symbols and half-life; Halliwell, 2001; Kohen and Nyska2002; Phaniendra et al., 2015)*

Reactive oxygen species	Symbol	Half-life
Radical		
Superoxide Anion	$O^{\bullet -}_2$	$10^{-6}s$
Hydroxyl Radical	$OH\bullet$	$10^{-10}s$
Nitric oxide	$NO\bullet$	s^*
Peroxyl Radical	$ROO\bullet$	$17\ s$
Non Radical		
Hydrogen Peroxide	H_2O_2	Stable
Singlet Oxygen	$^1O_2$	$10^{-6}s$
Ozone	O_3	s^*

s seconds; s^* in these cases the half life depends on the environmental medium; variation from few seconds to few minutes (Phaniendra et al., 2015).

Loschen (1971) and Nohl and Hegner (1978) reported already in the 1970's the generation of ROS during cellular metabolic respiration. The major

source of ROS production in a somatic cell is the electron-transport chain in the mitochondria (Sies, 2014; Skulachev, 2012). Other sources of ROS production are located in the plasma membrane (Lüthje et al., 2013), nuclear membranes (Vartanian and Gurevich, 1989), and the membranes of the endoplasmic reticulum (Brignac- Huber et al., 2011), respectively.

ROS are formed and degraded in all cells living under aerobic conditions as by-products of respiration. Molecular oxygen is the terminal electron acceptor in the mitochondrial electron transport chain. In 90 % of the cases molecular oxygen is reduced by cytochrome oxidase via a 4-electron mechanism into two molecules of water, without ROS formation or release (Figure 2.1red square; Ott et al., 2007, Skulachev 2012). Failure of this process leads to oxygen reduction by sequential one-electron transfers as shown in Figure 2.1. In this step-wise univalent process several intermediate molecules are produced (Fridovich, 1978). These necessary intermediate molecules are the ROS.

The step-wise molecular reduction of O_2 to two H_2O molecules can be summarized as follows (Nordberg and Arnér, 2001): a single electron addition to the dioxygen molecule (O_2) forms a superoxide anion radical ($O_2^{\cdot-}$) that contains one unpaired electron in its external orbital. The superoxide anion radical is one of the strongest ROS and is converted with another electron and two $H^+$to the less reactive hydrogen peroxide (H_2O_2). By accepting a third electron, hydrogen peroxide dismutates to form a highly reactive hydroxyl radical ($OH^{\cdot}$) and OH^-. Through the acceptance of a fourth electron and two more protons, $HO^{\bullet}$and OH^-form two molecules of water (H_2O; Figure 2.1).
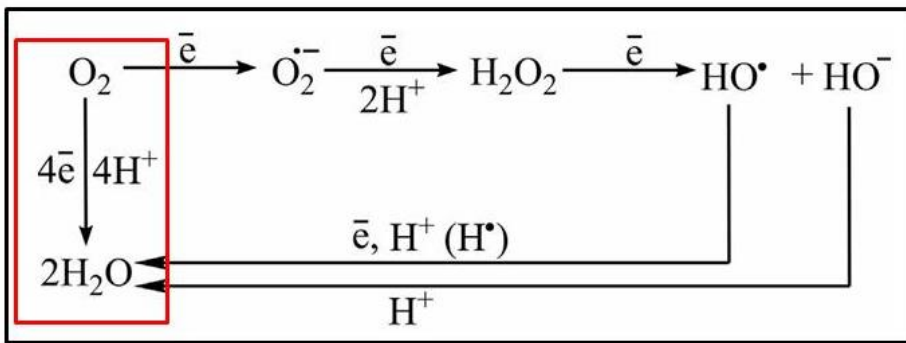
Figure 2.1 *Step-wise reduction of molecular oxygen via a four-electron transfer mechanism (red square) or sequential one-electron transfers which include ROS production (Lushchak, 2014).*

2.2 Historic background

Free radicals were first reported more than a century ago (Gomberg, 1900). The presence of these molecules in biological systems was initially discarded due to their high reactivity and consequently short half-life. In 1939, pioneering work in the field of reactive oxygen species was conducted by Michaelis (Michaelis, 1939). He proposed the involvement of free radicals in all oxidation reactions and stimulated other scientist to investigate the role of free radicals in biological processes (Lushchak, 2014). In the 1950's, free radicals were found in biological systems (Commoner et al., 1954). Since then, a continuously increasing knowledge on the role of free radicals in physiological and pathological processes has been accumulated.

For a long time, studies mainly suggested an exclusively deleterious role of free radicals in different diseases and in the process of aging (Gerschman et al., 1954; Harman, 1956). This knowledge was supported until 1969, when McCord and Fridovich (1969) discovered and described the first protective enzyme against free radicals which was called superoxide dismutase. Between 1970 and 1990, research on ROS suggested that free radicals have bactericidal roles in the immune system. (Babior et al., 1973, 1975; Rossi et al. 1975, Britigan et al., 1987). Later, it was discovered that nitric oxide is produced by L-argininein vascular endothelial cellsand nitric oxide was identified as the endothelium-derived relaxing factor (EDRF) which is responsible for endothelium-induced smooth muscle relaxation. (Furchgott et al., 1980, 1989; Palmer et al., 1987,1988). Those findings opened new perspectives in free radical research for looking now more into their positive role in physiological processes in living systems and their signaling function (Christman et al., 1985; Morgan et al., 1986; Tartaglia et al., 1989; Scandalios, 2005). Free radical research also focused on mechanisms that regulate the production and degradation of ROS and on ROS-induced cellular modifications. It has become clear that the presence of ROS is a prerequisite for various metabolic and physiological functions, such as mitochondrial respiration and oxidase catalyzed reactions, as long as low ROS concentrations are maintained (Andreyev et al., 2005; Dikalov, 2011; Kodama et al., 2013). Furthermore, ROS were identified as a mediators in a variety of signaling pathway, including growth factor signaling (Sundaresan et al., 1995; Bae et al., 1997), inflammation (Leto and Geiszt, 2006) engagement of integrins (Kheradmand et al., 1998; Werner and Werb 2002), and adhesion to extracellular matrix (reviewed by Chiarugi et al., 2003).

Production of ROS is among others regulated by hormones like insulin (Spagnoli et al., 1995; Shaikhali et al., 2008). The generation and degradation of ROS are physiological well-balanced by endogenous antioxidant defense systems. Any imbalance between these two processes leads to excessive concentrations of ROS and consequently results in oxidative stress (OS; reviewed by Lushchak, 2014).

2.3 Reactive oxygen species and equine spermatozoa

As early as 1943, MacLeod suggested for the first time that human spermatozoa are able to produce ROS and that ROS are involved in defective sperm function (MacLeod, 1943). This notion was confirmed by Tosic and Walton who demonstrated the production and deleterious effect of ROS in mammalian semen (Tosic and Walton, 1946;1950). Jones et al. (1979) showed that the vulnerability of sperm cells to oxidative stress is linked to their high polyunsaturated fatty acid (PUFA) content. Thereafter, the role of ROS in the biology of mammalian spermatozoa was investigated (Aitken et al., 1989; Aitken et al., 1999; Aitken and Baker, 2004). It was discovered that the presence of ROS in the male genital tract and in semen is not per se a negative phenomenon. Indeed, low concentrations of ROS are indispensable for the physiologic functioning of spermatozoa, e.g. during activation of their fertilizing capacity (Aitken, 1999). It has been found that both, spermatogenesis and Leydig cell steroidogenesis, are sensitive to excessive concentration of ROS in the testis. Consequently, ROS are involved in determining the production of immature and defected sperm (Hales et al., 2005). High concentrations of ROS create the already mentioned situation called oxidative stress (OS), a condition associated with an increased rate of cellular damage (Sikka et al., 1995). All the biomolecules of sperm cells, including lipids and proteins of the membrane and DNA, can be the target of an oxidative stress-induced sublethal cell injury (Aitken et al., 2010). Consequently, the attention has focused on the role of ROS in spermatozoa and the relationship between OS and male infertility (Aitken et al., 1989; Aitken et al., 1999; Aitken and Baker, 2004). In order to avoid excessive concentration of ROS, the testis are provided with an enzymatic and a non-enzymatic antioxidant system (Aitken and Roman, 2008). Furthermore, it has been discovered that human and equine seminal plasma are also endowed with antioxidants, which may protect spermatozoa from the moment of ejaculation to the oviduct (Smith et al., 1996; Ball et al., 2001b). Oxidative stress in the testis, epididymis and ejaculated semen is caused by both, an inefficient antioxidant barrier, or an excessive production of ROS by immature and damaged sperm cells (Aitken et al., 2012; Roca et al., 2013). The balance between generation and degradation of ROS should be kept during processing and storage of semen to preserve semen motility and the sperm's fertilizing ability. During either cooling or cryopreservation of semen, ROS production increases

dramatically and exceeds the antioxidant capacity of seminal plasma, thereby resulting in oxidative stress (reviewed by Ball, 2008).

2.3.1 Source of ROS in equine semen

The sources of ROS in semen are epithelial cells, leukocytes and mainly spermatozoa (Pagl et al. 2006; Makker et al., 2009; Mathur and D'Cruz., 2011). ROS production is clearly variable between sperm. This is probably related to different activities of enzymes such as the cytosolic enzyme glucose-6-phosphate dehydrogenase (Gomez et al. 1996, Aitken et al. 1997, Esfandiari et al. 2003, Aziz et al. 2004). This enzyme is associated with generation of *Nicotinamide adenine dinucleotide phosphate* (NADPH). NADPH seems to be the main source of ROS in spermatozoa. A sperm-specific NADPH oxidase system (NOX5) and a NADP-dependent oxidoreductase (diaphorase) are present in the plasma membrane of the sperm head (Aitken and Clarkson, 1987; Vernet et al., 2001; Sauber et al., 2006, 2007) and at mitochondrial level (Gavella and Lipovac, 1992), respectively. The number of mitochondria in spermatozoa to provide the required energy for their motility is high. Morphologically abnormal spermatozoa contain defective mitochondria that produce increased levels of ROS. The reactive oxygen species may affect membranes of other mitochondria and thereby induce a further increase in ROS production (Evenson et al., 1982). A cell-to-cell variation in ROS production in subsets of spermatozoa at different stages of maturation has been recognized (Gil-Guzman, 2001). Immature germ cells produce higher levels of ROS than mature germ cells. Spermatocytes, round and elongated spermatids produce low levels of ROS (Gil-Guzman, 2001). In equine ejaculates, immature spermatozoa with abnormal head morphology and retention of the cytoplasmic droplet, and dead spermatozoa have been recognized to be the major producers of ROS. Likewise, ejaculates with greater numbers of sperm with amorphous heads, damaged acrosomes, midpiece defects, cytoplasmic droplets, tail defects were correlated with higher ROS production (Gomez et al., 1996; Aziz et al. 2004).

Different authors suggest leukocytes, especially neutrophils and macrophages, as important source of ROS in the male reproductive tract and in ejaculated semen, because leukocytes possess a membrane-bound NADPH oxidase similar to that of spermatozoa (Aitken et al., 1992;1997; Sharma et al., 2001; Agarwal et al., 2004). However, other studies do not confirm a high correlation between leukocyte concentration and ROS levels

(El-Demiry et al., 1987; Fedder, 1996; Tomlinson et al., 1993). Leukocyte contamination is unusual in equine semen (Roberts, 1986; Ball et al., 2001a), except in cases of genital tract infection (Malmgren andSussemilch, 1992; Varner et al., 2000). In addition, equine spermatozoa are less sensitive to the deleterious effect of high concentration of leukocytes in the ejaculate than human spermatozoa (Baumber et al., 2002).

2.3.2 Physiological role of ROS in spermatozoa

Low concentration of ROS are a prerequisite for spermatozoa to obtain their full fertilizing ability. ROS are involved in maintenance of motility, capacitation, hyperactivation, the acrosome reaction, and fertilization, respectively (deLamirande and Gagnon 1993; deLamirande et al.,1998). It has been suggested that ROS may drive the physiological changes associated with sperm capacitation (de Lamirande et al., 1998). ROS promote capacitation through the redox regulation of tyrosine phosphorylation in different species, including men (Leclerc et al. 1997; Aitken et al., 1998) and horses (Baumber et al., 2003b). ROS may be involved in other mechanisms of sperm capacitation, e.g. the stimulation of cyclic adenosine monophosphate production and activation of protein kinase A, the activation of extracellular signal regulated kinase-like proteins and up-regulation of tyrosine phosphorylation in the sperm tail, or in the induction of sterol oxidation (reviewed by Aitken, 2017). It has been shown that the superoxide anion and small amounts of hydrogen peroxide are involved in signal transduction and tyrosine phosphorylation of sperm membrane proteins, thereby stimulating capacitation and the acrosome reaction (Bauskin et al., 1991; deLamirande and Gagnon 1993; Griveau et al., 1994). Superoxide anion and hydrogen peroxide also cause tyrosine phosphorylation that in turn promotes sperm membrane binding to the ZP-3 protein in the zona pellucida (Aitken et al., 1995).

ROS provoke lipid peroxidation in the plasma membrane which are usually associated with decreased sperm function and viability (Joneset al., 1979; Aitken et al., 1989, 1993; Griveau et al., 1995a). However, a lower degree of lipid peroxidation is necessary to facilitate the adhesion process of spermatozoa to homologous and heterologous zonae pellucidae (Aitken et al., 1989). In contrast, nitric oxide has no effect in zona pellucida binding, but is essential to activate the sperm's ability to fuse with oocytes (Zini et al., 1995; Francavilla et al., 2000).

2.3.3 Effect of oxidative stress on spermatozoa

Oxidative stress is a state characterized by excessive amounts of ROS, due to an imbalance in generation and degradation of ROS. High concentrations of ROS can damage critical sperm cell components, including lipids, proteins, nucleic acids and sugars. Hydrogen peroxide seems to be the most dangerous ROS for spermatozoa, because it is membrane permeable and can damage DNA, while another extrinsic ROS can cause mainly lipid peroxidation (Henkel et al., 2005). The detrimental effects of OS depend on the amount of ROS and on the duration of ROS exposure (de Lamirande and Gagnon, 1995; Agarwal and Prabakaran, 2005). Extracellular factors as temperature, oxygen tension and composition of the environment influence the extent of OS (Aitken and Fisher, 1994). The extent of OS in seminal plasma depends on the composition, such as concentration of ions, proteins and especially of ROS scavengers (Agarwal and Saleh, 2002).

Motility reduction of spermatozoa is a sensitive indicator of oxidative stress. Motility is the first parameter affected by excessive levels of ROS (Lenzi et al., 1993; Agarwal et al., 1994; Armstrong et al., 1999, Baumber et al., 2000). The exact mechanism underlying the link between OS and motility reduction is not clear. It has been hypothesized that extracellular hydrogen peroxide penetrates the spermatozoa and inhibits the activity of various enzymes, e.g. glucose-6-phosphate dehydrogenase (G6PD; Griveau et al., 1995). The G6PD regulates the intracellular glucose concentration and consequently the availability of NADPH (Aitken et al., 1997). The decreased availability of NADPH and the accumulation of oxidized and reduced glutathione affect the antioxidant defense of the sperm and peroxidation of membrane phospholipids. Lipid peroxidation is related to a loss of motility due the release of enzymes and ATP which are followed by a decreasing metabolic activity of the sperm cells (Storey, 1997). Any impairment in the ATP production process may have negative effect on motility. Such an ATP depletion decreases the available energy supplied by mitochondria, provoking decreased axonemal protein phosphorylation and sperm immobilization (deLamirande and Gagnon, 1992).

ROS have also a direct effect on mitochondria by disrupting inner and outer mitochondrial membranes, and inducing the release of apoptosis inducing factors (AIF). The AIF released are cytocrome-C protein and proteases, e.g. caspases 3 and 9, that directly interact with the DNA and lead to DNA fragmentation and apoptosis (Candé et al., 2002; Paasch et al., 2004). However, it has been suggested that in mammals species-specific

characteristics in the apoptotic mechanisms of sperm senescence are involved (Gallardo Bolanos et al., 2014). Indeed, cryopreservation of equine semen induces a premature senescence of the surviving spermatozoa due to oxidative stress resulting from mitochondrial malfunction (Pena et al., 2011; 2015). This mechanism recently termed spermptosis, does not provoke a direct DNA damage, but does implicate caspase 3 activation, reduced mitochondrial function and transposition of phosphatidylserine (PS) to the outer leaflet of the membrane (Ortega-Ferrusola et al., 2017).

Cooled storage and cryopreservation promote DNA damage in equine spermatozoa (Linfor and Mayers, 2002; Baumber et al., 2003a). OS is the primarily causes of DNA damage in sperm (Aitken and De Iuliis, 2007) in form of single- and double-strand DNA breaks (Duru et al., 2000). Modification of all bases, deletions, frame shifts, DNA cross links and chromosomal rearrangements were seen in sperm artificially exposed to ROS (Aitken and Krausz, 2001; Sharma et al., 2004; Bansal and Kaushal, 2014). When the degree of DNA fragmentation is small, DNA can be repaired by the oocyte. When the damage is extensive, sperm apoptosis can occur. High correlations between sperm DNA damage and decreased fertilization rates and poor embryo cleavage have been reported (Sakkas et al.,1998).

Spermatozoa require a high polyunsaturated fatty acid (PUFA) content to provide the plasma membrane with the essential fluidity for activation of the signal transduction pathways necessary for the fertilization processes (Wathes et al., 2007). The high amount of PUFA in the sperm plasma membrane makes them highly susceptible to peroxidative damage (Aitken, 1995). Initiation of PUFA peroxidation relies on the oxidation of Fe^{2+} to Fe^{3+}, to form a Fe^{3+}-dioxygen-Fe^{2+} complex (Ball and Vo, 2002). Oxidative stress leads to abstraction of a hydrogen atom from a PUFA, thereby initiatinga chain reaction of lipid peroxidation. A lipid alkyl radical reacts quickly with oxygen to form cytotoxic aldehydes. Membrane lipid peroxidation causes modifications of the integrity, permeability and fluidity of the sperm plasma membrane. The plasma membrane also loses its ability to fuse during acrosomal exocytosis. Semen preservation, i.e. refrigeration and cryopreservation, increase the sperm's susceptibility to oxidative stress especially in the midpiece region (Ball and Vo, 2002; Neild et al., 2005).

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Chapter 3

Antioxidants and male fertility

Mammals have evolved antioxidant defense systems which play a role in preventing ROS generation, inactivating oxidants and limiting the deleterious effects of oxidants by allowing repair of oxidative damage (Cheeseman and Slater, 1993). In order to alleviate the impact of oxidative stress on both, spermatogenesis and steroidogenesis, and to protect spermatozoa from excessive oxidative stress, the male reproductive tract is endowed with enzymatic and non-enzymatic antioxidant strategies (reviewed by Vernet et al., 2004). After ejaculation, in female genital tract or during storage/shipping, spermatozoa have to depend on antioxidants, metal ions and proteins provided by the seminal plasma and semen extender for protection (Wai-Sum et al., 2006). Understanding the mechanism that protect spermatozoa from oxidative stress (OS) through the manipulation of the intrinsic antioxidant mechanisms could constitute an ideal strategy for better preservation of sperm function. Human studies suggest an enhanced antioxidant defenses by exogenous reinforcement against OS with two different approaches: a dietary antioxidant supplementation to improve antioxidant status in tissue, seminal plasma and spermatozoa or an addition of antioxidants to semen extenders in order to increase the antioxidant status of seminal plasma (reviewed by Agarwal et al., 2004). In the last two decades, similar strategies of antioxidant supplementation in the diet or in the semen extender were proposed to improve quality of fresh, cooled, and frozen stallion semen (Ball, 2008; Peña et al., 2011).

3.1 Antioxidant defense systems

Different protective antioxidant systems interact with each other to ensure optimal protection against ROS in the male reproductive organs. Primarily, endogenous antioxidant enzymes, exogenous dietary antioxidants and metal binding proteins were recognized as compounds of those systems (Aitken and Roman, 2008; Makker et al., 2009; Dare et al., 2014). Endogenous antioxidants are produced by the testes and epididymides and are present in seminal plasma and spermatozoa. Concerning the enzymatic constituents of the endogenous defense, major ROS scavengers are the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase/ glutathione reductase (GPX/GRD). The antioxidant enzymes activity is supported by non- enzymatic molecules such as nutritional antioxidants and other molecules. Dietary antioxidants are commonly present in the form of vitamin C, vitamin E, carotenoids, and flavonoids. Metal chelators and metal-binding proteins, as a third member of the endogenous antioxidant system, provide an antioxidant activity by blocking the formation of new ROS. It seems that a deficiency in any part of the antioxidant defense system can cause a decrease in total antioxidant status in the testis, epididymis and in the semen (Walczak–Jedrzejowska et al., 2013).

3.1.1 Enzymatic antioxidants

The role of the enzymatic antioxidants SOD, CAT and GPX/GRD in the testis and epididymis is to create the microenvironment necessary for maturation and storage of mammalian spermatozoa, controlling ROS generation and degradation. The group of enzymatic antioxidants was found also in human and stallion seminal plasma (Zini et al., 1993; Ball et al., 2000; Baumber and Ball., 2005) and their activity was confirmed during semen storage at 5°C (Kankofer et al., 2005).

Oxidative stress in the testis triggers a response characterized by the NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) mediated induction of mRNA species for superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities (Kaur et al., 2006). As summarized in Figure 3.1, SOD, CAT and GPX are able to neutralize the ROS molecules by converting them into water and oxygen. SOD rapidly converts superoxide anion (O_2^-) into oxygen and hydrogen peroxide (H_2O_2), in order to prevent the formation of highly detrimental hydroxyl radicals. This H_2O_2 is less toxic than superoxide but it is a powerful membrane permeant oxidant

that has to be rapidly eliminated from the cell. In order to prevent the induction of oxidative damage to lipids, proteins and DNA, CAT and GPX complete the reaction started by SOD by degrading hydrogen peroxide to water and oxygen. GPX operates by using the reduced form of glutathione (GSH) as an electron donor. GSH is continuously oxidized to GSSG (oxidized glutathione) and then regenerated by GRD and NADPH to allow further antioxidant function (Figure 3.1; Agarwal et al., 2007; Kefer et al., 2009).
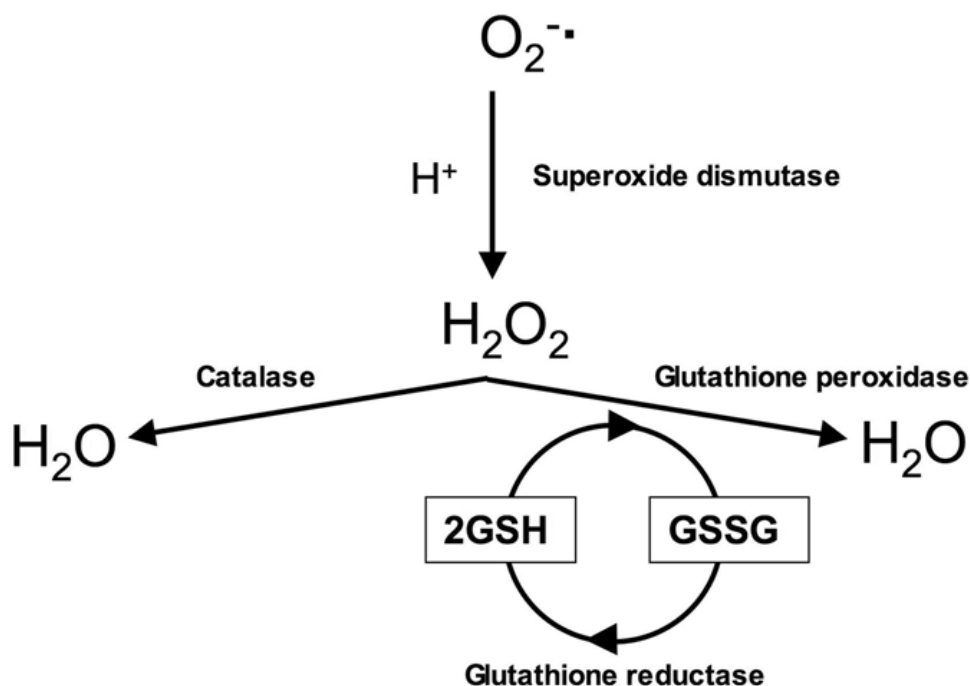
Figure 3 3 Antioxidant scavenging pathways of free radicals by superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX); Glutathione reductase catalyzes the reduction of oxidized glutathione (GSSG) to regenerate glutathione (GSH; modified by Aitken and Roman, 2008).

Within the spermatozoa, superoxide dismutases (SODs) constitute the first line of defense against ROS (Alscher et al. 2002). Three SOD isozymes were discovered in mammals. CuZnSOD is a cytosolic isoform containing Cu^{2+} and Zn^{2+} as metal cofactors, the mitochondrial isoform MnSOD that contains Mn^{2+}, and the extracellular form ECSOD which is similar to

CuZnSOD and also contains Cu^{2+} and Zn^{2+} as metal cofactors (reviewed by Mruk et al., 2002). ECSOD was found in the epididymis and in the lumen of the seminiferous tubules in the testis (reviewed by Mruk et al., 2002; Ookawara et al., 2002). The antioxidant function of SOD in the epididym is may be to protect the spermatozoa. Spermatozoa at this point of maturation are rich in unsaturated fatty acids and their cytoplasmic antioxidant status is diminished, due to the gradual shedding of the cytoplasmic droplet (Tramer et al., 1998). It has been hypothesized that SOD is implicated in the redox regulated, tyrosine phosphorylation events which are associated with the induction of motility and the initiation of capacitation (Lewis and Aitken, 2001). In stallion, the activity of SOD in stallion seminal plasma is 29.15 ± 6.64 U/mg protein and the principal sources of SOD are ampulla and prostate (Baumber and Ball, 2005).

CAT is responsible for the dismutation of H_2O_2 to O_2 and H_2O and is involved in sperm capacitation, which is acomplex mechanism involving in turn H_2O_2 (de Lamirande and Gagnon, 1995). CAT localization is limited to the peroxisome and major source are prostatic secretions. The presence of this enzyme was not found in rabbit spermatozoa (Holland and Storey, 1981), mouse spermatozoa (Alvarez and Storey, 1984) and bull spermatozoa despite a high level in semen (Bilodeau et al., 2000). Only low levels of CAT were found in human and rats spermatozoa (Jeulin et al., 1989). CAT activity was detected in testes, accessory sexual glands and cauda epididymal fluid of stallion (Ball et al. 2000). Furthermore, CAT activity in seminal plasma is 98.7 ± 29.2 U/mg protein for catalase (Ball et a., 2000).

Mammalian GPX are selenium-containing enzymes and were found in spermatozoa and seminal fluid (Alvarez and Storey, 1984; Alvarez et al., 1987). GPX is involved in redox regulation and in the protection of membranes from oxidative stress, interrupting the cascade of radical formation (Ursini et al., 1982). The best know mammalian GPXs are cytosolic GPX, phospholipids hydroperoxide GPX, plasma GPX and gastrointestinal GPX (Brigelius-Flohe, 2006). Amongst all peroxidases, the major enzyme present in the testes is phospholipid hydroperoxide GPX (Brigelius-Flohe, 1999a). GPX is involved in stallion sperm maturation and differentiation (Pagl et al., 2006). Phospholipids hydroperoxide GPX participates in the condensation of chromatin by the oxidation of sulfhydryl-groups on protamines from epididymal rat sperm in the presence of H_2O_2 (Godeas et al. 1997).GPX activity in the stallion seminal plasma is 0.87 ± 0.06 µM NADPH oxidized/min/mg protein (Baumber and Ball, 2005).This enzymatic antioxidant system contained in stallion seminal

plasma seems to protect spermatozoa against ROS at least until 24 hours of storage at cooling temperature (Kankofer et al., 2005).

3.1.2 Non-enzymatic antioxidants

3.1.2.1 Nutritional antioxidant

Active antioxidant components which are taken up with the diet form an essential part of the protective antioxidant defense system in the testis and in seminal plasma Nutritional antioxidants are present in the form of vitamin C, vitamin E, selenium, carotenoids, and flavonoids. Vitamin E (α-tocopherol) and C (ascorbic acid) are chain break antioxidant that probably act synergistically to protect against a peroxidative attack (Baker et al.,1996). Vitamin E was recognized in the 1940s as a powerful lipophilic antioxidant that supports mammalian spermatogenesis. High amounts of vitamin E were found in Sertoli cells and pachytene spermatocytes and to a lesser extent in round spermatids (Amara et al., 2007). Vitamin E is able to interrupt lipid peroxidation and enhance the activity of various antioxidants (e.g. Vitamin C, β-carotene; Brigelius-Flohe et al., 1999b), thereby preserving sperm vitality and motility (de Lamirande and Gagnon, 1992).
Vitamin C is highly concentrated in seminal plasma and helps in protecting spermatozoa against endogenous oxidative damage and preventing sperm agglutination (Fraga et al., 1991). In seminal plasma, high levels of ROS are correlated with reduced concentration of Vitamin C in men (Lewis et al., 1997). Deficiencies of vitamins C or E leads to a state of oxidative stress in the testes that disrupts spermatogenesis and the production of testosterone (Turner and Lysiak, 2008). Vitamin C reduces oxidized α-tocopherol and it maintains this antioxidant in an active state, in order to support spermatogenesis (Yoganathan et al., 1989). Vitamin C itself is maintained in a reduced state by a GSH-dependent dehydroascorbate reductase.
Selenium (Se) may protect against DNA damage which is induced by oxidative stress. Furthermore, Seacts as a cofactor for antioxidant enzymes maintaining normal testicular development and spermatogenesis (Ursini et al., 1999; Ozturk et al., 2002). Selenium contributes to the protection of sperm DNA and cell membranes, thereby preserving sperm motility and function. Apart from Se, other metals are needed to support the function of antioxidant enzymes, such as superoxide dismutase. The antioxidant role of zinc is attributable to its ability to bind ROS, whereas manganese enhances

sperm motility and viability (Lombardo et al., 2011; Bansal and Bilaspuri, 2008; 2011).

Vitamin A and its precursors (carotenoids) have short half-lives. They work synergistically with Se and vitamin E as ROS quenchers (Sies and Stahl, 1995). High concentration of β-carotene which prevent lipid peroxidation have been found in the testis (Kaplan et al., 1990; Agarwal and Sekhon, 2010). However, the most potent carotenoid is lycopene. It prevents peroxidation processes in the seminal plasma (Lampiao, 2012). Flavonoids, a group of polyphenolic compounds, have also antioxidant or free radical scavenging properties. More than 4000 flavonoid derivatives were described. Their antioxidant properties differ, depending on the number and position of hydroxyl groups in the molecule (Farkas et al., 2004).

3.1.2.2 Metal chelators or metal binding proteins

Transitional metal ions are involved in the generations of highly reactive oxygen species (Ochsendorf, 1999). Metal chelators and metal-binding proteins (e.g. albumin, metallothionein) reduce the formation of new ROS, by inactivation of transitional ions. In the seminal plasma, metal chelators such as transferrin, lactoferrin, and ceruloplasmin protect sperm integrity by controlling lipid peroxidation of plasma membrane (Sanocka and Kurpisz, 2004). The addition of other metal chelators to semen extender such as ethylene diamine tetraacetic acid, 1,10-phenanthrolin, DL-penicillamine and neocuproine have been shown to reduce sperm DNA damage and increase sperm motility (Henkel and Schill, 2003; Wroblewski et al., 2003; Agarwal et al., 2005).

3.1.2.3 Other non-enzymatic antioxidants

Other antioxidants molecules such as glutathione (GSH), N-acetyl L-cysteine, N‑acetyl-5‑methoxytryptamine, coenzyme Q 10 and carnitines provide as well excellent antioxidative support (Aitken and Roman, 2008; Agarwal and Sekhon, 2010).

Glutathione is the most abundant non-thiol protein in mammalian cells. It is a peptide composed of glutamate, cysteine and glycine. GSH, vitamin E and Se play essential roles in the formation of GPX. N-acetyl L-cysteine is a precursor of glutathione and acts as well as antioxidant by eliminating ROS and blocking ROS production (Gressier et al., 1994; Agarwal and Said, 2004).

The antioxidant activity of the pineal hormone melatonin (N‑acetyl-5‑methoxytryptamine) is associated with two different features of the hormone. First, melatonin is soluble in lipid and aqueous environments and can readily cross the blood testes barrier to protect the germinal epithelium. Second, melatonin is a ROS scavenger, undergoing a two electron oxidation and avoiding a generation of other ROS. Low melatonin levels in seminal plasma were correlated with poor motility and infertility in rats (Mogulkoc et al., 2006).

A testes-specific form of cytochrome C is another ROS scavenging molecule. Additionally, it is an activator of apoptosis and exerts a protective role in the testes by eliminating damaged germ cells (Liu et al., 2006).

Coenzyme Q-10 is found endogenously in human seminal fluid and in the miedpiece of spermatozoa (Mancini and Balercia, 2014). Coenzyme Q-10 is an energy promoting agent, able to recycle vitamin E and controlling its pro-oxidant capability. Furthermore, coenzyme Q is associated with low density lipoproteins (LDL) and reduced lipid peroxidation of spermatozoa. *In vitro* and *in vivo* supplementation with coenzyme Q-10 increases sperm motility improves sperm motility (Lewin and Lavon, 1997; Mancini and Balercia, 2014).

High concentrations of Carnitine were found in human semen, produced by the seminal vesicle and the epididymis (Lewin et al., 1976). Carnitine plays an important role in sperm maturation and development (Lenzi et al., 2003; 2004). Dietary supplementation with carnitine promotes membrane stability, protecting sperm cell from ROS damage and apoptosis (reviewed by Lombardo et al., 2011).

3.2 Antioxidant strategies

Since the detrimental effect of oxidative stress on spermatozoa is reported as primary cause of subfertility in men (Tremellen, 2008), antioxidant strategies have been proposed to reduce oxidative stress levels and thereby enhance male fertility (Agarwal et al., 2004; Agarwal and Sekhon, 2010; Lombardo et al., 2011; Elmussareh et al., 2015). Although a general beneficial effect of antioxidants on the quality of semen has been demonstrated, only few studies reported an improvement in pregnancy rates (reviewed by Ross et al., 2010; Showell et al., 2011).

The goal of antioxidant supplementations is to improve antioxidant defense mechanism in the male genital tract and in semen and spermatozoa after ejaculation. Antioxidant strategies may indicate both antioxidant diet supplementation (*in vivo* studies) and antioxidants addition into semen diluents used to prepare fresh, cooled and frozen semen (*in vitro* studies; Lombardo et al., 2011). The effect of *in vivo* and *in vitro* supplementation of antioxidants on men semen has been reported in the Table 3.1 (Lombardo et al., 2011; Mora-Esteves et al., 2013).

Table 3.1 *An overview of antioxidants tested in human male fertility: natural source, dosages, the in vivo or in vitro methodology and the observed effect (modified from Lombardo et al., 2011; Mora-Esteves et al., 2013).*

Antioxidant	Natural sources	In vivo / in vitro	Effect and dosage	Studies
Carnitine	Red meat, poultry, fish, and dairy	In vivo	Improved motility, morphology and concentration (3 g /day L-carnitine for 2 months); Increased motility and viability; reduced ROS quantity; no effects on concentration or morphology (2 g/day carnitine, 1 g/day acetylcarnitine, for 3 and 4months); Increased concentration and motility (2 g/day carnitine for 6 months); Increased sperm count and motility (carnitine 2 g/day and acetyl-L-carnitine 1 g/day for 6 months); Improved concentration, motility and morphology (carnitine 2 g acetyl-L-carnitine 1 g daily for 4 months); Increased velocity (L-carnitine 3 g/day, or acetyl-L-carnitine 3 g/day, or L-carnitine 2 g/day + acetyl-L-carnitine 1 g/day , for 6 months)	Costa et al., 1994; Vicari and Calogero, 2001; Vicari et al., 2002; Lenzi et al., 2003; Lenzi et al., 2004; Cavallini et al., 2004; Balercia et al., 2005
Coenzyme Q-10	Oily fish (mackerel, sardines), organ meats, whole grain, rice bran, soybeans, nuts, vegetables	In vitro; In vivo	Improved motility (50 μmol/l); Increased fertilization rate; no effects on motility, morphology or concentration (60 mg/day for 103 days); Improved motility (200 mg/day for 6 months)	Lewin and Lavon, 1997; Lewin and Lavon, 1997; Balercia et al., 2009
Glutathione	Fresh meat, fruits, and vegetables	In vitro; In vivo	Reduced ROS-induced DNA damage (10 mmol/l); Preserved tail-beat frequency (5-10 mmol/l); Preserved motility (1-10 mmol/l); Improved motility and morphology; no effects on concentration (600 mg alternative/day for 2 months); Improved motility, morphology and concentration (600 mg alternative day for 2 months)	Griveau and Le Lannou, 1994; Lopes et al., 1998; Hong et al., 1994; Baker et al., 1996; Lenzi et al., 1993; Lenzi et al., 1994
SOD	-	In vitro	Preserved motility; reduced lipid peroxidation (87.5-500 IU/ml); Preserved motility (1 mg/ml); No effects on lipid peroxidation (100, 250 or 500 IU/ml)	Kobayashi et al., 1991; Aitken et al., 1993; Griveau and Le Lannou, 1994; Lopes et al., 1998. Kovalski et al., 1992; Twigg et al., 1998
Catalase	-	In vitro	Preserved motility; reduced lipid peroxidation; reduced DNA damage (0.008-0.1 mg/ml; 50-2000 IU/ml, in vitro); No effects on lipid peroxidation (250, 500 or 2000 IU/ml, in vivo)	Kovalski et al., 1992 Griveau and Le Lannou, 1994 Lopes et al., 1998 Twigg et al., 1998
N acetylcysteine	Dietary sources are not adequate	In vitro; In vivo	Reduced ROS production; improved motility (1-10 mmol/l); Preserved motility (0.1-15 mg/ml/l); Reduced ROS-induced DNA damage (0.1 mmol/l); Reduced ROS quantity; no effects on semen (600 mg/day for 3 months); Improved sperm count, motility and morphology (600 mg/day N-acetyl-cysteine, or 600 mg/day N-acetyl-cysteine and Se 200 μg /day, or Se 200 μg /day daily, for 6 months); Improved sperm motility, volume, viscosity and oxidative status (600 mg/day N-acetylcysteine for 3 months)	Baker et al., 1996; Oeda et al., 1997; Lopes et al., 1998; Comhaire et al., 2000; Safarinejad and Safarinejad, 2009; Ciftci et al., 2009
Vitamin A	Fruits and vegetables, eggs, meats, oil and salt water fish	In vivo	Increased sperm count (0.06 IU/kg/day Vit A, 3 mg/kg vitamin C, 0.2 mg/kg vitamin E, 10 mg/kg N-acetyl-cysteine, 0.01 mg/kg zinc and others, daily, for 3 months)	Paradiso Galatioto et al., 2008

Antioxidant	Natural sources	In vivo / in vitro	Effect and dosage	Studies
Vitamin C	Fruits and vegetables. Ubiquitous in processed foods and drinks	In vitro	Reduced DNA damage after Percoll preparation (300–600 µmol/l vitamin C) Increased DNA damage after Percoll preparation (300–600 µmol/l vitamin C+ 30–60 µmol/l vitamin E) Preserved motility (800 µmol/l) Reduced H2O2-induced ROS production and DNA damage (300–600 µmol/l) Reduced H2O2-induced DNA damage (100–200 µmol/l)	Hughes et al., 1998 Hughes et al., 1998 Verma and Kanwar, 1998 Donnelly et al., 1999 Sierens et al., 2002
		In vivo	Reduced DNA fragmentation (1 g/day vitamin C+1 g /day vitamin E, for 2 months) Improved motility and fertilizing capacity (10 mg/day vitamin C+20 mg /day vitamin E+ 400 mg ZnSO4 or 20 mg/day vitamin E+ 400 mg ZnSO4 or 400 mg ZnSO4 daily for 3 months) Increased sperm count (0.06 IU/kg/day vitamin A, 3 mg / kg vitamin C, 0.2 mg /kg vitamin E, 10 mg /kg N-acetyl-cysteine, 0.01 mg/kg zinc and others, daily, for 3 months)	Greco et al., 2005 Omu et al., 2008 Paradiso Galatioto et al., 2008
Vitamin E	Vegetable oils, wheat germ, cereals/grains, meat, poultry, eggs, dairy products, fruits, vegetables	In vivo	Increased motility, morphology, viability; no effects on concentration (400 mg/day+ 100–200 µg /day Se for 6 months) Increased pregnancy rate, increased motility, reduced lipid peroxidation (300 mg/day for 6 months) Increased concentration, reduced DNA damage (200 mg vitamin C + 200 mg vitamin E+ GSH 400 mg every day for 2 months) Reduced ROS quantity; no effects on semen parameters (30 β-carotene, ~180 mg vitamin E daily for 3 months) Increased motility (400 mg vitamin E+ 225 µg Se daily, for 3 months) Reduced DNA fragmentation (1 g /day vitamin C, +1 g /day vitamin E, for 2 months) Improved motility and fertilizing capacity (10 mg /day vitamin C+20 mg /day vitamin E+ 400 mg ZnSO4 or 20 mg/day vitamin E+ 400 mg ZnSO4 or 400 mg ZnSO4 daily for 3 months) Increased sperm count (0.06 IU/kg/day vitamin A, 3 mg / kg vitamin C, 0.2 mg /kg vitamin E, 10 mg /kg N-acetyl-cysteine, 0.01 mg/kg zinc and others, daily, for 3 months)	Vezina et al., 1996 Suleiman et al., 1996 Kodama et al., 1997 Comhaire et al., 2000 Kessikes-Ammar et al., 2003 Greco et al., 2005 Omu et al., 2008 Paradiso Galatioto et al., 2008
		In vitro	Reduced DNA damage after Percoll preparation (30–60 µmol/l vitamin E) Increased DNA damage after Percoll preparation (300–600 µmol/l vitamin C, 30–60 µmol/l vitamin E) No effects on NADPH-induced lipid peroxidation (1 mmol/l) Reduced H2O2-induced ROS production and DNA damage (40–60 µmol/l) Reduced H2O2-induced DNA damage (1–100 µmol/l)	Hughes et al., 1998 Hughes et al., 1998 Twigg et al., 1998 Donnelly et al., 1999 Sierens et al., 2002
Selenium	Absorbed from soil: plants and meat products (kidney in particular); Brazilian nuts, cereals, meat products, seafood, eggs	In vivo	Increased motility, morphology, viability; no effects on concentration [100 µg /day (1 month)+200 µg /day (5 months)+400 mg vitamin E for 6 months] Improved motility; no increase in concentration (100 µg/day Se, or Se +1 mg vitamin A, 10 mg vitamin C, 15 mg vitamin E daily, for 3 months, in vivo) Increased motility (400 mg vitamin E+ 225 µg Se daily, for 3 months, in vivo) Improved sperm count, motility and morphology (600 mg /day N-acetyl-cysteine, or 600 mg /day N-acetyl-cysteine and Se 200 µg /day, or Se 200 µg /day daily, for 6 months)	Vezina et al., 1996 Scott et al., 1998 Kessikes-Ammar et al., 2003 Safarinejad and Safarinejad, 2009
Zinc	Absorbed from soil: plants such as wheat and seeds (sesame, sunflower, pumpkin, etc.); in beef products	In vivo	Improved sperm count, motility and membrane integrity (500 mg /day for 3 months) Improved concentration and sperm count (5 mg folic acid and/or 66 mg ZnSO4 daily for 6 months, Improved motility and fertilizing capacity (10 mg /day vitamin C+20 mg /day vitamin E+ 400 mg ZnSO4 or 20 mg/day vitamin E+ 400 mg ZnSO4 or 400 mg ZnSO4 daily for 3 months)	Omu et al., 1998 Wong et al., 2002 Omu et al., 2008

(GSH, glutathione; NADPH, nicotinamide adenine dinucleotide phosphate, ROS, reactive oxygen species; SOD, superoxide dismutase)

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3.2.1 Dietary supplementation of antioxidant

An improvement in semen quality after dietary supplementation of antioxidants in humans, rats, pigs, turkey, sheep and fish has been detected (Audet et al., 2004; Eskenazi et al., 2005; Sönmez et al., 2005; 2007; Akmal et al., 2006; Eid et al., 2006; Yue et al., 2010). The success of dietary supplementation with antioxidants is due to an enhanced antioxidative protective mechanisms in the epithelial lining and in the secretions of the male reproductive tract. This results in reduced ROS-induced damages of spermatozoa in testis, epididymis, and in ejaculated and processed semen. Diet supplementation with PUFA added with vitamin C or E, as antioxidant in the food, has resulted in an increase in sperm quality of e.g. rabbit (Castellini et al., 2004), broiler chicken (Surai, 2000), cockerels (Cerolini et al., 2005), boars (Liu et al., 2015) japanese quail (Al-Daraji et al., 2010); ram (Alizadeh and Shaabani, 2014; Jafaroghli et al., 2014); goat (Dolatpanah et al., 2008), bull (Kaka et al., 2015).

Dietary supplementation with nutraceutical containing antioxidants has become routinely used in men infertility as result of widespread availability. It has been demonstrated that most herbs, fruits and vegetables have antioxidant properties and may effect sperm production and function (reviewed by Ko EY and Sabanegh, 2014). Different nutraceuticals were also tested in animal reproduction (reviewed by Arruda et al., 2010). For example, the use of an Andean crop, *Lepidium meyenii* (maca) has been tested in different species, e, g, human, rodents, and cattle, respectively. *Lepidium meyenii* (Maca) grows between 3,800 and 4,500 m altitude. Clinical trials showed that Maca administration increases the number and the motility of spermatozoa and enhances the sexual function in humans (Gonzales et al., 2001; 2002). Several studies conducted in rats demonstrated the beneficial effect of Maca administration on spermatogenesis, thereby improving sperm count and motility (Gonzales et al., 2003; 2004; 2006; 2013; Chung et al., 2005; Gasco et al., 2007; Yucra et al., 2008). The dietary supplementation with Maca flour improved sperm count and motility and reduced the percentage of DNA fragmentation index in bulls (Clément et al., 2010). This plant has positive effects on the reproductive tract, semen quantity and quality of mammals (reviewed by Clément et al., 2012; and by Gonzales, 2011). Therefore, it would be interesting to investigate its effect on stallion semen production and quality.

3.2.2 Antioxidant addition in semen extender

Antioxidant addition to media for semen handling and storage aims at counterbalancing the decrease in antioxidant defense capacity of semen *in vitro* that happens during semen processing and chilled or frozen semen storage. Whether addition of antioxidants to semen extender has a beneficial or detrimental effect depends on the dosage, the type of antioxidant, the combination of antioxidants and in which context the antioxidant is used. Several antioxidant agents have already been tested in *in vitro* studies with human, bovine, porcine, rabbit and equine semen (Alvarez and Storey, 1983; Beconi et al., 1993; Kessopoulou et al., 1995; Baker et al., 1996; Aurich et al., 1997; Donnelly et al., 1999; Ball et al., 2001a, 2001b; Bilodeau et al., 2001; Peña et al., 2003) with controversial efficacy and usefulness.

For example, Vitamin E has a detrimental effect on fresh human semen (Donnelly et al., 1999) and liquid ram semen (Upreti et al., 1997) and little or no effect in equine chilled semen (Aurich et al., 1997; Ball et al., 2001b) and in frozen-thawed human semen (Askari et al., 1994). On the contrary, addition of Vitamin E or B16 in dog chilled or frozen semen extender improves semen parameters and inhibits ROS production (Michael et al., 2007; 2009). In the boar, vitamin E addition to semen results in a positive effect on sperm motility and mitochondrial membrane potential and membrane integrity after cryopreservation, but depends on the portion of ejaculate (Peña et al., 2003; 2004).

The supplementation of diluents with enzymatic antioxidants has been tried individually to counteract oxidative stress during cooling and freezing of semen. In previous studies, CAT addition in semen extender has been shown to improve the post-thaw sperm functions in red deer (Fernández-Santos et al., 2007), dogs (Michael et al., 2007), boars (Roca et al., 2005) and bulls (Asadpour et al., 2012). Furthermore, CAT provides a protective effect against DNA damage in equine sperm (Baumber et al., 2003a). SOD addition to semen extender results in controversially effects. A protective role of SOD on sperm was reported in men and mouflon (Kobayashi et al., 1991; Berlinguer et al., 2003), but a deleterious role was reported in equine sperm during cryopreservation (Baumber et al., 2005).

3.2.3 Antioxidant treatment in stallion

Several studies have analyzed how antioxidants in equine diets affect semen parameters and whether they interfere with the quality of stored, cooled or frozen, semen (Franceschini et al., 2003; Stradaioli et al., 2004; Deichsel et

al., 2008, Contri et al., 2011; Schmidt-Lausigk and Aurich., 2014; Freitas et al., 2016). Likewise, the addition of antioxidants to cooling or freezing extenders were tested (Aurich, 2008). The effect of different antioxidants, alone or in combination, on stallion semen quality was controversial and is summarized in the tables 3.2 and 3.3.

Table 3.2 *An overview of antioxidants tested in stallion semen: columns indicate the dose, the way of supplementation (in vivo or in vitro), semen processing and observed effects.*

Antioxidant	Dose and duration	In vivo/In vitro	Effects on semen	Semen process	Studies
Ascorbic acid	0.45 g /l	In vitro	Increase membrane integrity	Cooled (72h)	Aurich et al., 1997
	0,9 g/l	In vitro	Reduced motility	Cooled (72h)	Aurich et al., 1997
	10mM	In vitro	No positive effect	Frozen-thawed	Baumber et al., 2005
Butylated hydroxyanisole (BHA)	2 mM	In vitro	Preserved sperm motilities, viability and plasma functionality by minimizing oxidative stress	Frozen-thawed	Seifi-Jamadi et al., 2016b
Butylated hydroxytoluene (BHT)	0,5-1-2 mM	In vitro	No positive effect, reduced motility	Cooled (72h)	Ball et al., 2001 a
	1 mM	In vitro	Preserved sperm motilities, viability and plasma functionality by minimizing oxidative stress	Frozen-thawed	Seifi-Jamadi et al., 2016b
Catalase (CAT)	0,45 -1,8 x 10^6 units/l	In vitro	Reduced motility and membrane integrity	Cooled (72h)	Auriche t al., 1997
	150 U/ml	In vitro	Preserved motility	Incubation with xanthine (X, 0.6mM), xanthine oxidase (XO, 0.05 U/mL) 30 minutes at room temperature	Baumber et al., 2000
	100-200 U/ml	In vitro	No positive effect	Cooled (72h)	Ball et al., 2001 a
	200 U/ml	In vitro	No positive effect	Frozen-thawed	Baumber et al., 2005
	200U/ml	In vitro	No positive effect	Frozen-thawed	Gibb et al., 2013
Cysteine	0,2 mg/ml	In vitro	No positive effect	Frozen-thawed	Gibb et al., 2013
Glutathione	1 mM	In vitro	Preserved motility	with xanthine (X, 0.6mM), xanthine oxidase (XO, 0.05 U/mL) 30 minutes at room temperature	Baumber et al., 2000
	10mM	In vitro	No positive effect	Frozen-thawed	Baumber et al., 2005

Antioxidant	Dose and duration	In vivo/In vitro	Effects on semen	Semen process	Studies
L-carnitine	10 g/day for 90 days	In vivo	No positive effect	Fresh	Rosas Filho et al., 2001
	Dietary supplementation	In vivo	Positive effect on mithocondria activity, improved motility	Frozen-thawed	Franceschini et al., 2003
Quercitin	0,15 mM	In vitro	Increased motility and zona binding ability	Frozen-thawed	Gibb et al., 2013
	0,1 mM	In vitro	Preserved motility parameters	Frozen-thawed	Seifi-Jamadi et al., 2016a
Superoxide dismutase (SOD)	150 U/ml	In vitro	No positive effect	with xanthine (X, 0.6mM), xanthine oxidase (XO, 0.05 U/mL) 30 minutes at room temperature	Baumber et al., 2000
	200 U/mL	In vitro	No positive effect	Frozen-thawed	Baumber et al., 2005
	25 -50 IU/ml	In vitro	Increased viability and motility	Cooled (72h)	Cocchia et al., 2011
Tempo (lipid- and water-soluble antioxidant)	0,5-1-2 mM	In vitro	No positive effect	Cooled (72h)	Ball et al., 2001 a
Trehalose	150 mM	In vitro	Increased motility and viability Reduced morphologically abnormal spermatozoa	Cooled	Ghallab et al., 2017
	150 mM	In vitro	Preserved sperm motility, viability and membrane integrity	Frozen-thawed	Ghallab et al., 2017
VitaminE	1-2-4 mM	In vitro	No positive effect	Cooled (72h)	Ball et al., 2001 a
Zinc sulphate	200 μM	In vitro	Preserved sperm motility, viability Reduced morphologically abnormal spermatozoa	Cooled	Ghallab et al., 2017
	200 μM	In vitro	Preserved sperm motility, viability Reduced morphologically abnormal spermatozoa	Frozen-thawed	Ghallab et al., 2017
α-tocopherol	25, 50, 100, or 500μM or 1mM	In vitro	No positive effect	Frozen-thawed	Baumber et al., 2005

Different diet supplementations with L-carnitine showed an improved total motility in cryopreserved sperm after thawing, possibly due to an optimization of mitochondrial activity (Franceschini et al., 2003). In a study in pony stallions, supplementation of diet with tocopherol (Vitamin E), ascorbic acid, l-carnitin and folic acid did not show any effects on fresh and cooled semen (Deichsel et al., 2008). Dietary intake with a combination of organic Se, vitamin E, and zinc resulted in enhanced quality of fresh semen and in an improved quality during preservation of cooled semen (Contri et al., 2011). In general, the diet's antioxidants seem to protect sperm cells during spermatogenesis and/or epididymal maturation. Besides, an increase in the antioxidant power of seminal plasma may preserve semen motility during processing.

Recently, dietary supplementation of stallions with linseed oil and a combination of the antioxidants vitamin E, selenium, and superoxide dismutase, attenuated a decline in motility and membrane integrity of cooled-stored semen during winter (Schmidt-Lausigk and Aurich, 2014). Supplementing stallion's diet with antioxidants combined with PUFA may reduce oxidation of the sperm plasma membrane and, thus, contribute to the maintenance of semen quality during cooled storage. However, a supplementation of only PUFA did not show a beneficial effect on fresh semen quality and on longevity during storage (Brinsko et al., 2005; Deichsel et al., 2008). Dietary supplementation with docosahexanoic acid resulted in improving motion parameters after cooling and cryopreservation (Brinsko et al., 2005; Deichsel et al., 2008). However, in stallions with poor cooling-ability of their spermatozoa, a higher preservation of semen quality during cold storage could be obtained (Brinsko et al., 2005). Therefore, improving sperm quality and the longevity of preserved semen with a dietary supplementation of antioxidants seems to be an interesting option.

Table 3.3 *An overview of antioxidant combinations tested in stallion semen: columns indicate the dose, the way of supplementation (in vivo or in vitro), semen processing and observed effects.*

Antioxidant combination	Dose and duration	*In vivo/ In vitro*	Effects on semen	Semen process	Studies
Trolox Tempo Vitamin C BSA	2,0 mM 1 mM 0,45 mg/ml 3% w/v	*In vitro*	No positive effect	Cooled (72h)	Ball et al., 2001 a
Vitamin E (tocopherol) Ascorbic acid L-carnitin Folic acid	OS 8 weeks : 300 mg/day; 300 mg/day 4000 mg/day 12 mg/day	*In vivo*	Reduced morphologically abnormal spermatozoa No effect on motility and acrosome integrity	Cooled (24h)	Deichsel et al., 2008
Vitamin E (α-tocopherol) Zinc organic Selenium	OS 16 weeks: 15 g/kg 3.6 g/kg 0.025 g/kg	*In vivo*	Increased viability, progressive motility, VAP, STR and total seminal plasma antioxidants levels Reduced morphologically abnormal spermatozoa	Fresh semen	Contri et al., 2011
Linset Oil (60% α-linolenic acid and 15% linoleic acid) Myostem Protect (magnesium HCL 114 g/L, Promutase 3.3 g/L, Selplex 3.4 g/L, vitamin E 83.7 g/L, lysin 83.3 g/L)	OS for 84 days : 100ml/day 30 ml/day	*In vivo*	Attenuated decreasing in motility and membrane integrity during winter	Cooled (72h)	Schmid-Lausigk and Aurich,2014
		In vivo	No effect	Frozen-thawed	Schmid-Lausigk and Aurich,2014
Vitamin A(800,859 IU) Vitamin B12 (17,292 µg) Vitamin B6 (720 mg) Vitamin E (22,000 IU) Folic acid (1326 mg) Beta-carotene (500 mg) L-carnitine(330.005 g) Glutamine (1,500 mg) Aspartic acid (280 mg) Glutamic acid (2800 mg) Arginine 28,41 g) Phenylalanine (370 mg) Glycine (4,600 mg) Lysine (740 mg) Omega-3 (110g) Omega-6 (55g) Oleic acid (57.072 g) Proline (2,330 mg) Taurine (1,500 mg) Valine (460 mg) Selenium (150 mg) Zinc (3,303 mg) Copper (574 mg) Chromiu (221mg)	OS for 10 weeks (50 mL/day)	In vivo	Increased total motility, trajectory speed, as well as plasma and acrosomal membrane integrity	Fresh	Freitas et al., 2016
		In vivo	Increased total motility, speed, and membrane integrity	Cooled	
		In vivo	Increased total motility, speed, and membrane integrity	Cooled	

Cooling and cryopreservation of equine spermatozoa are associated with elevation in ROS levels and ROS-induced damages in sperm (Ball et al., 2001a). Consequently, the addition of antioxidants to extenders has been tested. In order to preserve equine sperm quality during storage, the addition of either enzyme scavengers or non-enzymatic antioxidants in semen extender has been proposed (Baumber et al., 2005). The first study on antioxidant addition in stallion semen was performed by Aurich et al. in 1997. The addition of ascorbic acid and CAT showed a protective effect of ascorbic acid on sperm membrane integrity in diluted stallion semen (Aurich et al., 1997).

Controversial results were obtained from addition of quercetin (flavonoid) as antioxidant in stallion semen extender. Gibb et al. (2013) reported that quercetin supplementation of stallion sperm diluents used during sex-sorting and cryopreservation reduced DNA damage and improved motility of semen. In a study conducted by Seifidi-Jamadi et al. (2016a) only the use of lower concentrations of quercetin improved stallion sperm post-thaw motility. Higher concentrations of quercitin improve production of ROS and thereby had a negative effect on viability and membrane integrity (Seifidi-Jamadi et al., 2016a).

Seifidi-Jamadi et al. (2016b) demonstrated that supplementation of extender with 2 mM of BHA or 1 mM of BHT, i.e. synthetic analogues of vitamin E, may improve sperm total and progressive motility, viability and plasma functionality by minimizing oxidative stress during frozen storage (Seifidi-Jamadi et al., 2016b). Similarly, an improvement in quality of chilled and frozen/thawed stallion spermatozoa was found after addition of non-enzymatic antioxidants, trehalose or zinc sulphate to extender media (Shahat et al., 2017).

Attempts were made to increase the capacity of the intrinsic antioxidant defense mechanisms by individual addition of enzymatic antioxidants, i.e. catalase, reduced glutathione (GSH), or SOD to semen extenders (Aurich et al., 1997; Baumber et al. 2000, 2003a, 2003b; Cocchia et al., 2011). Addition of CAT in semen extender did not show any improvement in semen quality (Aurich et al., 1997). However, Baumber et al. (2000) tested the effect of CAT, SOD and GSH on ROS-related decreases in motility. ROS were artificially induced by the xanthine–xanthine oxidase system. The authors demonstrated that CAT and to a lesser extent GSH, prevented a decline in motility (Baumber et al., 2000) In the same study, SOD did not preserve semen motility. In contrast, another study showed that the addition of SOD to a semen extender preserves semen quality parameters, such as

vitality, motility and acrosome integrity during cold storage (Cocchia et al., 2011). No studies tested so far the effects of a combination of two or more enzymatic antioxidants in stallion semen extender. Since in the physiological situation equine spermatozoa and seminal plasma contain all three antioxidant systems at the same time, it would be interesting to investigate the effect of a combination of SOD, CAT and GPX added to semen extender on the quality of cooled-stored.

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Chapter 4

Influence of dietary supplementation with Lepidium meyenii
(Maca) on stallion sperm production and on preservation of
sperm quality during storage at 5°C

Submitted for pubblication

4.1 Introduction

Over the past decades, artificial insemination (AI) has become a major breeding tool in many horse breeds (Aurich and Aurich, 2006). Cooled-transported semen has been used worldwide, allowing breeders tobenefit from the best stallions of most breeds and avoiding animal transportation (Aurich, 2008). A limiting factor is that cooling and storage of spermatozoa is associated with a reduction in cell viability, motility and fertilizing capacity (Yoshida, 2000). One of the underlying mechanisms causing such damage is related to an imbalance in the production and scavenging of reactive oxygen species (ROS; reviewed by Pagl et al., 2006a).

The presence of ROS in the male genital tract and in spermatozoa is not per se a negative phenomenon, in fact ROS are produced in the testes during normal spermatogenesis and steroidogenesis (Makker et al., 2009; Mathur and D'Cruz, 2011). Spermatogenesis and Leydig cell steroidogenesis are sensitive to excessive concentrations of ROS in the testis (Hales et al., 2005), which consequently result in the production of immature and defected sperm. In order to avoid excessive concentration of ROS, the testes are equipped with enzymatic and non-enzymatic antioxidant systems. However, they are not always sufficient to counteract an increase in ROS concentration. Cold storage of equine semen increases ROS production (Aurich et al., 2005), thereby being able to provoke damages to all biomolecules of spermatozoa such as lipids andproteins ofthe plasma membrane orthe DNA (Ball, 2008; Aitken et al., 2010). Low concentrations of ROS are indispensable for several important functions of spermatozoa, such as capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion (reviewed by Pagl et al., 2006a). An inefficient antioxidant concentration in semen, an excessive production of ROS by immature and damaged sperm cells, or both provoke oxidative stress (OS) and induce damage to the other sperm cell (Aitken et al., 2012; Roca et al., 2013).

In order to maintain the quality and fertility of stallion semen for prolonged time periods, semen processing has been optimized and the supplementation of extenders with antioxidants has been tested with variable success (Aurich JE et al., 1997; Bruemmer et al., 2002; Kankofer et al. 2005; Aurich C, 2008; Brogan et al., 2015). An alternative strategy to prevent oxidative damage to spermatozoa in the testis and epididymis and concomitantly improve the antioxidative capacity of ejaculated spermatozoa during storage has been sought in food supplementation with antioxidants and/or poly-unsaturated

fatty acids (PUFAs; Brinsko et al., 2005; Deichsel et al., 2008; Contri et al., 2011; Schmid-Lausigk et al., 2014; Aurich, 2014; Freitas et al., 2016).

Studies in humans (Hunt et al., 1992; Eskenazi et al., 2005) suggest that a dietary supplementation with antioxidants reduces seminal oxidative stress and improves semen quality, especially in subfertile males (Wong et al., 2000, Ross et al., 2010). Furthermore, Yue et al. (2010) demonstrated that a supplementation with the natural antioxidant vitamin E improves the quality of sheep semen by protecting the plasma membrane and mitochondria of testicular cellsfrom oxidative stress. The mechanism underlying the improvement of the oxidative status in semen are not fully clear. Probably, the diet's antioxidants improve testicular concentration of antioxidants that contribute to balance the testicular oxidative status. The positive correlation between dietary consumption and semen concentration of PUFAs has been reported in different species (Blesbois et al., 1997, Drokin et al., 1998, Conquer et al., 2000). A dietary intake of PUFAs increasedthe quality of fresh and cooled stallion semen (Brinsko et al., 2005; Contri et al., 2011) probably due to a change in lipid composition which has been observed in other species (Zaniboni et al., 2006; Cerolini et al., 2006; Mourvaki et al., 2010; Radomil et al., 2011). Changes in lipid composition of the sperm membranesmay improveplasma membrane stability and reduce the vulnerability of membrane components to oxidative stress during storage.

Lepidium meyenii (Maca) is an Andean crop that grows between 3,800 and 4,500 m altitude. Maca has many traditional therapeutic uses, including the treatment of rheumatism, respiratory problems, premenstrual and menopausal disorders and is used as laxative (Kilham, 2000). The interest in this plant is based on its positive effects on the reproductive tract, semen quantity and quality of mammals (reviewed by Clément et al., 2012 and by Gonzales, 2012). Clinical trials showed that Maca administration increases the number and the motility of spermatozoa and enhances the sexual function in men (Gonzales et al., 2001; 2002). Several studies in rats demonstrate the beneficial effect of Maca administration on spermatogenesis, thereby improving sperm count and motility (Gonzales et al., 2003a; 2004; 2006; 2013a,b; Chung et al., 2005; Gasco et al., 2007; Yucra et al., 2008).The dietary supplementation with Maca flour improved sperm count and motility and reduced the percentage of sperm with a high DNA fragmentation index in bulls (Clément et al., 2010).

The effect of Maca on the reproductive system has been related to the lipid fraction of the plant, which contains mainly fatty acids and macamides (Zheng et al., 2000; Hudson, 2008). Macamides and macaenes are secondary

metabolites and represent a novel group of long-chained saturated fatty acids, PUFAs and their amides which are characteristic for this plant (Zheng et al., 2000). In addition to a range of other components from Maca (reviewed by Wang et al. 2007), several authors have demonstrated that preparations from Maca have antioxidant properties (Valentova et al., 2001; Sandoval et al., 2002; Lee et al., 2005). The mechanism of action how Maca enhances semen parameters is still unknown. It has been proposed that macamides and the lipid-extractable fraction of Maca act directly on the reproductive tract by affecting the antioxidant-oxidant balance (Gasco et al., 2007; Melnikovova et al., 2015).

The aim of the present study was to evaluate the effects of dietary supplementation with *Lepidium meyenii* on the quantity of fresh stallion sperm and the quality, i.e. motility, viability, acrosome integrity and lipid peroxidation, of diluted stallion semen during storage at 5°C for up to 72 hours.

4.2 Materials and methods

4.2.1 Animals

Ten active breeding stallions (9 to 16 years of age, median: 14 years) weighing 400 to 600 kg were included in the study. They were clinically healthy and had a moderate body condition score of 3 (scale 1 to 5) at the start of the experiment. Stallions were housed in box stalls in the same local stud farm in Sala Consilina (Salerno, ITALY) and were fed hay twice daily and concentrate twice daily at a total amount of 2% of the body weight per day. Water was freely available.

4.2.2 Source and supplementation of Maca

The yellow Maca hypocotyls used for this experiment were harvested in the district of Junin in the Andean highlands of Peru (4,100 m above sea level), and milled to a flour with a particle size of 0.8 mm. Maca flour was obtained from hypocotyls exposed for two months to the extreme temperature cycles, strong light conditions, and atmospheric pressure typical of the high altitude environment (> 3,500 m).After a traditional open-field drying outdoors, hypocotyls were selected, washed, powdered, and packaged for further use. Animals of the control group (C) and a treatment group (M) group received an identical diet based on hay and concentrate pellets. In the M group, the concentrate was supplemented daily with 20 g of yellow Maca powder, resulting in an average dosage of 4g Maca/100 kg body weight (minimum: 3g Maca/100 kg, maximum: 5 g Maca/100 kg). The Maca powder was supplemented for a period of 60 days, starting after the first basal semen collection. The dosage was based on human studies (Gonzales et al., 2001, 2002) and in accordance with the minimum effective dosages reported for rats (Zheng et al., 2000; Cicero et al., 2001; 2002, Gonzales et al. 2004).

4.2.3 Experimental Design

The animals were divided into a control group (C; n = 5) and a treatment group (M; n=5). Group assignment for all animals was determined randomly before the study began. Theassignment of the animals to the groups was unknown to any of the investigators analyzing samples. Maca was administered after the first basal sperm collection (day 0 = D0), for a period of 60 days, because a full spermatogenic cycle (spermatocytogenesis, and

spermiogenesis) in the horse takes 57 days. (Johnson et al., 1997). Blood samples were collected at 5:00 a.m. for all treated stallions and at the same time as each semen collection and used to evaluate testosterone concentrations. Semen was collected with an artificial vagina three times a week in all stallions. Collections started one month before the study period began and were continued during the whole study period. For analysis, one ejaculate per month was used (D0 = the day of starting the Maca diet supplementation, followed by D30, D60, D90, and D120) resulting infive ejaculates/stallion and in a total of 50 samples.

4.2.4 Blood processing

Blood samples were obtained by venipuncture from the jugular vein of each treated stallion. In total 5 samples for each animal were obtained. After incubation, blood samples were centrifuged at 330 xg for 10 min and sera were placed in 1ml Eppendorf tube and stored at -20°C for subsequent analysis.
At the end of the study all sera were sent to the reference laboratory (Di.Lab. Veterinary Service, Naples, Italy) for testing. Determination of testosterone concentration was carried out by chemiluminescence assay (Leme et al., 2012).

4.2.5 Semen processing

Immediately after collection, the gel fraction was removed bya nylon semen filter (Minitube, Germany) and the semen was filtered through semen filter pouch (Minitube, Germany). The sperm concentration was determined by a Burkerhemocytometer chamber. The volume of ejaculate was determined for each semen sample by a graduated laboratory bottle (Sigma, Italy). Finally, the total sperm count (TSC) was calculated by multiplying sperm concentration and semen volume.
The collected semen underwent routine processing procedures (Nunes et al., 2008) using a Kenney semen extender with antibiotics (EZ-Mixin®) as described below. Semen was centrifuged at 1200 g for 10 min, and 75% of the supernatant was removed to optimize storage at +5°C. The pellet was diluted to obtain a final concentration of 500×10^6 sperm/mLin the Kenney semen extender. The samples were immediately refrigerated and brought to the laboratory within three hoursfor semen evaluation and storage at +5°C. Measurements of total and progressive motility were done 3h (T0), 24h

(T24), 48 h (T48), and 72 h (T72) after semen collection. At each time, an aliquot of every sample was pelleted and frozen at -20°C andprocessed within two months to evaluate semen lipid peroxidation. A second aliquot was pelleted and fixed in 4% paraformaldehyde overnight for immunohistochemically staining with PNA-FITC. After fixation, samples were washed, transferred into a 70% ethanol solution, stored at -20°C, and processed within two months.

4.2.6 Assessment of motility

Progressive motility was evaluated using a phase contrast microscope, witha heated stage at 37°C, at 100x magnification. Each sperm sample was pre-incubated at 37°C for ten minutesbefore assessment. The proportion of total and progressive motile spermatozoa from eight randomly selected fields in each sample was evaluated subjectively in a Makler chamber at 37°C.

4.2.7 Acrosome status

Acrosome integrity was evaluated by a fluorescent-labeled peanut agglutinin (PNA-FITC, Vector Laboratories, FL, USA; Tamuli and Watson, 1994), as shown in the Figure 4.1. An aliquot of the fixed sperm sample was spread on microscope slides and air-dried. The spermatozoa were permeabilized with methanol for 15 minutes at room temperature, washed once with 25mMTris-buffered saline (pH 7.6) for five minutes, and then washed twice with H_2O at five minutes intervals. The slides were air-dried, incubated with PNA-FITC (60 µg/mL) for 1 hour, washed twice with H_2O at five minutes intervals, and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). At least 100 cells per slide were evaluated. Cells with green staining over the acrosomal cap were considered acrosome intact (AI); cells with green staining in the equatorial region or no staining were considered acrosome reacted (AR) (Cocchia et al., 2011).
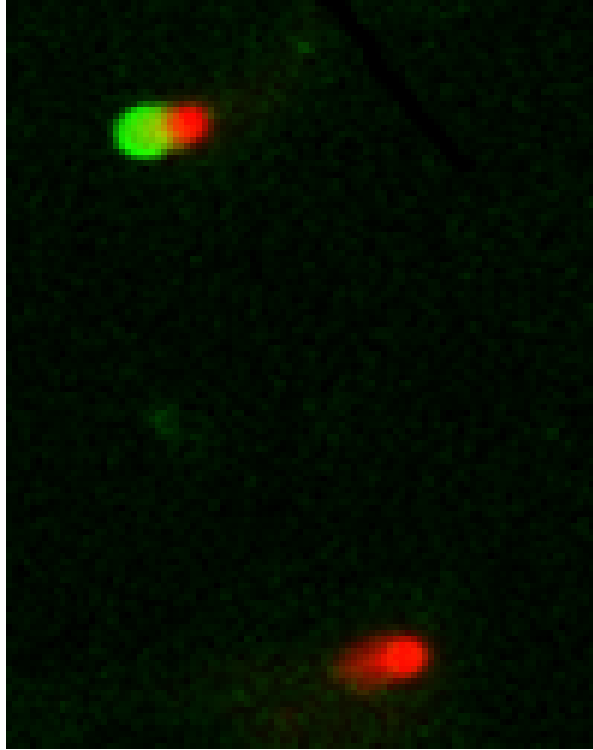
Figure 4.4 *Representative image of equine spermatozoa stained with FITC-PNA staining methods using confocal microscope at 400 x magnification. Spermatozoa with green fluorescent acrosome are classified as intact acrosome (AI); spermatozoa without this green fluorescent region are classified as acrosome reacted (AR).*

4.2.8 Lipid Peroxidation

Lipid peroxidation (LPO) was evaluated by a commercially available assay kit (LP SpermTest, Diacron International, Grosseto, Italy). The assay is based on the ability of peroxides to promote the oxidation of Fe^{2+} to Fe^{3+}. Fe^{3+}binds to thiocyanate, thereby developing a colored complex whose absorbance can be measured photometrically at 505 nm. The increase in absorbance was directly proportional to the concentration of peroxides in the sample. LPO was expressed as millimoles LPO/10^6spermatozoa. Briefly, an aliquot of a sperm pellet which had been stored at -80° C waswashed three times in distilled water, diluted 1:1 in PBS and then

vortexed for 5 min to homogenize the sample (Tafuri et al., 2015). The reagent 1 (1 mL) was transferred to a cuvette to which 3 drops of reagent 2 and the sample (40 µl) were added. The mixture was shaken after each addition and incubated for 5 min at 37°C. Then, the cuvette was positioned in the reading chamber for the absorbance measurement.

4.2.9 Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Data were recorded using a computerized spreadsheet (Microsoft®Excel® 2011) and imported into a statistical analysis software package (IBM® SPSS®StatisticsVersion 22.0, IBM Corporation, Armonk, New York). Normality was not tested due to the small sample size of both groups. Nonparametric tests were used for statistical analysis.

Semen volume, concentration and total sperm count (TSC) of raw semen were compared between groups by the Mann-Whitney testfor each day of the experiment. Total and progressive motility, acrosome integrity, and lipid peroxidation at the initial (T0) and the final (T72) storage time were compared between groups by the Mann-Whitney testat the different days during and after Maca supplementation (D0 to D120). In order to quantify the decrease of each parameter from T0 to T72, mean differences were calculated. These mean differenceswere compared between the two groups for each day of the experiment (D0 to D120) by the Mann-Whitney test and within each group by the Friedman ANOVA test. Significance was set at P≤0.05 and P<0.01.

4.3 Results

4.3.1 Serum testosterone levels

Results for serum testosterone concentration of each treated stallion throughout the experiment are reported in Figure 4.2. There were no significant changes in serum testosterone concentrations during the experimental period (mean±SD; D0 = 0.52± 0.29 ng/ml; D30= 0.49 ± 0.21ng/ml; D60= 0.60±0.23 ng/ml; D90=0.77±0.26 ng/ml; D120= 0.47± 0.34 ng/ml).
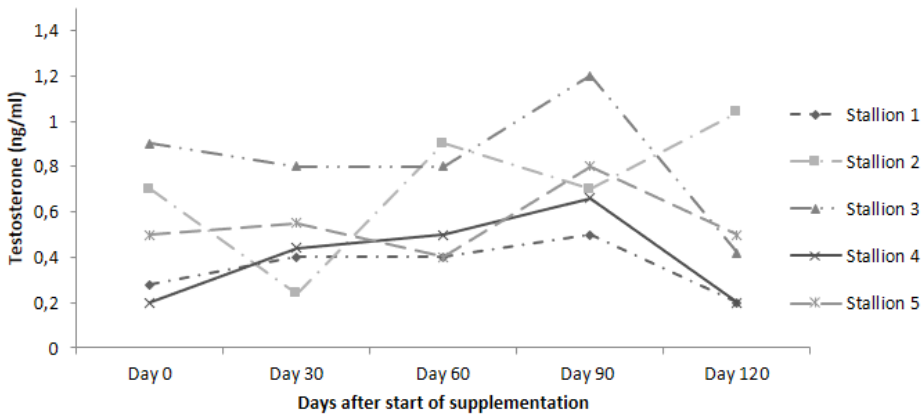
Figure 4.2 *Blood testosterone concentration in treated stallions during the experimental period, from D0 to D120.*

4.3.2 Quantitative parameters

The ejaculate volume was similar in both groups at the start of the experiment and did not change throughout the study (P>0.05; Figure 4.3A). Sperm concentration and TSC did not differ between both groups at the start of the experiment (D0; P>0.05; Figure 4.3B, C). Over time, the sperm concentration and TSC increased for stallions treated with Maca from 213± 80.4 x10^6spz/ml (T0) to 447± 73.1x10^6spz/ml (T120) and from 10880± 4377 x10^6spz (T0) to

24783± 4419 x10^{6}spz (T120), respectively. In the control group, sperm concentration and TSC did not change throughout the experiment (166.7 ± 15.3x10^{6}spz/ml (T0) vs 193.3± 51.3 x10^{6}spz/ml (T120) and 8300± 2914x10^{6}spz(T0) vs 9933± 4317x 10^{6}spz (T120); P>0.05). The sperm concentration and TSC were higherin the M group compared to the C group at 30 days (D90; 377± 62.3 vs. 180 ± 20x 10^{6}spz/ml and 20090± 3534 vs 9307± 3675 x 10^{6}spz; both P<0.05) and 60 days (D120;447 ± 73.1 x 10^{6}spz/ml vs193.3 ± 51.3x 10^{6}spz/ml and 24783± 4419 x 10^{6}spz vs 9933± 4317 x 10^{6}spz; both P<0.05) after the end of diet supplementation (Figure 4.3).
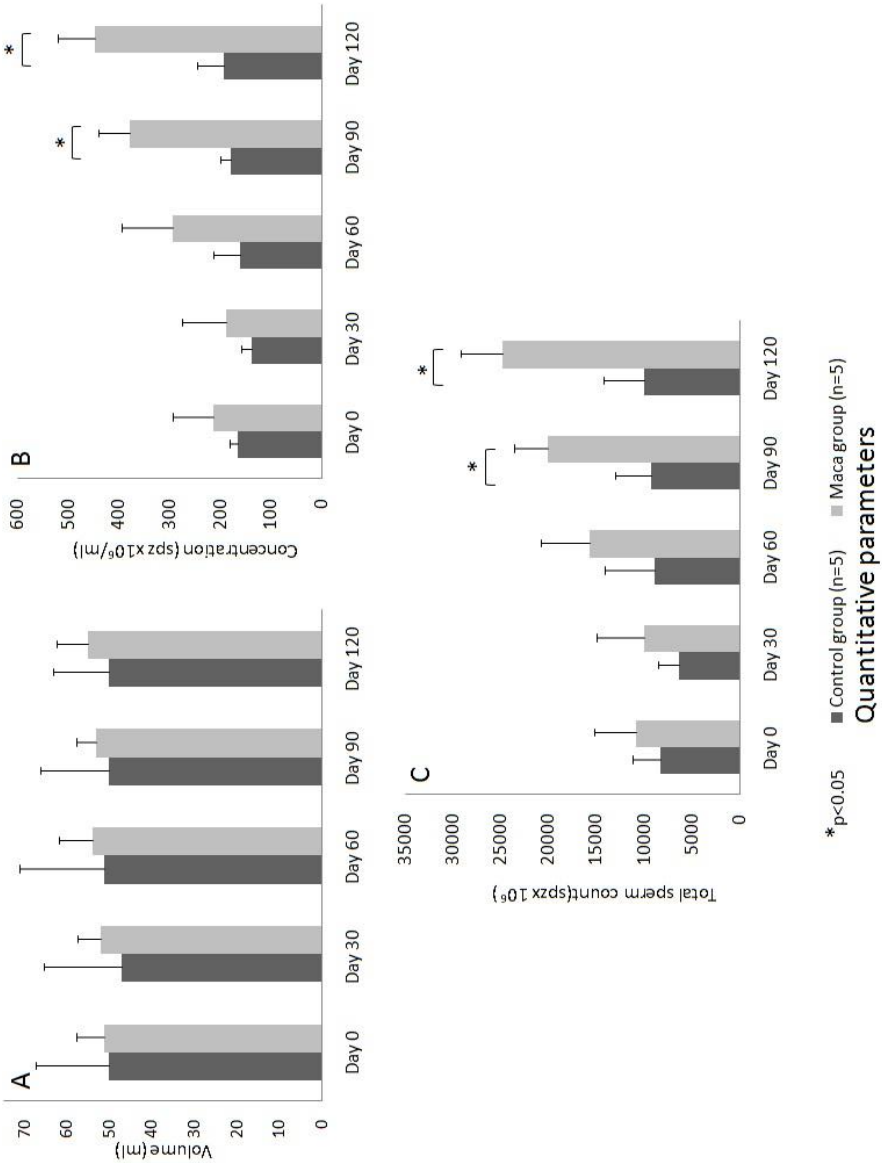
Figure 4.3 *Quantitative parameters of semen collected every 30 days during the experiment from D0 to D120.Ejaculate volume (A), sperm concentration (B) and total sperm count (TSC; C) in control group (n=5, dark bars) and Maca group (n=5; light bars; *p <0.05).*

4.3.3 Motility

Total and progressive motility of spermatozoa in fresh diluted semen (T0) collected at D0, D30, D60, and D90 di
d not show any significant difference between the C and M group (P>0.05). Total and progressive motility decreased significantly during cold storage in both groups (P<0.05). At T72 there was no difference between groups at D0, D30, and D60. As shown in Figure 4.4A and 4.4B, total and progressive motility were higher in group M thanin group C at D90 and D120 of the experiment (both P<0.05).

The mean differences (T72-T0) in total motility or progressive motility evaluated at the initial and final storage time did not differ between both groups at D0, D30, or D60, respectively. At D90 and D120, the mean differences for progressive motility or total motility significantly differed between the two groups (P<0.05; Figure 4.5A, B).

In the control group, the mean differences of total and progressive motility between storage times(T72-T0) did not change throughout the experimental period, while in the M group these differences were getting significantly lower throughoutthe experiment (P<0.05). This shows that the decrease oftotal and progressive motility during cooled storage (i.e., from T0 to T72) was lower in semen of the treated group as early as 30 days (D90) after the end of Maca administration.

4.3.4 Acrosome integrity

The percentage of spermatozoa with intact acrosomes did not differ between groups in fresh diluted semen (T0) at any day of the study (D0 to D120). Storage of the stallion semen resulted in a significant decrease of acrosome integrity in both groups (P<0.01). At T72 there were no significant differences at D0 and D30. The percentage of acrosome intact spermatozoa was significantly higher in the M group than in the C group at D60, D90, and D120 (P<0.01; Figure 4.4C).

As shown in Figure 4.5C, the average decrease in acrosome integrity at the beginning and end point of storage (T0-T72) did not differ between both groups at D0 and D30. From D60 until the end of the experiment, the average decreasewas lower in M group (P<0.01).
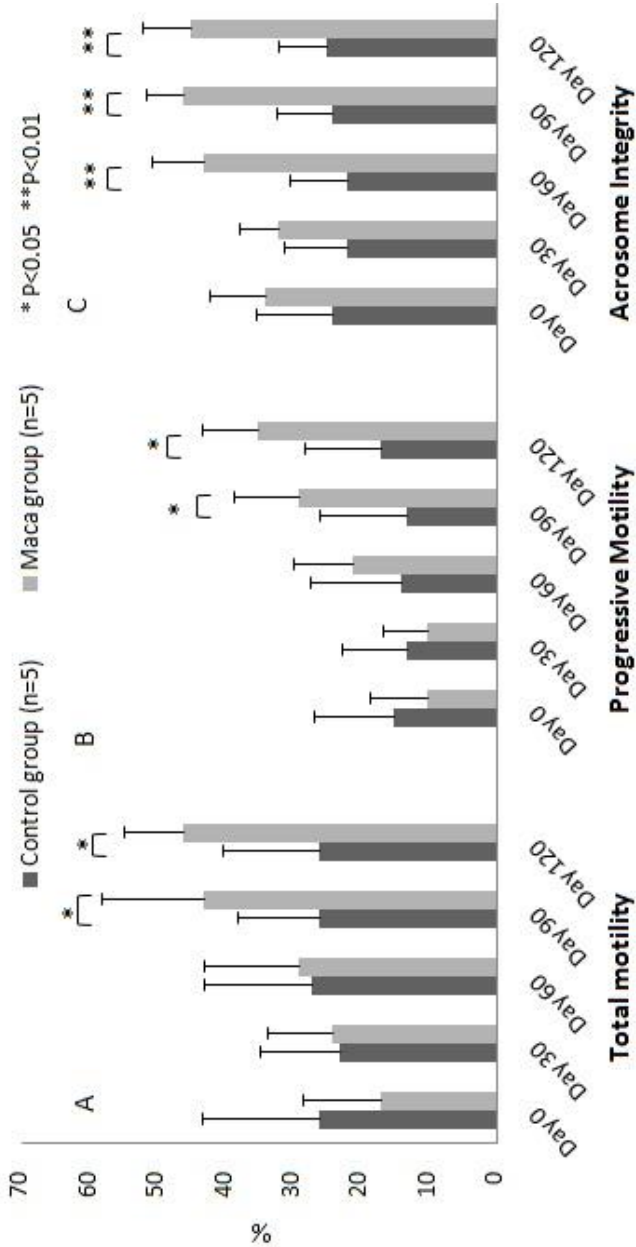
Figure 4.4 *Semen quality parameters in fresh diluted semen: total (A) and progressive motility (B) and acrosome integrity (C) at 72 hours of storage at 5° C in control group (C) and Maca-treated (M) group at different days of the experiment (D0-D30-D60-D90-D120). (*: P<0.05; **: P<0.01)*
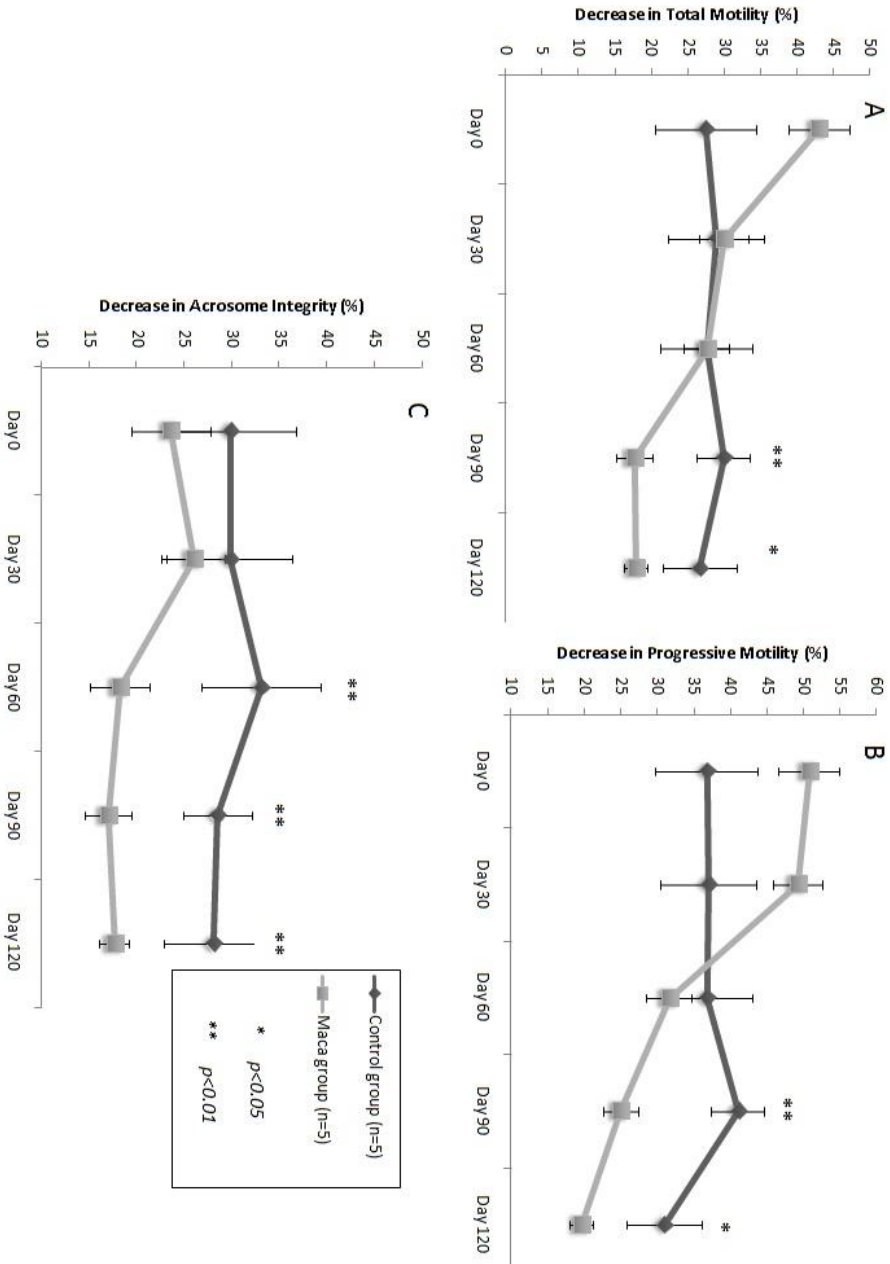
Figure 4.5 Mean difference(T0-T72) in semen quality parameters for both groups (C and M) during cooled-storage time at each collection day: total (A) and progressive (B) motility and acrosome integrity (C; *: P<0.05; **: P<0.01)

4.3.5 Lipid peroxidation

As can be seen in Table 4.1, results relating to lipid peroxidation of spermatozoa did not show a consistent trend in either group during the Maca supplementation (from D0 to D120) or during cooling time (from T0 to T72). Comparison of these data between the groups (C, M) did not reveal any statistically significant results in either collection day or storage time.

*Table 4.1. Lipid peroxidation was evaluated within three hours(T0) after collection and after 72 hours (T72) of cooled-storage in the control group and Maca-treated group throughout the experimental period. There were no differences between groups and no difference within each group between storage times or collection days.*

LIPID PEROXIDATION (Mm LPO/10^6)	Fresh semen (T0)					72 hours of refrigeration (T72)				
Day after feed supplementation	Day 0	Day 30	Day 60	Day 90	Day 120	Day 0	Day 30	Day 60	Day 90	Day 120
Control group (n=5)	382 ±99.3	247 ±74.9	427 ±100.9	374 ±79.5	254 ±85.6	382 ±89.6	308 ±98.7	448 ±110.9	413 ±110.7	444 ±118.5
Maca group (n=5)	368 ± 78.2	311±110.1	403 ±79.6	374 ±84.8	247 ±98.8	277 ±99.5	442 ±98.6	467 ±83.6	293 ±75.5	501 ±112.1

4.4 Discussion

This study provides the first results on a positive effect of diet supplementation with yellow Maca powder on stallion semen quantity and quality. Beneficial effects on raw semen and subsequently chilled and diluted semen started as early as supplementation lasted for one full spermatogenic cycle.

4.4.1 Sperm quantitative parameters and Maca's androgenic activity

During the experimental period, ejaculate volume was constant. At the same time, the concentration of spermatozoa and total sperm count in the raw semen was increased from the last date of treatment with Maca (D60) until the end of the experiment (D120). Total sperm output was approximately two-times higher at the end of the experiment than at the beginning. A similar increase in total sperm count was reported in adult rats (Gonzales et al., 2004; Gonzales et al. 2006), peripubertal bulls (Clément et al., 2010; 2012), and adult men (Gonzales et al., 2001). Compared to studies in animals, studies involving men also noted increased ejaculate volume (Gonzales et al. 2001; Melnikovova et al., 2015). This may be partially explained by an observed increase in sexual desire for men (Gonzalez et al. 2002) which was not observed in bulls (Clément et al. 2010). Previous studies reported that yellow and black Maca enhance sperm count and sperm motility (Gonzales et al., 2013a, b) and spermatogenesis (Clément et al., 2010; 2012) without influencing hormone levels (Balick and Lee, 2002; Melnikovova et al., 2015). However, recently Ohta et al. (2016) found that Leydig cells in male rats increasedin steroidogenic ability after treatment with a hydroalcoholic extract of different ecotypes of Maca. The mechanism of action of Maca and its different ecotypes is not clear yet, but some actions are related to Maca's lipidic fraction, which contains fatty acids and macamides (Melnikovova et al., 2015). In order to clarify whether Maca increases sperm production by enhancing testosterone levels, serum concentrations of testosterone were measured in treated stallion. In agreement with previous findings in humans and in mice (Gonzales et al., 2002, 2003b; Zenico et al., 2009; Leme et al., 2012), dietary supplementation with Maca did not alter blood testosterone concentration in stallions. In contrast to the latter results, *in vivo* studies in rats report increased levels of testosterone after dietary supplementation with

hydroalcoholic extract powder of Maca (Ohta et al., 2016; Yoshida et al., 2017).

Maca hypocotyls are present in nature in different colors or phenotypes. Several studies show the differences in biological response to the three major ecotypes of *Lepidium meyenii* (yellow, red, and black Maca). Published reports suggest that different phenotypes are associate with different chemical composition of the hypocotyls. At the same time, different ecotypes are associated with different biological effects and medical target. For instance, red Maca is the variety that reverses benign prostatic hyperplasia in mice and experimentally induced osteoporosis (Gonzales et al., 2014) and is reported to be useful in stimulatingsperm count (Gonzales et al., 2006). Black and yellow Maca show the best results on spermatogenesis, memory and fatigue, and increasememoryand learning in mice (Rubio et al., 2007). In addition, black Maca reduces glucose levels, and its consumption is related to the lowering of blood pressure and an improved health score (Gonzales et al., 2013b). The Maca used in the present study was a powder from the yellow Maca ecotype.

Studies investigating the *in vitro* and *in vivo* effect of Maca on different cultured cells, e.g. macrophages, hepatocytes and neurons, have confirmed its cytoprotective effect (Sandoval et al., 2005; Valentova et al., 2006; Pino-Figueroa et al., 2010; 2011). Sandoval et al. (2005) investigate the antioxidant activity of Maca. It has been shown that Maca has the ability to scavenge free radicals and provides cytoprotection during oxidativestress conditions (Sandoval et al., 2005). Recent investigations showed that methanol extracts of Maca display an antioxidative effect on neurons, thereby increasing cell viability (Rodríguez-Huamán et al.,2017). The proposed mechanism to explain this protective role is that Maca enhances the oxidation-reduction balance of enzyme mechanism by increasing the activity of the enzyme superoxide dismutase (Rodríguez-Huamán et al.,2017). Previous study by Večeřa et al. (2007) demonstrated the positive effect of Maca on systemic antioxidant status, improving the activity of enzymatic ROS scavengers (superoxide dismutase, glutathione peroxidase, and glutathione). An improvement in systemic antioxidant capacity after Maca supplementation may explain the beneficial effect on the quality of fresh semen. Further evaluation of systemic antioxidant capcity after stallion dietary supplementation with Maca may confirm this potential effect. It will be also interesting to investigate the antioxidant status of seminal plasma after Maca supplementation, because an improved antioxidant capacity of

the seminal plasma may explain the improved preservation of semen quality during cooling semen storage.

4.4.2 Effect of Maca on stored semen: possible mechanisms and perspectives for frozen semen

The benefit of Maca is directly obvious as it results in an increased amount of total motile sperm for the production of insemination doses. The cost for production and importation of Maca may be offset by a more efficientuse of stallions. Moreover, in semen from stallions that had been fed for 60 days with Maca, the reduction in total and progressive motility and in spermatozoa with acrosome integrity, was lower during cooled storageat 5°C. Similar effects on the preservation of stallion semen quality have been noted in studies with a diet supplementation of poly-unsaturated fatty acids (PUFA) from fish oil (Brinsko et al. 2005). Indeed, macamides and macaenes in Maca powder represent a diverse group of saturated and poly-unsaturated fatty acids (Wu et al., 2013; Wang et al., 2007). Spermatozoa require a high PUFA content in order to provide the plasma membrane with the fluidity necessary for activation of the signal transduction pathways that are required for the fertilization process (Wathes et al., 2007). Oxidative stress-induced changes in the PUFA composition of sperm, especially in the n-6 and n-3 ratiocause changes in the membrane architecture along with altering its permeability and the fluidity of its functions. (Cross, 2003; Maldjianet al., 2005). Diet supplementation with PUFA added to Vitamin C or E, as antioxidants in the food, resulted in an increase in sperm quality in rabbits (Castellini et al., 2004), chickens (Surai, 2000), cockerels (Cerolini et al., 2005), boars (Liu et al., 2015) Japanese quail (Al-Darajietal., 2010); rams (Alizadeh et al., 2014; Jafaroghli et al., 2014); goats (Dolatpanah et al., 2008), and bulls (Kaka et al., 2015). PUFA administration, with no added antioxidant to preserve food from oxidation, resulted in contrasting results (de Graaf et al., 2007; Grady et al., 2009; Fair et al., 2014), probably due to peroxidation of the food's lipid content (Cerolini et al., 2006).

In the present study, the refrigeration and storage of the semen in the control and Maca group caused a progressive and significant reduction in semen quality. Refrigeration is associated with damage to sperm function due to various factors, including excessive ROS production (Wang et al., 1997; Ball, 2008; Thomson et al., 2009) and/or alteration of the antioxidant defense systems in semen. The imbalance between oxidant and antioxidant

systems, can induce changes in the membrane structure and consequently changes in its fluidity (Martinez-Soto et al., 2013; Tafuri et al., 2015).

The lipidomic composition of the sperm plasma membrane can influence its fluidity. It has been demonstrated that PUFA feeding modifies membrane composition of sperm cells (Moallem et al., 2015). Martinez-Soto et al., (2013) reported that the level of saturated fatty acids in stallion spermatozoa were correlated with the incidence of membrane-damaged spermatozoa. In contrast, ahigh percentage of unsaturated fatty acids was positively correlated with the percentage of sperm with intact membranes after freezing and thawing. In our study, dietary supplementation of Maca alleviated the decrease of sperm quality caused by refrigeration of stallion sperm, especially with respect to sperm with intact acrosomes. Maca, cointaining macamides and mecaenes, is made up of PUFA. Thereby, it may improve the resistance of sperm to sperm cold shock (Giraud et al., 2000; Clément, 2012).

Another initial hypothesis was that Maca supplementation might increase the intrinsic resistance of the ejaculated spermatozoa to lipid peroxidation. This hypothesis was rejected because no significant difference in lipid peroxidation was detected between both experimental groups at any time of the experiment. No increase in lipid peroxidation during equine semen storage corresponds with previous findings showing that equine spermatozoa appear relatively more resistant to membrane peroxidation than sperm of other domestic animals during storage at 5°C (Baumber et al., 2000; Neild et al., 2005; Kankofer et al., 2005). According these authors, lipid peroxidation did not increase during cold storage of equine semen despite a decrease in sperm motility and acrosome integrity.

We cannot exclude that the method used for detecting lipid peroxidation in our study was not sensitive enough. Alternatively, lipid peroxidation and consequently oxidative stress levels in equine semen canbe investigated by using lipid peroxidation end-products such as malondialdehyde (Stradaioli et al., 2001; Ball et al., 2001) which can be determined by measuring the amount of thiobarbituric acid reactive species (TBARS). Another option would be the labeling of sperm with the fluorescent probe C11-BODIPY for detecting lipid peroxidation. Such lipid peroxidation has been related to apoptotic-like changes of stallion sperm (Ball and Vo, 2002; Neild et al., 2005, Ferrasuola et al., 2009).

The findings from our study may further suggest that molecules other than lipids are major targets of ROS in chilled and stored stallion semen. Oxidative stress damages cell membranes, and also breaks down DNA. ROS

are the primary cause of DNA damage in sperm (Aitken and De Iuliis, 2007). Wnuket al. (2010) found a negative correlation between total antioxidant capacity of stallion and sperm DNA damage, postulating that the redox status of seminal plasma may be an additional important parameter for evaluation of equine semen quality. Furthermore, after ROS exposure, a dose-dependent increase in sperm DNA damage was seenin equine sperm (Baumber et al., 2003). In order to understand the beneficial effect of Maca, further analysis to evaluate apoptosis-related parameters and DNA strand breaks should be performed. Currently, major tests of sperm DNA fragmentation are the Comet assay (single-cell gel electrophoresis), the terminal deoxynucleotidyl transferase-mediated dUDP nick end-labeling (TUNEL) assay and the SCSA (Sperm chromatin structure assay) (Colebrander et al., 2003; López-Fernándezet al., 2007; Varner et al., 2008). Other potential targets for ROS in sperm can be mitochondria or proteins involvedin the acrosome reactions. The detection of activated apoptosis marker such as caspase 3, 7 and 9 by flow cytometer analysis or Western blotting may help to provide more information on potential ROS actions and protective mechanisms of Maca on the mitochondrial level (Ortega-Ferrusola et al., 2008; Brum et al., 2008). The preservation of acrosome integrity maybe associated with the ability of Maca to alleviate the effect of ROS on phosphorylation of proteins involved in the acrosome reaction mechanism (Cocchia et al., 2011). Irrespective of the underlying mechanism, our findings support the possibility for using Maca as a food supplement for stallions in order to produce semen in higher quantity and of better quality. Future research will determine whether similar positive effect on semen quality can be obtained when spermatozoa are cryopreserved.

4.4 Conclusion

Despite the limited number of animals in the present study, we showed, for the first time, that Maca diet supplementation in stallions improves semen production, resulting in more artificial insemination (AI) doses per ejaculate. Furthermore, the semen from Maca-treated stallions showed a better resistance to cooling and storage, thus preserving acrosome integrity and total and progressive sperm motility. The underlying mechanisms that promote the effect of *Lepidium meyenii* (Maca) on stallion semen is still unclear. Further research is necessary to verify and quantify Maca's oral absorption in horses, elucidate the mechanisms of *Lepidium meyenii* on semen production, and to explore better the Maca's potential effect on the systemic antioxidant capacity in horses.

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Chapter 5

Combined addition of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPX) to a cooling extender for equine semen
Submitted for publication

5.1 Introduction

Since the late 1980s, artificial insemination with cooled semen has become the major breeding tool in equine reproduction (Aurich and Aurich, 2006). During cooled storage or transport, viability, motility and fertilizing capacity of semen is progressively declining, especially after more than 24 hours storage (Jasko et al., 1992). The mechanism underlying this decline in semen quality is related to a dramatical increase in reactive oxygen species (ROS) production by spermatozoa (Ball et al., 2001; Johannisson et al., 2014). High concentrations of ROS create a situation called oxidative stress, a condition associated with an increased rate of cellular damage (Sikka et al., 1995).

Oxidative stress in sperm cells is associated with lipid peroxidation, loss in motility and viability and DNA fragmentation (reviewed by Ball, 2008). Furthermore, oxidative stress is the primary cause of DNA damage in spermatozoa (Aitken and De Iuliis, 2007) in form of single- and double-strand breaks (Duru et al., 2000). Sperm DNA damage is correlated with decreased fertilization rates, poor embryo cleavage and slow embryo and fetal development (Sakkas and Alvarez, 2010). In equine spermatozoa, the increase in DNA damage has been found to be dependent on ROS concentration (Baumber et al., 2003a).

Although mammalian sperm cells are very sensitive to peroxidative damage, due to their high concentration of polyunsaturated fatty acid (PUFA) in the plasma membrane (Aitken, 1995), equine spermatozoa seem to be more resistant to lipid peroxidation (Baumber et al., 2000; Neild et al., 2005). Lipid peroxidation does not increase during the first 24 hours of refrigeration at 5°C (Kankofer et al., 2005). However, an increase in lipid peroxidation has been detected after more 24 hours of storage at 5°C (Ball and Vo, 2002). Motility of equine semen is the first parameter to be modified by oxidative damage (Lenzi et al., 1993; Agarwal et al., 1994; Armstrong et al., 1999, Baumber et al., 2000) and may be used as sensitive indicator of oxidative stress (OS; Ball, 2008). A loss in motility is related to ROS-mediated lipid peroxidation which decreases metabolic activity of sperm cells due to permeation of enzymes and ATP to the extracellular environment (Storey, 1997). Such an ATP depletion decreases the available energy from the mitochondria. It provokes a decreased axonemal protein phosphorylation and consequently sperm immobilization (de Lamirande and Gagnon, 1992). ROS have also a direct effect on mitochondria by disrupting inner and outer membranes, and inducing the release of apoptosis inducing factors (AIF).

The AIF released are cytocrome-C protein and proteases, e.g. caspases 3 and 9, that directly interact with the DNA and lead to DNA fragmentation and apoptosis (Candé et al., 2002; Paasch et al., 2004).

Despite their destructive potential at high concentrations, low concentrations of ROS are involved in many physiological sperm functions such as capacitation-related increases in tyrosine phosphorylation (Baumber et al., 2003b). In order to avoid oxidative stress-induced effects on spermatozoa during cooled storage, i.e. premature capacitation, motility loss and DNA damage, ROS concentration in diluted semen should be keep low. Equine spermatozoa and seminal plasma are endowed with enzymatic and non-enzymatic antioxidants to maintain the levels of ROS low. The major enzymatic ROS scavengers are the superoxide dismutase (SOD; Mruk et al., 2002), catalase (CAT; Ball et al., 2000) and glutathione peroxidase (GPX; Brigelius-Flohe R, 1999). The activity of those antioxidants in equine seminal plasma were 98.7±29.2 U/mg protein for catalase (Ball et a., 2000), 29.15±6.64 U/mg protein for SOD and 0.87±0.06 µM NADPH oxidized/min/mg protein for GPX, respectively (Baumber and Ball, 2005). The antioxidant systems in seminal plasma seem to protect spermatozoa against ROS until 24 hours of storage at cooling temperature (Kankofer et al., 2005). Attempts were made to increase the capacity of the intrinsic antioxidant defense mechanisms by individual addition of catalase, reduced glutathione (GSH), or SOD to semen extenders (Baumber et al. 2000, 2003b, Cocchia et al., 2011). Baumber et al. (2003b) demonstrated that only the addition of catalase (150 IU/mL) or reduced glutathione (GSH; 1.5 mM), but not SOD (150 IU/mL), reduced DNA fragmentation and a decline in motility secondary to an induced oxidative stress. However, addition of lower concentrations (25-50 UI/ml) of SOD to a semen extender preserved not only acrosome integrity but also sperm vitality and motility during cold storage (Cocchia et al., 2011).

In the physiological situation equine spermatozoa and seminal plasma contain all three antioxidant systems at the same time. Therefore, the aim of the present study was to investigate the effect of a combination of SOD, CAT and GPX added to a milk-based semen extender on the quality of cooled-stored semen for 72 hours.

5.2 Material and Methods

5.2.1 Semen collection and processing

Two ejaculates from each of seven stallions (2-15 years old) were included in the study. Stallions were on a regular semen collection schedule (3 times/week) and ejaculates contained a minimum of 50% motile spermatozoa. Semen collection was performed with a pre-warmed, lubricated Missouri type artificial vagina with an in-line nylon filter (Minitube, Germany) to eliminate the gel fraction. Immediately after collection, the semen was filtered through a semen filter pouch (Minitube, Germany) and the ejaculate volume was recorded. The sperm concentration was measured with a Burker hemocytometer chamber. Each ejaculate was split in two aliquots of 8 mL each and diluted with semen extender (INRA96, IMV technologies, Italy) without (Control=C) or with the addition of antioxidants (Treated=T) to obtain a final concentration of 50×10^6 spermatozoa/mL and sent to the laboratory within three hours. The final concentration of antioxidant which were added to the diluted semen were15 IU/ml of superoxide dismutase (SOD from bovine erythrocytes),15 IU/ml of catalase (CAT from bovine liver) and 15 IU/ml of glutathione peroxidase (GPX from bovine erythrocytes), respectively. All antioxidant preparations were purchased from Sigma–Aldrich, Germany. Both aliquots were placed in a syringe without air and stored at 5 °C for 72 hours. At intervals, subsamples were removed from the syringes for analysis.

5.2.3 Semen evaluation

Semen evaluation was performed within 3 hours after semen collection (T0) and every 24 hours during cooled storage (T24, T48, T72). At each time point, viability and motility were immediately evaluated. In addition,500 µL aliquots of each control and treated subsample were taken and washed twice with 4.5 ml of PBS solution (600g, 10 minutes) in 15 mL Falcon centrifugation tubes. The supernatants were discarded, and the pellets were dissolved in 300 µL PBS. Subsequently, the dissolved pellet was split and placed in three Eppendorf cups (100µL/Eppendorf). One pellet was fixed in 2%paraformaldehyde in 0.1 M PBS (pH 7.6) for 15 min at RT. Then, the cells were washed in PBS and maintained in 5 ml of 70% methanol at -20°C. The other two pellets were frozen at -80°C until processing for SCSA

(Sperm chromatin structure assay) and for Western blotting of cleaved caspase-3.

5.2.3.1 Viability

Sperm viability was evaluated at each time point by eosine staining (Viability stain-Europath, Naples, Italy). A diluted sperm sample (5 μl) was mixed with the Eosine stain (10μl) at 37°C, incubated for 30s, smeared on a slide and dried on a warm plate at 37°C. Slides were evaluated using bright field microscopy at 400x magnification. Live spermatozoa remained unstained, whereas the dead spermatozoa were dull pink. At least 200 sperm cells were scored for each sample. The percentage of live, i.e. eosin negative, spermatozoa was expressed as viability (%).

5.2.3.2 Motility evaluation with a computer-assisted sperm analysis (CASA) system

Motility was assessed immediately after collection (T0) and at each time point during refrigeration at 5°C (T24, T48, T72). Semen (100 µL) was incubated with 600 µL of PBS for10 minutes at 37°C and 10µL were placed in a pre-warmed Makler chamber with a depth of 20 µm. Samples were analyzed by SCA (Sperm Class Analyzer by Microptic S:L, Barcelona, Spain) at 100x magnification. At least five representative fields were evaluated at a frame rate of 25 Hz. Total and progressive motility (%) were measured according to the manufacturer's instructions. Furthermore, rapid (VCL ≥ 90 µm/s), medium (90 µm/s > VCL≥ 45 µm/s), slow (45µm/s>VCL ≥ 25 µm/s), and static (VCL < 10) spermatozoa were classified. Kinematic parameters such as VCL (curvilinear velocity; µm/s), VSL (straight-line velocity; µm/s), VAP (average path velocity; µm/s), LIN (linearity; VSL/VCL, %), STR (straightness; VSL/VAP, %), WOB (wobble; VAP/VCL, %), ALH (amplitude of lateral head displacement; µm), and BCF (beat cross frequency; Hz) were evaluated.

5.2.3.3 Assessment of sperm DNA fragmentation

Sperm DNA damage was evaluated with two different methods. The APO-BrdUterminal deoxynucleotidyl transferase-mediated dUTP-nick end-labelling (TUNEL) assay was used to detect single- and double-strand

breaks in the DNA, while the sperm chromatin structure assay (SCSA) was performed to detect single-strand breaks.

> *-TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-nick end-labelling) assay*

For recognition of DNA fragmentation in apoptotic cells, an APO-BrdU™ TUNEL Assay Kitwith Alexa Fluor 488®anti-brdU (Invitrogen, Molecular Probes) was used. This kit allows detection of30-hydroxyl ends that serve as starting points for TdT, which adds deoxyribonucleotides in a template-independent fashion. Addition of the deoxythymidine analogue BrdUTP to the TdT reaction labels the break sites. Once incorporated into theDNA, BrdUTP is detected by an anti-BrdUTP antibody, which is conjugated to Alexa Fluor 488 (excitation: 495 nm, emission:519 nm). The DNA is visualized by propidium iodide staining (PI; excitation: 535 nm, emission: 617 nm). The spermatozoa were washed twice in a wash buffer and resuspended in the DNA-labelling solution for 60 min at 37 °Cin a water bath. The suspensions were washed twice in rinse buffer, and the Alexa Fluor 488-labelled anti-BrdU antibody was added to each suspension. The samples were incubated for30 min at RT in the dark. Finally, 0.5 ml of PI RNase staining buffer were added. After 30 minutes additional incubation in the dark at RT, the sperm suspension was placed on three microscope slides and mounted with Vectashield (Vector Laboratories). At least 100 sperm cells were scored per slide under a Nikon 90 upright fluorescence microscope equipped with a mercury lamp (100 W) using a 488 nm excitation filter and a 530 long-pass filter. The heads of all sperm cells were stained red by PI and only spermatozoa with fragmented DNA were stained also in green, as shown in the Figure 5.1. The mean of the results for the three slides was considered to be the final value of TUNEL positive spermatozoa.
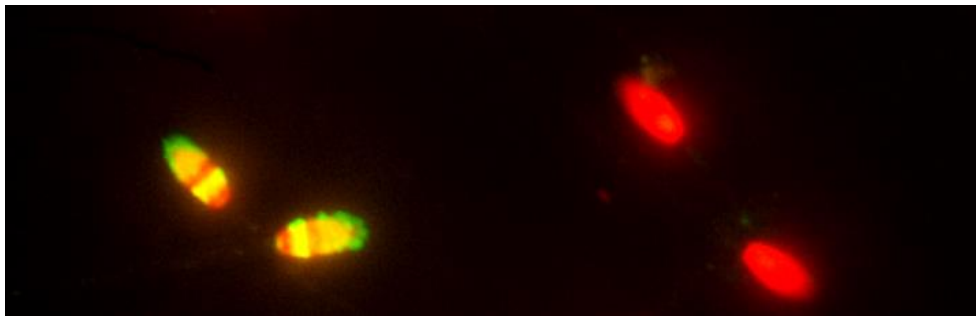
Figure 5.5 *Representative image of equine spermatozoa stained with APO-BrdU™ TUNEL Assay Kit with anti-brdU Alexa Fluor 488® using fluorescence microscope at 400x magnification. Propidium iodide (PI) stains the nucleus of all sperm cells red and anti-brdU Alexa Fluor 488® the spermatozoa with fragmented DNA in green.*

-SCSA (Sperm chromatin structure assay)

The SCSA is a flow cytometric technique to detect spermatozoa with abnormal chromatin structure. The assay evaluates the susceptibility of sperm DNA to undergo acid-induced DNA denaturation *in situ*, by staining with the fluorescent DNA-bindig dye acridine orange (AO). The metachromatic shift of AO from green (stable, double-stranded DNA) to red (denatured, single-stranded DNA) is detected by flow cytometry (Evenson et al. 1980). The protocol developed and described in detail by Evenson et al. (1980; 2013) was followed. Briefly, the samples were removed from the freezer (-80°C) and thawed with a water bath set at 37°C. Each pellet was diluted with Tris-NaCl-ethylenediaminetetra-acetic acid (TNE) buffer (0.15 m NaCL, 0.01 m Tris-HCL, 1 mm ethylenediaminetetra-acetic acid (EDTA), pH 7.4) to achieve a final sperm concentration of 1 to 2×10^6 cells/ml. In order to obtain a partial DNA denaturation, we added 400μlacid solution, prepared with a combination of 20.0 ml 2.0 N HCl (0.08 N), 4.39 g NaCl (0.15 M), and 0.5 ml Triton X-100 (0.1%) in H_2O, with a low pH (~1.2).After exactly 30 seconds,1.2 ml of AO solution (6 μg/mL in 0.1 mol/L citric acid, 0.2 M/L Na_2HPO_4, 1 mM/L EDTA and 0.15 M/L NaCl, pH 6.0)were added.The stained samples were processed after exactly 3 minutes on the flow cytometer (FACSCalibur™, BD Biosciences, San Jose, CA).For each sample 10,000 events (200-300 cells/sec) were analyzed. Acridine orange was excited with an argon ion laser at 488 nm (200 mW). As shown in the Figure 5.2, spermatozoa with intact DNA (double-stranded)

emit green fluorescence (530± 30 nm BP, FL 1 detector), but in the presence of fragmented DNA (single-stranded) emit an increasing intensity of red fluorescence (630 nm LP, FL 3 detector). The DNA fragmentation index (DFI; formerly: alpha t) was calculated by the ratio between red fluorescence intensity to total sperm fluorescence intensity (i.e. red and green). The DFI is used to discriminate spermatozoa with mainly single-stranded or increased amounts of double-stranded DNA (Evenson et al. 2002). The percentage of spermatozoa with an increased amount of singled stranded DNA is expressed as %DFI (formerly: comp alpha t). A reference sample was run every six samples to control machine performance.
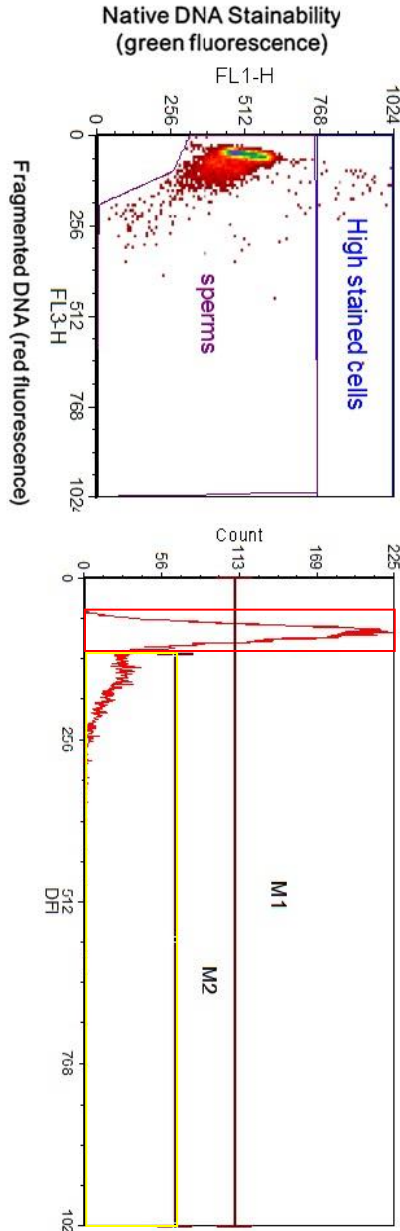
Figure 5.6 *Left: Representative dot plot from SCSA analysis of stallion semen showing each of 10000 spermatozoa as a dot. Y axis represents green fluorescence (FL1-H) intensity and X axis represents the red fluorescence (FL3-H) intensity of acridine orange. Right: Representative histogram of SCSA analysis of data from left scattergram showing the peak of normal population of spermatozoa (red box) and the sperm cells with fragmented DNA (yellow box). The ratio between the spermatozoa with fragmented DNA red and the total sperm count is reported as the %DFI.*

5.2.3.4 Assessment of the apoptosis marker cleaved caspase 3 by Western blotting

Activation of the apoptosis pathway was detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot analysis of activated (cleaved) caspase 3. Sperm pellets were resuspended in a lysis buffer (50 mMTris pH 7.5, 150 mMNaCl, 1 mM EDTA, 1mM EGTA, 0.25% deoxycholic acid, 1% Triton X-100, 2 mMphenylmethylsulfonyl fluoride, 0.1 mg/ml aprotinin, 10 mM sodium orthovanadate, 20 mM sodium pyrophosphate and 50mMsodium fluoride) and then homogenized using the TissueLyser LT (Qiagen). Equal amounts of lysate samples were boiled and loaded on bis/acrylamide gels, separated by electrophoresis and proteins were blotted from the gel onto PVDF membranes. After determining the protein concentration with Bio-Rad protein assay, based on the Bradford dye-binding method (Bio-Rad laboratories). Equal amounts of lysate samples were boiled and loaded on acrylamide gradient gel (Mini-PROTEAN® TGX™ Precast Gel, Biorad) and electrophoresis was carried out. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. The membrane blocked with 5% w/v non fat dry milk in Tris-buffered saline (TBS: 12.5 mM Tris-HCl pH 7.4; 125 mM NaCl) at RT for one hour, washed with TBS-0.1% Tween and incubated overnight with a primary antibody at 4°C. The primary antibody was rabbit anti-cleaved caspase 3 (Cell Signaling Technologies catalog no. 9662; 1:1000 dilution). Membranes were incubated at least for one hour with a secondary anti-rabbit IgG antibody (GE Healtcare, UK, catalog no. NA934; 1:1000 dilution). The blots were stripped and reprobed against rabbit anti-actin antibody (Calbiochem, San Diego, CA; catalog no. CP10) at 1:2000 dilution to confirm equal loading of proteins in each lane. Protein expression levels were quantitatively estimated by densitometry using a Gel Doc scanner (BioRad) equipped with a densitometric work station. The protein concentrations were normalized to the actin level and expressed as relative band density (arbitrary units). Values obtained for samples immediately after collection (T0) were considered 100%.

5.2.4 Statistical Analysis

Statistical analysis was performed using a commercial software for statistical analysis (IBM® SPSS®StatisticsVersion 22.0, IBM Corporation, Armonk, New York). For each semen parameter the average value of both

ejaculates per stallion was calculated (n=7). All data are expressed as mean±SD. Normality of the distribution for all data was tested with Shapiro-Wilk test. Data were not normal distributed and non parametric tests were used. The effect of storage time on sperm analysis data was evaluated in each group (C and T) by Friedman test. If significant, analysis was followed by Wilcoxon signed rank test as post hoc test. Differences between C and T samples at each time point were analyzed using Wilcoxon signed rank test. The significance level was set at $p<0.05$.

5.3 Results

5.3.1 Viability

The viability of sperm cells in control samples and treated semen is illustrated in Figure 5.3. The percentage of live cells was statistically decreasing with storage time in both groups (p<0.01). Viability between control and treated semen differed only at 72 hours of storage (p<0.05).
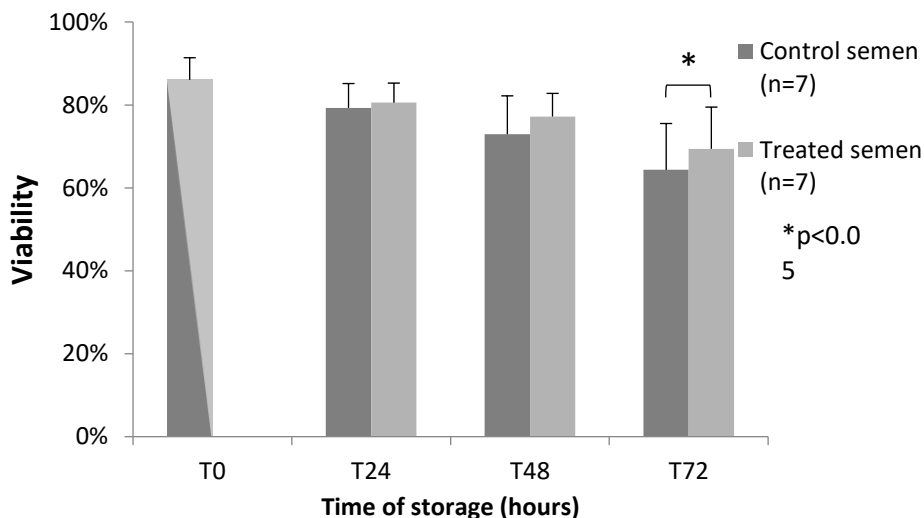

Figure 5.7 *Percentage of live sperm cells (% viability) in control and treated samples at each time point; * significant difference between control samples and treated semen (p<0.05).*

5.3.2 Assessment of motility

Total and progressive motility of control samples and treated semen for each time point are reported in Figure 5.4. As expected, total and progressive motility significantly decreased during cold storage (p<0.01). Total motility was higher in treated samples than in control samples at 48 hours and 72 hours of storage (p<0.05). Furthermore, at 48 hours of cooling, progressive motility tended (p=0.063) to be higher in treated semen then in control semen.
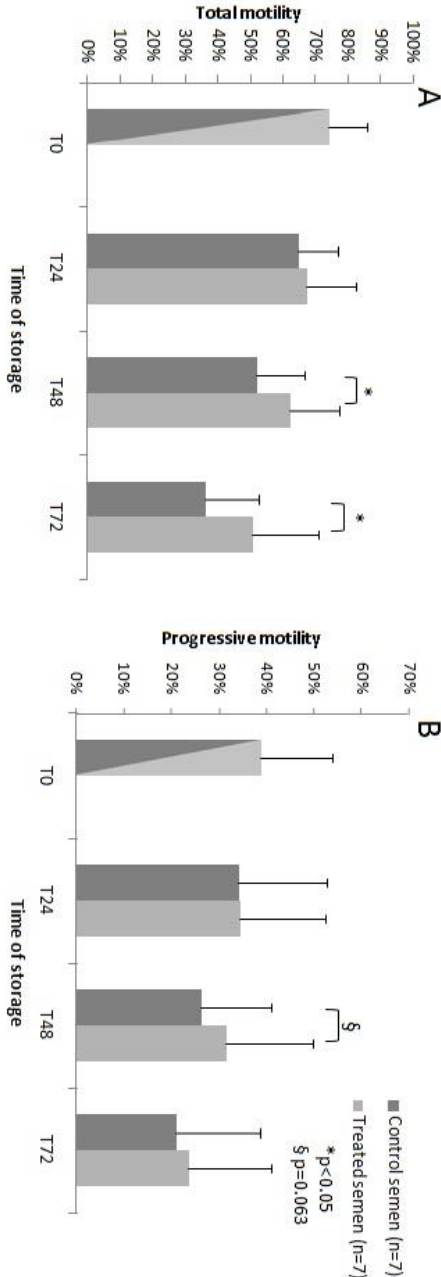
Figure 5.8 *(A)Total motility and (B) progressive motility in control samples and treated semen at each time point of cold storage (T0-24-48-72). * Significant differences between control and treated semen (p<0.05). § tendency for difference between control and treated semen (p=0.063).*
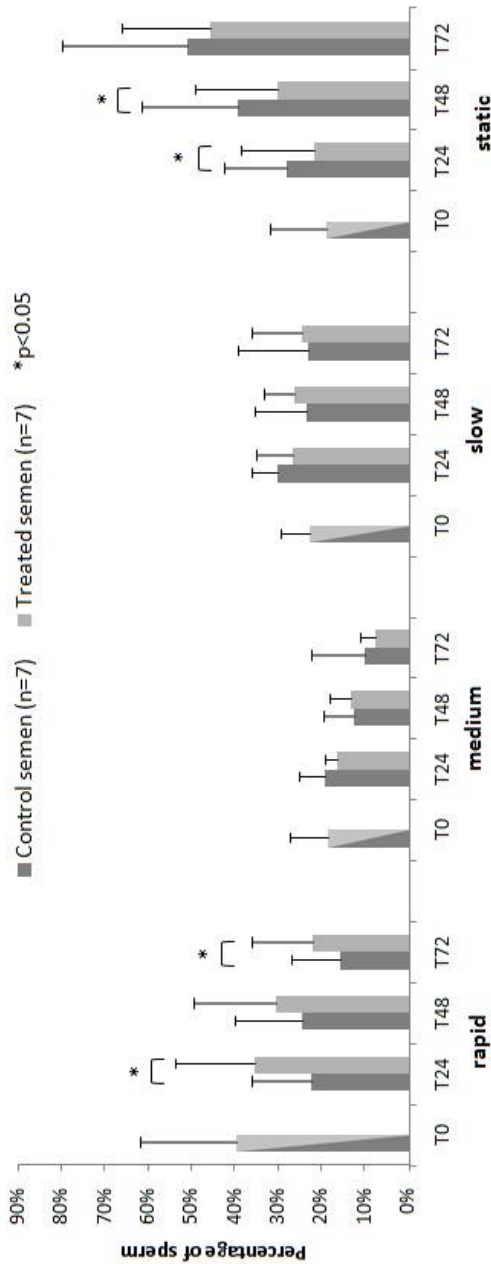
Figure 5.5 Rapid, medium, slow and static cells in control samples and treated semen at each time point during storage; * significant differences between control and treated semen (p<0.05).

The percentage of rapid, medium, slow and static sperm cells in both groups are illustrated in Figure 5.5. The percentage of rapid sperm cells significantly decreased in both groups (p<0.01) during cooled storage. Furthermore, the percentage of rapid spermatozoa was significantly higher (p<0.05) in treated semen then in control semen at 24 hours and 72 hours of cooled storage. There was an effect of storage time on the sperm subpopulation with medium speed in treated semen (p<0.05), but not in control semen. There was no time effect on the subpopulation slow moving spermatozoa in control or treated samples. No differences in the percentage of medium and slow sperm cells were observed between both groups. The percentage of static sperm cells significantly increased in control and treated samples during the storage(p<0.01). However, in treated semen the percentage of static sperm cells was significant lower (p<0.05) than in control semen at 24 hours and 48 hours of storage.

Kinetic motility parameters of the spermatozoa for each time point are shown in Table 5.1. VCL, STR and ALH were not modified by cold storage. VSL, VAP and BCF decreased significant (p<0.01) only in C semen during storage. LIN and WOB significantly decreased in both, control and treated samples, during cold storage. No differences in VSL, LIN, STR, WOB and BCF were found between control sample and treated semen samples. However, VCL and VAP were significantly higher (p<0.05) in treated semen than in the control group at 24 hours and 72 hours of cooled storage. ALH of treated semen was significantly higher than in control samples only at 24 hours (p<0.05).

Table 5.1. *Motility parameters of control samples and treated semen at each time point of cold storage.*

Kinematic parameters	Time of cooling storage						
	T0	T24		T48		T72	
	Control	Control	Treated	Control	Treated	Control	Treated
VCL(μm/s)	106.8 ± 45.3	77.3± 30[a]	94.9±34.3[b]	93.1±40.4	96±48.7	85.5±51.3 [a]	99.5±51.3[b]
VSL(μm/s)	60.6±31.0	44.1±17.7	50.9±24.1	45.3±23.4	48.8±28.5	36.8±25.4	43.3±24.8
VAP(μm/s)	76.5±34.6	56±21.9 [a]	67.4±27.6[b]	60.4±29.2	82.1±68	49.6±31.9 [a]	58.7±29.6[b]
LIN (%)	52.8±10.3	53±7.9	50.6±9.7	43.5±10.6	46.4±11.6	39±10.4	40.1±12.4
STR (%)	74.4±6.2	75.1±6.1	73.1±9	70.3±9.7	71.8±10.5	69.2±8.6	67.6±10.3
WOB (%)	68.8±8.6	67.8±7	67.3±5.5	59.1±8	61.9±7.8	54.3±8.3	56.4±10.2
ALH(μm)	3.8±1.3	3±0.8[a]	3.8±0.8[b]	3.7±1.3	3.8±1.5	3.4±1.6	4.2±1.5
BCF(Hz)	14.2±6.9	12.5±5.2	12.8±5.1	10.8±3.6	13±4.4	9.2±5.3	9.4±2.5

VCL = curvilinear velocity. VSL = straight line velocity. VAP = average path velocity. LIN= linearity. STR = straightness coefficient. WOB= wobble. ALH = amplitude of lateral head displacement. BCF=beat/cross frequency. [a,b] significant differences in a row between control and treated semen (p<0.05).

5.3.3 DNA damage

The TUNEL test (A) and SCSA (B) showed different percentages of spermatozoa with fragmented DNA (Figure 5.6). The percentage of sperm cells with DNA damage significantly increased during time storage at 5°C in control samples and treated semen as indicated by TUNEL test ($p<0.01$). On the other hand, the %DFI by SCSA statistically increased during cooling storage only in control samples ($p< 0.01$), and not in samples treated with antioxidants. Furthermore, the percentage TUNEL positive spermatozoa was significantly ($p<0.05$) higher in control semen than in treated semen at 72 hours of storage. No differences in %DFI between both groups were found by SCSA.
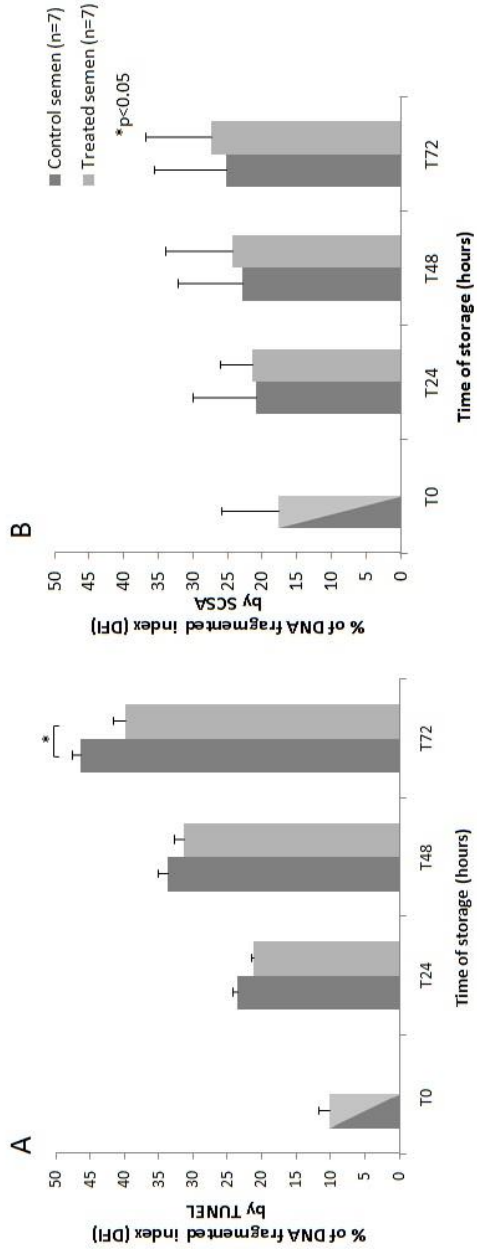
Figure 5.6 Percentage of spermatozoa with fragmented DNA in control samples and treated semen at each time point; DNA fragmentation was evaluated with two different methods. APObrdU- TUNEL test (A) and Sperm Chromatin Structure Assay (SCSA; B). * significant differences between control and treated semen (p<0.05)

5.3.4 Quantification of cleaved caspase 3

Cold storage at 5°C caused a significant increase (p<0.01) in activated caspase 3 in control semen, but not in treated semen (Figure 5.7). The amount of activated caspase 3 is statistically higher (p<0.050) in control samples than in treated semen at 48 hours of cold storage. There was a tendency (p=0.065) for this difference to be maintained at 72 hours of cold storage.
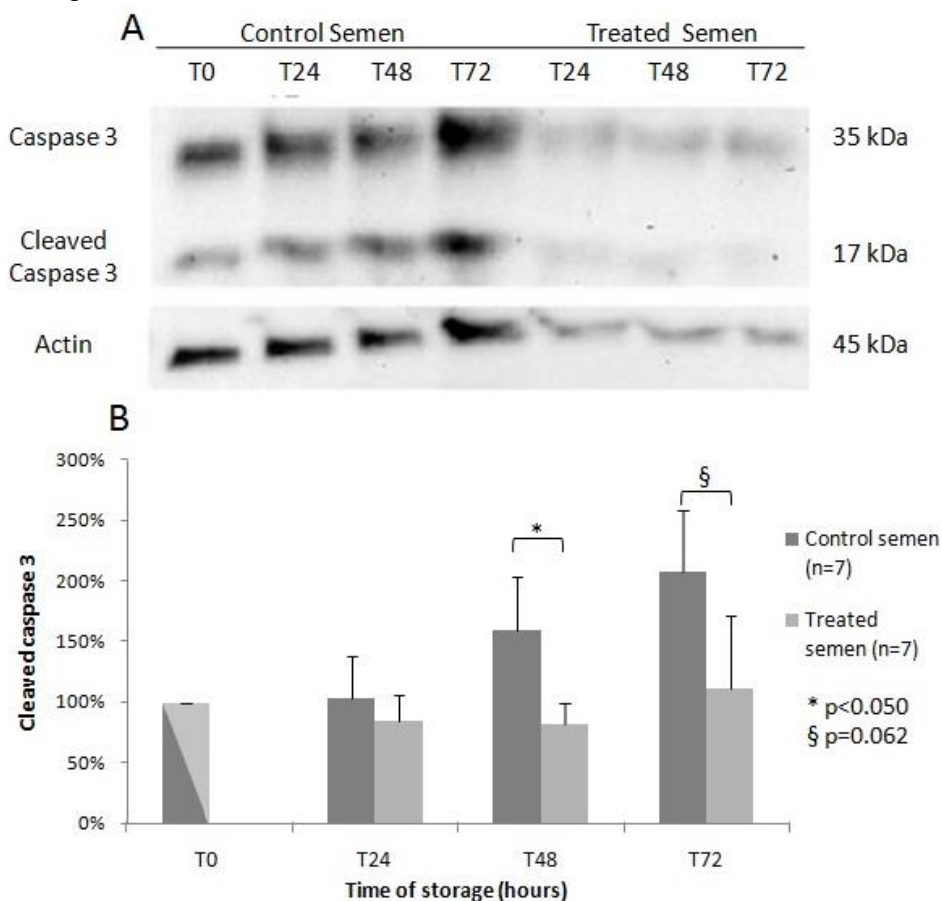

Figure 5.7 *Representative Western blot analysis of control and treated semen of stallion at each time point. Whol eand cleaved caspase 3 were detected using a rabbit anti-cleaved caspase-3 antibody diluted 1 1000 (Mr 35 and 17 kDa protein band) (A). Bar chart of cleaved caspase-3 in control and treated semen at each time point (T0-24-48-72); values are expressed as relative intensities in relation to beta-actin signal. The ratio obtained at T0 was considered 100%; * significant difference between control and treated semen (p<0.05); § tendency for difference between control and treated semen (p=0.062).*

5.4 Discussion

In this study, the effect of adding a combination of SOD, CAT and GPX on the quality of stallion semen stored at 5°Cfor 72 hours was investigated. The major parameters that are indicative of oxidative stress-induced damages to spermatozoa during cooled storage of stallion semen were monitored, i.e. total and progressive motility, motility kinetic parameters, DNA fragmentation and activation of caspase-3 (Ball, 2008). The combined addition of SOD, CAT and GPX inhibited the activation of activated caspase 3 and concomitantly maintained total motility and the percentage of rapid moving sperm cells at a higher level. A storage-dependent increase in DNA damage as detected by the TUNEL assay was alleviated only to a minor extend after prolonged storage time.

In the first 24 h of storage, the diluted equine spermatozoa and the remaining seminal plasma seem to be sufficiently equipped for fending off any ROS challenges. Almost no difference between control samples and treated samples was evident. This is in line with a previous study (Kankofer et al., 2005). Only the percentage of rapid cells in the control samples was lower than in the supplemented samples. However, this might already be a first indicator of subtle, sub-lethal ROS related influence on sperm movement. After 24 hours storage, a supplementation with exogenous antioxidants came into effect. Beneficial effects of a combination of antioxidant on quality of semen were evident after 48 hours and 72 hours of cooled storage. Whether one or all of the enzymatic components of the antioxidant defense system in semen get exhausted is not clear. However, the synergistic action of SOD, CAT and GPX is necessary to neutralize the ROS molecules by converting them into water and oxygen. Within the spermatozoa, superoxide dismutases (SODs) constitute the first line of defense against ROS (Nordberg and Arnér, 2001). SODs rapidly convert superoxide anion (O^{2-}) into oxygen and hydrogen peroxide (H_2O_2). Hydrogen peroxide seems to be the major ROS responsible for the damage in equine spermatozoa (Baumber et al., 2000; Ball, 2008). Due to its higher membrane permeability, hydrogen peroxide affects intracellular enzyme systems, induces DNA fragmentation and a reduction in motility (Baumber et al; 2003b; Ball, 2008). In order to prevent the induction of oxidative damage to lipids, proteins or DNA, CAT and GPX complete the reaction started by SOD by degrading hydrogen peroxide to water and oxygen. GPX operates by using the reduced form of glutathione (GSH) as an electron donor.

In addition to the negative effects on motility and viability, ROS can also damage the DNA of equine spermatozoa during cooling and storage (Love et al., 2002; Baumber et al., 2003a). DNA fragmentation was evaluated with two different methods, i.e. TUNEL and SCSA. A high correlation exists between the two tests (Evenson et al., 1980a, b; Gorezyeca et al., 1993) although the SCSA test is assumed to have a greater sensitivity for measuring DNA strand breaks in contrast to the TUNEL test (Evenson, 2016). However, in our study, there was a substantial discrepancy between the results of these two methods. This result differs from other studies showing a strong relationship between SCSA and TUNEL results for mammalian sperm DNA fragmentation (Sailer et al., 1995; Aravindan et al., 1997; Chohan et al., 2006). Meanwhile, our results sustain the assumption that SCSA and TUNEL assays are not correlated because measure different aspects of sperm DNA integrity (Alvarez, 2005; Stahl et al., 2015). TUNEL assay detects actual single and double DNA strand breaks, whereas SCSA measure potential DNA damages (Henkel et al., 2010). Furthermore, the evaluation of real double-stranded DNA breaks should provide a higher predictive value in assisted reproduction technologies than tests that detect potential DNA damage (Alvarez et al., 2005).

As expected, an increased DNA fragmentation was detected in our experiment during cold storage with both assay. Increasing values for TUNEL-positive spermatozoa and an increasing amount in activated caspase 3 confirmed that apoptosis-like changes were occurring in spermatozoa, because the activation of caspase 3 is a hallmark for the point of no return in the process of apoptosis (Said et al., 2004). Activated caspase-3 levels were lower in the treated group than in control group, from 48 hours of cold storage onwards. The antioxidant prevented the activation of caspase-3 starting at 48 hours and thereby the DNA fragmentation at 72 hours. This supports the hypothesis of a mitochondrial origin of oxidative stress-induced damage after sperm cooling (Peña et al., 2015). Indeed, it has been shown that cooling and rewarming of equine semen induces a premature senescence of the surviving spermatozoa due to oxidative stress resulting from mitochondrial malfunction (Ortega-Ferrusola et al., 2008; Peña et al., 2011; 2015). This mechanism recently termed spermptosis, does not provoke a direct DNA damage, but does implicate caspase 3 activation, reduced mitochondrial function and transposition of phosphatidylserine (PS) to the outer leaflet of the membrane (Ortega-Ferrusola et al., 2017).

One of the limitation of this study is that data about ROS production during semen storage are missing. A decreased presence of ROS in treated semen

would confirm that the mechanism underlying the beneficial effect of antioxidant is indeed in ROS scavenging. However, when assuming no side effects of SOD, CAT and GPX, it can be concluded that there is at least an indirect evidence that ROS-mediated processes may play a significant role in the storage-related decline in semen quality.

The capability of semen to be processed for cold storage differs between stallions (Brinsko et al., 2000). This significant variation is linked, among others, to the composition of their seminal plasma (Aurich, 2005) and the respective inter-stallion difference in the activity of each enzymatic ROS scavenger (Ball et al., 2000; Baumber and Ball, 2005). We tested only one fixed concentration of antioxidants ass supplement to a semen extender in our study. Data about the concentration of enzymatic antioxidant levels in seminal plasma and systemic antioxidant status may be useful to distinguish stallion with low and higher antioxidant status. Based on stallion antioxidant status, different concentration of antioxidant as additive to the diluted semen may be chosen. This might be especially of benefit for stallions with low antioxidant status. Semen from stallions with low antioxidant status may require higher supplementation with exogenous antioxidants to improve semen quality during cold storage.

In conclusion, our results suggest that the tested combination of SOD, CAT and GPX added to a cooling extender improves viability, motilities and kinetic features and reduce DNA fragmentation in semen stored for more than 24 hours. The result indirectly confirm that equine spermatozoa are affected by an increasing imbalance between ROS production and degradation during cold storage.

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