Sveriges lantbruksuniversitet
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Department of Clinical Sciences

The effect of the natural antioxidants catalase and epigallocatechin added after thawing on the quality of dromedary camel spermatozoa

Johanna Grundin

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The effect of the natural antioxidants catalase and epigallocatechin added after thawing on the quality of dromedary camel spermatozoa

Effekten av de naturliga antioxidanterna katalas och epigallocatechin tillsatta efter upptining på spermiekvaliteten hos kameler

Johanna Grundin

Supervisor: *Prof. Jane Morrell Department of Clinical Sciences*

Assistant Supervisor: *Dr. Clara Malo, Camel Reproduction Centre*

Examiner: *Prof. Eva Axnér*

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SUMMARY

The objective of this study was to evaluate the effect of adding two natural antioxidants (Catalase and Epigallocatechin) to the thawing media on the quality of cryopreserved dromedary camel spermatozoa post-thaw. Also, an indirect measure of ROS production was performed by measuring malondialdehyde (MDA) production (a product of oxidative damage) post-thaw. Ejaculates from 6 adult dromedary camel males (3 ejaculates/male) were included in this study. Ejaculates were obtained using an artificial vagina, frozen in liquid nitrogen vapour (1 cm above the liquid) for 15 min and then plunged into liquid nitrogen for storage. Samples were thawed at 60 ºC for 10 s. Semen evaluation was performed immediately after thawing and 3 aliquots were prepared: control, catalase and epigallocatechin. Semen samples where then evaluated for total motility, progressive motility, kinematics (Computer Assisted Sperm Analysis, CASA), acrosome integrity and membrane integrity at 1.5 and 3h post-thaw. The MDA concentration was measured 3 hours after thawing in the different treatments. Significantly ($p<0.05$) better values were obtained for the catalase group compared to the epigallocatechin or control groups for total motility (TM) and progressive motility (PM) and some kinematic parameters. None of the treatments showed any significant effect on vitality (VIT) or acrosome damage (AD) after thawing. For MDA production 3h after thawing, no significant effect could be demonstrated for the different treatments and no significant male effect was found. A significant difference was found between males regarding TM, PM and some kinematic parameters, as well as for VIT and AD after thawing. In conclusion, according to this study, addition of catalase to the thawing media can prove beneficial for the survival of dromedary camel sperm following cryopreservation by improving post-thaw sperm quality. The beneficial effects of addition of antioxidants in the cryopreservation media suggest that ROS are likely to be important contributors to the reduced viability of spermatozoa following cryopreservation.

SAMMANFATTNING

Syftet med denna studie var att utvärdera effekten av att tillsätta två naturliga antioxidanter (Katalas och Epigallocatechin) i upptiningsmediet på kvalitén hos kryokonserverad kamelsperma efter upptining. Det utfördes även en indirekt utvärdering av ROS produktionen genom att mäta malondialdehyde (MDA) produktionen (en produkt av oxidativ skada) efter upptining. Ejakulat från 6 kamelhanar (3 ejakulat/hane) användes i denna studie. Ejakulaten samlades med hjälp av en artificiell vagina och frystes ner genom att placera dem 1 cm ovanför flytande kväve i 15 min och förvarades sedan i flytande kväve. Proven tinades vid 60 ºC i 10 s. En första utvärdering av spermierna gjordes direkt efter upptining och provet delades sedan i 3 alikvoter; kontroll, katalas och epigallocatechin. Spermierna utvärderades med avseende på total och progressiv motilitet, kinematiska parametrar (Computer Assisted Sperm Analysis, CASA), membran- och akrosomintegritet 1.5 och 3 timmar efter upptining. MDA koncentrationen mättes 3 timmar efter upptining i de olika behandlingarna. Signifikant ($p<0.05$) bättre värden erhölls för katalas jämfört med epigallocatechin eller kontroll med avseende på total (TM) och progressiv motilitet (PM) samt några kinematiska parametrar. Ingen behandling visade någon signifikant effekt på vitalitet (VIT) eller akrosomskada (AD) efter upptining. Ingen signifikant effekt på MDA produktionen 3 timmar efter upptining kunde påvisas för de olika behandlingarna, inte heller sågs någon signifikant skillnad mellan hanar för MDA produktionen. En signifikant skillnad påvisades dock mellan hanar för TM, PM, några kinematiska parametrar samt även för VIT och AD efter upptining. Sammanfattningsvis, enligt denna studie, kan tillsättande av katalas till upptiningsmediet visa sig vara fördelaktigt för överlevnaden av kryokonserverad kamelsperma genom att förbättra kvalitén på sperman efter upptining. De fördelaktiga effekterna med att tillsätta antioxidanter till kryokonserveringsmediet tyder på att ROS kan vara en bidragande orsak bakom den minskade viabiliteten hos spermier efter kryokonservering.

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INTRODUCTION

Artificial insemination (AI) is considered to be the most important practice contributing to the progress of animal production (Bailey *et al*., 2003). The advantages of AI are augmented with the use of sperm cryopreservation. The use of cryopreserved semen, however, is limited in most mammals. The freezing and thawing processes produce physical and chemical stresses on the sperm and may reduce sperm fertilization ability (Salamon & Maxwell, 1995).

Cryopreservation of camelid semen has met with poor success as camelid sperm are generally not tolerant to freezing and thawing (Morton *et al*., 2010; Bravo *et al*., 2000). However, as interest grows in trying to improve genetic traits in these species, cryopreservation and AI are being used more frequently in camelids such as the dromedary camel (Skidmore *et al*., 2013). Hence, work aimed at improving the fertility of cryopreserved camel spermatozoa is becoming increasingly important.

The mechanisms behind cryoinjury to spermatozoa are relatively well understood. During recent years cryopreservation was found to be associated with excessive formation of reactive oxygen species (ROS) with injury to spermatozoa due to oxidative stress (OS) (Chatterjee & Gagnon, 2001). There is also evidence of a decrease in the antioxidant defence mechanisms of sperm following cryopreservation (Bilodeau *et al*., 2000). An imbalance between ROS levels and the antioxidant systems protecting spermatozoa against ROS is now considered as one of the factors causing sperm damage during cryopreservation (Alvarez & Storey, 1995).

Studies have shown that addition of certain antioxidants to liquid stored semen and to semen during the cryopreservation process can be beneficial to post-thaw sperm quality in several species such as bovine (Tvrdá *et al*., 2016), boar (Gadani *et al*., 2017; Hu *et al.,* 2014; Zhang *et al*., 2012) and ram (Souza *et al*., 2016). In the dromedary camel it was demonstrated that certain antioxidants added to the cryopreservation extender before freezing could maintain post-thaw sperm quality after thawing (Malo *et al., unpublished data*). However, the influence of antioxidants added after thawing has not been reported previously.

The aim of this study was to determine the effect of adding two natural antioxidants (Catalase and Epigallocatechin) to the thawing media on sperm quality parameters after 1.5 and 3 hours by evaluating motility parameters (total and progressive motility), membrane integrity and acrosome integrity. Also, an indirect measure of ROS production was performed by measuring MDA production (a product of oxidative damage) 3 hours after thawing.

LITERATURE REVIEW

The dromedary camel

The dromedary camel, *Camelus dromedarius* (figure 1)*,* belongs to the Camelidae family. There are two genera within the Camelidae family, *Camelus* and *Lama* (Bravo *et al*., 2000). The genus *Camelus* consists of the dromedary camel, which has one hump, and the bactrian camel, *Camelus bactrianus,* which has two humps. These two species are also known as Old World camelids. The genus *Lama* consists of four species: llama, alpaca, guanaco and vicuna. These four species are also known as New World camelids. All species within the Camelidae family are generally referred to as camelids.

Fig. 1. *The dromedary camel, Camelus dromedarius.*

Dromedary camel semen

The development of artificial reproduction technologies in the dromedary camel, as in other species of the Camelidae family, has been challenging due to the characteristics of the male ejaculate and also due to aspects of the female's reproductive cycle (see below) (Crichton *et al.,* 2015). Dromedary camel semen has a highly viscous consistency. The coagulum, which is a product of the prostate and bulbourethral glands, plays a role in lubrication for coitus, but also traps the spermatozoa. The entrapment of spermatozoa in the viscous seminal plasma makes handling, diluting and cryopreservation difficult (Bravo *et al.,* 2000).

The camelid ejaculate (figure 2), besides being highly viscous, is also of low volume and low sperm concentration (Kershaw-Young & Maxwell, 2012). In addition, spermatozoa have low progressive motility.

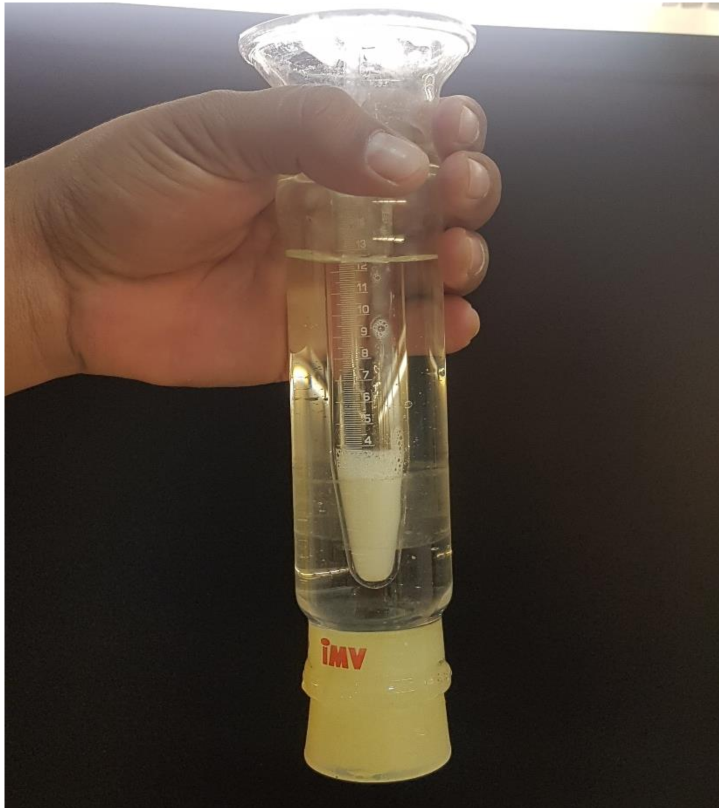

Fig. 2. *Dromedary camel semen immediately after collection.*

Semen evaluation

Normal spermatozoa can be defined as those that successfully undergo the necessary steps for oocyte fertilization (Hammadeh *et al*., 2009). The first step is maturation, initiated in the male genital tract, and the final step is capacitation in the female genital tract. When fully matured the spermatozoa must be able to reach the oocyte, undergo the acrosome reaction, penetrate the zona pellucidae and fuse with the pronucleus in the oocyte forming a zygote. Fertilization is a complex process. Due to this complexity a set of tests assessing different sperm functions has to be selected to evaluate potential fertility.

In the camel, conventional methods for semen assessment include macroscopic evaluation of volume and colour, and the microscopic characteristics of spermatozoa (El-Bhrawi, 2005). Skidmore *et al.* (2013) described the methods used for semen evaluation in the dromedary camel including volume, colour, viscosity, sperm concentration, motility, sperm viability, sperm morphology, sperm plasma membrane integrity and sperm acrosome membrane integrity. The same sperm quality tests have been described for semen assessment in most mammals, for example in the bull (Tanghe *et al.,* 2002).

Different opinions exist on the definition of sperm quality; there might also be species differences in the importance of the individual parameters. However, it is generally considered that sperm quality can be described in terms of sperm number, motility and morphological normality (Colenbrander *et al.,* 2003). Additional parameters can be added such as membrane integrity and chromatin integrity. Oxidative damage to spermatozoa, due to excessive production of free radicals or decrease of the natural antioxidant mechanisms, can also be used to assess sperm quality (Sikka *et al.,* 1995).

Motility is one of the most important qualities of spermatozoa for them to reach the site of fertilization. Motility can be assessed by visual examination under the microscope; however, this method is subjective and is not reliable for predicting fertility (Pena-Martinez, 2004). Computer assisted sperm analysis (CASA) gives an objective motility assessment. With CASA several motility parameters can be calculated such as total and progressive motility and sperm kinematics including average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), amplitude of lateral head displacement (ALH, µm), straightness (STR, %), linearity (LIN, %), and beat cross frequency (BCF, Hz).

The ability of the sperm to succeed with fertilization is derived from several functions such as viability, morphology and acrosomal status. These assays are useful diagnostic tests on their own although the incorporation of *in vitro* gamete interaction tests such as the zona pellucida binding assay and the oocyte penetration assay are of greater diagnostic relevance.

Reports on the evaluation of camel spermatozoa are scarce. The method used to evaluate acrosome and membrane integrity (vitality) to date is by using vital stains such as eosin-nigrosin (Deen *et al., 2003;* Malo *et al.,* 2017a; Malo *et al.,* 2017b) or Coomassie blue staining (Crichton *et al.,* 2015). During the last decade many fluorescence probes have been used for semen assessment in other species. The fluorescence of these compounds can be assessed using fluorescence microscopy or flow cytometry. Unfortunately, these techniques are not commonly applied in camel research.

The zona pellucida binding assay, a good tool to assess the integrity of receptor proteins on the spermatozoa to bind to the zona pellucida, has not been reported in camels. The oocyte penetration assay permits observation of pronuclear formation. Crichton *et al.* (2016) incorporated a heterologous penetration test using zona-free goat oocytes in their evaluation of dromedary camel sperm quality after cryopreservation. Successful penetration and pronuclear formation were obtained with this system.

Semen selection

In vivo, spermatozoa migrate away from seminal plasma after deposition in the female reproductive tract (Morrell & Rodriguez-Martinez, 2009). The presence of seminal plasma is believed to be beneficial to sperm function, containing so-called decapacitation factors which help to maintain spermatozoa in a non-capacitated state until they can be inseminated. However, seminal plasma is also considered detrimental to the long-term survival of spermatozoa containing substances such as sperm motility inhibiting factor and ROS. Because of the inhibiting factors in seminal plasma it is essential that the spermatozoa are removed from their

influence before reaching the oocyte. It has been shown that removal of seminal plasma can improve sperm survival during cool storage and cryopreservation in some species such as stallion and boar (Moore *et al.,* 2005; Roca *et al.,* 2006: see Morrell & Rodriguez-Martinez, 2009 p. 3), this has also been shown to be true in the dromedary camel (Malo *et al.,* 2017a).

The female reproductive tract has a dual filtering action allowing spermatozoa to be separated from seminal plasma and also by providing various barriers to the progress of abnormal, poor quality spermatozoa (Morrell & Rodriguez-Martinez, 2009). Only spermatozoa which remain viable longer and are more motile progress through the female reproductive tract. The cervix represents the first barrier in species with vaginal deposition of semen. In species such as the camel, semen is deposited in the uterus; thus selection by the cervix is lacking in either natural mating or AI in this species, leaving the uterus and the uterotubal junction to act as a barrier. Another selection process may occur through interaction of the spermatozoa with the oviductal epithelial cells. The result of these barriers is that only highly motile, viable spermatozoa arrive at the fertilization site.

It is unknown whether poor quality sperm samples result in low pregnancy rates because the natural filtering systems remove all abnormal spermatozoa, leaving insufficient number of spermatozoa for fertilization, or the filtering systems are overwhelmed by the number of abnormal spermatozoa leading to fertilization by abnormal sperm and thus failure of zygotic development (Morrell & Rodriguez-Martinez, 2009).

Several mechanisms can be used to mimic this *in vivo* selection of viable spermatozoa in the female reproductive tract. In a review by Morrell & Rodriguez-Martinez (2009) these mechanisms are described in detail. Malo *et al.,* (2017a) showed that sperm quality parameters and *in vitro* fertilization ability of dromedary camel spermatozoa was improved by using single layer centrifugation through a colloid (SLC) compared with simple sperm washing. Typically, when using density gradient centrifugation as a selection technique, two or more layers of colloid of different densities are used to separate live motile sperm from seminal plasma, dead cells, debris and extender. With the SLC technique only a single layer of colloid is needed to select functional spermatozoa; this technique has been used successfully for viable sperm selection in many different species.

Cryopreservation

Cryopreservation is a process to preserve living cells and tissues using very low temperatures (Pegg, 2007). At low temperatures cell metabolism decreases which makes long-term preservation possible (Yeste, 2016; Gao & Critser, 2000).

Semen cryopreservation augments the advantages of assisted reproduction technologies such as AI (Bailey *et al.,* 2003). There are numerous benefits of AI such as better herd health, improved safety, less costs resulting from fewer male animals and most importantly the possibility to inseminate multiple females with the semen from one genetically superior male making an accelerated rate of genetic improvement possible. With the use of cryopreservation, transportation of semen over long distances is made possible, providing access to a greater population of animals (Bailey *et al*., 2003). Also, cryopreservation enables the use of semen for

AI long after the sire has died and it is an important aspect of genome resource banking making preservation of genetic material possible for the future.

The use of cryopreserved semen, however, is limited in most mammals other than cattle. The cryopreservation process, freezing and thawing, produces physical and chemical stress on the spermatozoa and may reduce sperm fertilization ability due to ultra-structural, biochemical and functional damage (Salamon & Maxwell, 1995). Reduced fertilizing potential of spermatozoa due to a negative impact on sperm quality parameters following cryopreservation is well recognised in many different species including boar (Suhee *et al*., 2011), equine (Baumber *et al*., 2003), bull (Tanghe *et al*., 2002), ram (Salamon & Maxwell, 1995) and human (Alvarez & Storey, 1992).

Damage to cells at low temperatures is called cryoinjury (Gao & Critser, 2000). It is this damage that results in the decrease in sperm quality seen following cryopreservation, resulting in decreased fertilizing ability following AI with cryopreserved semen.

There are species differences regarding the success of AI with cryopreserved semen resulting from variations in the physiology and biochemistry of the spermatozoa and from variations in the anatomy and physiology of sperm transport in the female reproductive tract (Holt, 2000).

Cryopreservation and AI in the dromedary camel

In camelids, the use of AI has been reported but insemination trials are rare (Skidmore *et al.,* 2013). During the last 25 years sperm cryopreservation and AI has started to be used more frequently as the interest grows in trying to improve genetic traits such as milk, meat and wool production as well as racing ability.

The advantages of AI and cryopreservation in the dromedary camel are the same as in other species. Additionally, it helps overcome behavioural problems as often a male camel will refuse to mate a particular female, or will act aggressively towards her (Skidmore *et al*., 2013).

However, cryopreservation of camelid semen has met with poor success as camelid sperm are generally not tolerant to freezing and thawing (Bravo *et al*., 2000; Morton *et al*., 2010; Crichton *et al.,* 2015; Malo *et al.,* 2017b). Another complicating factor is that camelids are induced ovulators which means that the females have to be induced to ovulate when using AI (Bravo *et al*., 2000). There are problems associated with the collection and handling of camel semen as camels mate with the female in sternal recumbency (figure 3) and have a long duration of copulation. The ejaculates, as previously described, are very viscous with a low volume and low sperm concentration (Skidmore *et al.,* 2013).

Studies report lower post thaw motility and fewer pregnancies (1/13) with AI using frozen thawed semen compared with whole semen (Deen *et al*., 2003). Using fresh sperm for AI results in a pregnancy rate of approximate 50% (Skidmore *et al*., 2013). Crichton *et al*., (2015) reported lower total and progressive motility following cryopreservation in dromedary camel sperm compared to fresh semen, and the cryopreservation process resulted in a significant decline in sperm acrosomal integrity. Binding studies of cryopreserved dromedary camel sperm to

heterologous (zona-pellucida free) goat oocytes showed that cryopreservation resulted in a sharp decline in sperm-oocyte interaction compared with fresh sperm (Crichton *et al*., 2016).

Progress with AI and semen cryopreservation has been slow in the dromedary camel in comparison to other livestock species (Bravo *et al.,* 2000). Camel semen cryopreservation is possible, but improvements in the methods used are needed.

Fig. 3. *The Dromedary camel mates with the female in sternal recumbency, making semen collection difficult.*

Mechanisms of cryoinjury

The most important mechanisms behind cryoinjury are associated with the phase change of water in the extra-and intracellular environments (Gao & Critser, 2000).

When cooled to about –5 ^{o}C both cells and the surrounding media remain unfrozen because of supercooling and depression of the freezing point by protective solutes (Mazur, 1984). Between –5 and –15 ^{o}C ice crystals form in the external medium, but the contents of the cell remain unfrozen because the plasma membrane blocks the growth of ice crystals in the cytoplasm. As ice formation occurs in the external media the concentration of solutes in the remaining unfrozen fraction increases and water flows out of the cell osmotically and freezes externally (Mazur, 1984; Gao & Critser, 2000).

Mazur *et al*. (1972) hypothesised that at least two factors dependent on cooling rate interact to determine the ultimate survival of a frozen-thawed cell. Cells that are cooled slowly are killed by solution effects i.e. altered solution properties of the extracellular and intracellular solutions induced by ice formation (solute/electrolyte concentration, severe cell dehydration and reduction of the unfrozen fraction in the extracellular space). Cells cooled faster than optimum are killed by the formation of intracellular ice and its recrystallization during slow warming

(Mazur, 1984; Mazur *et al.,* 1972). If cells are being cooled too fast, the intracellular water is not lost fast enough to maintain equilibrium, they become increasingly supercooled, eventually attaining equilibrium by freezing internally (Mazur, 1984). With sufficiently slow cooling, the cell is able to lose water fast enough to concentrate intracellular solutes sufficiently to eliminate supercooling and maintain the intracellular water in equilibrium with the extracellular water.

Membranes, both surface and internal membranes, seem to be the main targets of cryoinjury during both slow and rapid freezing. Sperm membranes affected by cryopreservation include the plasma membrane, the outer acrosomal membrane and mitochondrial membranes (Watson, 1995). Cooling induces changes in the lipid composition and organisation of the membrane. Behaviour and function of membrane lipids and proteins may be affected by temperature, for example, cooling induces phase transition of membrane lipids from the liquid crystalline to the gel phase (Drobnis *et al.,* 1993) accompanied by leakage of solutes across membranes, resulting in damage.

During recent years cryopreservation was found to be associated with excessive formation of reactive oxygen species (ROS) and injury to spermatozoa due to oxidative stress (OS). A study by Chatterjee & Gagnon, (2001) provided strong evidence that ROS are generated during the various stages of the freeze-thaw cycle of bull spermatozoa, suggesting that these ROS may be a cause of the decrease in sperm function following cryopreservation. One of the main detrimental effects of ROS is a state called oxidative stress. Oxidative stress causes lipid peroxidation of the sperm membrane. In the same study the authors demonstrated that lipid peroxidation was higher in thawed spermatozoa, a likely consequence of an increase in ROS production.

The effects of ROS are limited by the presence of various regulatory antioxidant systems in seminal plasma and spermatozoa which maintain a balance between the production and metabolism of ROS (Griveua & Le Lannou, 1997). Beneficial effects of adding antioxidants provide indirect evidence that ROS production and oxidative stress occurs during cryopreservation (Chatterjee & Gagnon, 2001).

Reactive Oxygen Species (ROS)

Oxygen is an essential molecule for most animals and plants that produce their energy by the oxidation of biological molecules, but under certain conditions, oxygen can also be potentially highly toxic (Griveua & Le Lannou, 1997). Initially inert oxygen may react with biological molecules and thereby lead to the formation of ROS.

ROS represent a broad category of molecules including radicals (hydroxyl ion, superoxide, nitric oxide, peroxyl, etc.) and non-radical (ozone, single oxygen, lipid peroxides, hydrogen peroxide) oxygen derivates (Agarwal & Prabakaran, 2005). In addition, there is another class of free radicals that are nitrogen-derived, called reactive nitrogen species (RNS). They are considered a subclass of ROS.

Free radicals are short-lived reactive chemical intermediates, which contain one or more unpaired electrons (Bansal & Bilaspuri, 2011). They give rise to cellular damage by passing

this unpaired electron on to nearby cellular structures, resulting in oxidation of cell membrane lipids, amino acids in proteins or in nucleic acids. In the reproductive tract, free radicals also play a dual role and can modulate various reproductive functions.

Mechanisms of ROS formation

ROS are produced in the body by mitochondria, phagocytes, arachidonate pathways and other physiological processes in which they act as vital signalling molecules (Hammadeh *et al.*, 2009). They are products of natural oxygen metabolism and represent approximately 1 to 2% of metabolized oxygen. Additionally, their production is induced by external factors, such as cigarette smoke and ultraviolet light radiation.

Oxygen in the atmosphere has two unpaired electrons, and these unpaired electrons have parallel spins (Agarwal & Prabakaran, 2005). Oxygen is usually non-reactive to organic molecules that have paired electrons with opposite spins. This oxygen is considered to be in a ground (triplet or inactive) state and is activated to a singlet (active) state by two different mechanisms: absorption of sufficient energy to reverse the spin on one of the unpaired electrons or monovalent reduction (acceptance of a single electron).

Monovalent reduction in oxygen molecules produces the superoxide anion (O_2^-), the primary free radical generated (Griveua & Le Lannou, 1997). Production of this superoxide anion *in vivo* is continuous and directly linked to oxygen metabolism. This superoxide radical is relatively inert, but its presence may lead to the appearance of other powerful oxidant molecules. The superoxide anion undergoes further reduction to form hydrogen peroxide (H_2O_2) and hydrogen peroxide is further reduced to hydroxyl radicals in the presence of ferrous salts (Fe2+) (Agarwal & Prabakaran, 2005). The hydroxyl radicals are very powerful initiators of lipid peroxidation (Griveua & Le Lannou, 1997).

ROS production by spermatozoa during cryopreservation

Different types of cells are present in semen; mature and immature spermatozoa, round cells from different stages of the spermatogenic process, leukocytes and epithelial cells (Agarwal & Prabakaran, 2005). The two main sources of ROS in the ejaculate are the spermatozoa themselves and leukocytes (Griveua & Le Lannou, 1997; Agarwal & Prabakaran, 2005).

Like all cells living under aerobic conditions, spermatozoa produce ROS, mostly originating from normal metabolic activity (De Lamirande *et al.,* 1997). The production of ROS is higher in immature spermatozoa, as well as in morphologically abnormal or damaged spermatozoa (Hammadeh *et al*., 2009).

Leukocytes have been associated with excessive ROS production (Agarwal & Prabakaran, 2005). ROS produced by leukocytes form the first line of defence in any infectious process. Leukocytes are present in the ejaculate as a result of their role in the immunological defence against pathogens (Hammadeh *et al*., 2009). Sperm damage from ROS, produced by leukocytes, occurs if seminal leukocyte concentrations are abnormally high (leukocytospermia), or by removing seminal plasma during sperm preparation for assisted reproduction.

Several studies have been performed associating cryopreservation with excessive production of ROS, suggesting that these ROS may be a contributing cause for cryoinjury and the decrease in sperm function and fertilizing ability following cryopreservation. Strong evidence exist that ROS are generated during the various stages of the freeze-thaw cycle of bull spermatozoa (Chatterjee & Gagnon, 2001). Wang *et al.,* (1997) showed that the process of cryopreservation can cause a significant increase in ROS generation in human sperm cells, particularly if the semen sample is contaminated with a significant number of leukocytes. These authors suggested that it is crucial to remove leukocytes from semen samples being prepared for cryopreservation.

The initial increase in ROS production associated with exposure to decreasing temperatures may be the result of increased enzymatic activity or changes in the structural or functional integrity of the cell membrane (Wang *et al.,* 1997). Research studies have shown that repeated cycles of centrifugation in the process of sperm preparation can induce the production of ROS by spermatozoa (Hammadeh *et al.,* 2009).

Physiological and detrimental effects of ROS

ROS are known mostly for their detrimental effects on sperm function. However, very low and controlled concentrations of ROS participate in signal transduction mechanisms and play an important role in sperm physiology (Lamirande *et al.,* 1997). They induce sperm hyper-activation, capacitation, acrosome reaction and sperm-oocyte fusion *in vitro*. In a review by Lamirande *et al.,* (1997) studies demonstrated that these physiological processes occurring in spermatozoa are oxidative and that low concentrations of ROS, exogenously added or generated by spermatozoa, are needed to trigger these events *in vitro*. The addition of different antioxidants reduces these processes *in vitro*, giving further evidence that ROS may be needed to promote these events. One study showed that hydrogen peroxide (H_2O_2), generated *in vivo*, triggered the acrosome reaction of capacitated human spermatozoa and that the antioxidants catalase and superoxide dismutase (SOD) reduced or totally prevented the acrosome reaction, indicating the involvement of ROS in this process (Lamirande *et al.,* 1998).

Normally there is a fine balance between the production and scavenging of ROS, where the cellular antioxidant mechanisms present in almost all tissues and their secretions reduce these ROS and protect against oxidative damage (Hammadeh *et al.,* 2007). The detrimental effects of ROS occur when oxidants outnumber antioxidants giving rise to a state called oxidative stress (OS). During OS, the elevated levels of ROS damage cells, tissues and organs and may be a mediator of sperm dysfunction (Hammadeh *et al.,* 2007).

One of the major consequences of OS is the degradation of membrane structures by ROS by a process known as lipid peroxidation (LPO) (Griveua & Le Lannou, 1997). To stabilize their electronic structures, free radicals react with most organic compounds, behaving either as oxidants or reducers, starting a chain of events. This chain reaction brings about modification of some biological structures, particularly those rich in polyunsaturated fatty acids (PUFA). Owing to the high proportion of PUFA present in the phospholipids that make up membranes, radical degradation primarily concerns membrane structures. The presence of double-bonds allows ROS to attack the carbon chain of UFA producing a lipid radical, initiating the LPO

process which spreads over the membrane, causing deterioration of its properties as well as disorganization.

However, LPO is not the only mechanism by which ROS can lead to impaired sperm function. In addition to direct effects on cellular components, ROS could produce OS by decreasing the enzymatic defences of the spermatozoa (Griveua & Le Lannou, 1997). It is not only membranes that are affected - all cellular components can be targets for potential ROS-attack including lipids, proteins, nucleic acids and sugars (Bansal & Bilaspuri, 2011).

It is generally accepted that ROS production and OS are associated with cryoinjury and poor sperm function in several species (boar: Awda *et al.,* 2009, equine: Baumber *et al*., 2003, mouse: Chen *et al.,* 2016; goat: Gadea *et al.,* 2013; Shafiei *et al.,* 2015; Kargar *et al.,* 2017, human: Wang *et al.,* 1997; Moustafa *et al*., 2004; Hammadeh *et al.,* 2007). In semen, ROS production has been associated with loss of sperm motility, reduced capacity for sperm-oocyte fusion and loss of fertility (Griveua & Le Lannou, 1997). The most common ROS with potential implications in sperm oxidative damage include the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Zhang *et al*., 2012). Excessive ROS formation by spermatozoa during the cryopreservation process has been associated with a decrease in the function of thawed spermatozoa (Chatterjee & Gagnon, 2001). However, in camels, no studies have been made concerning ROS production and OS and their involvement in sperm damage during semen cryopreservation.

Antioxidants and their role in the protection against ROS

Antioxidants, present in almost all tissues and their secretions (Hammadeh *et al*., 2007), reduce OS by breaking the oxidative chain reaction started by ROS (Bansal & Bilaspuri, 2011). They scavenge and suppress the formation of ROS or they oppose their actions.

There are two types of antioxidants (Bansal & Bilaspuri, 2011; Hammadeh *et al.,* 2009; Bathgate, 2011; Kefer *et al.,* 2009). Enzymatic antioxidants, also known as natural antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) represent one group. SOD catalyzes the conversion of the superoxide anion (O_2^-) to oxygen and hydrogen peroxide (H_2O_2). CAT transforms H_2O_2 to water and oxygen eliminating the potential ROS toxicity. GPx also degrades H_2O_2. The other group of antioxidants are the non-enzymatic antioxidants, also known as synthetic antioxidants or dietary supplements, which contain vitamins and minerals such as vitamin C, vitamin E, taurine, hypotaurine and glutathione. All these antioxidants, enzymatic or non-enzymatic, maintain a balance between the production and metabolism of ROS.

In semen, both natural and synthetic antioxidants work to maintain a low level of OS to avoid oxidant-induced cell damage, allowing for oxidant-dependent cell signaling processes and normal sperm function (Kefer *et al.,* 2009). In the cytoplasm of most cells CAT, SOD and GPx are found in high concentrations, but this is not the case in the sperm cell. Spermatozoa discard most of their cytoplasm during differentiation and therefore contain only minimal amounts of these critical ROS-scavengers (Bansal & Bilaspuri, 2011). Spermatozoa possess primarily enzymatic antioxidants, SOD being the most predominant (Makker *et al.,* 2009). The majority

of the antioxidant protection is instead contained in the seminal fluid which holds both the enzymatic antioxidants SOD, CAT and GPx as well as a wide range of non-enzymatic antioxidants (Makker *et al.,* 2009; Kefer *et al.,* 2009). Antioxidants present in the seminal plasma compensate for the deficiency of cytoplasmic enzyme in the sperm cell (Hammadeh *et al.,* 2009) and provide spermatozoa with crucial protection against oxidative attack by different forms of ROS (Hammadeh *et al*., 2007).

As stated before, the main target for LPO and oxidative damage are PUFA present in membrane phospholipids. Sperm have a high proportion of PUFA in their membranes (Kefer *et al.,* 2009). This susceptibility to LPO together with a low intracellular defense mechanism make spermatozoa extra sensitive to oxidative damage. During cryopreservation it is customary to remove the seminal plasma, causing spermatozoa to lose the antioxidant protection that this component provides (Roca *et al.,* 2005). As a consequence, during cryopreservation, spermatozoa are especially susceptible to oxidative damage because of their relatively high proportion of PUFA, the removal of the scavenging potential of SP before freezing and the generation of ROS by defective and dead spermatozoa during the freezing-thawing process, leading to membrane LPO and damage. (Roca *et al.,* 2005; Baumber *et al.,* 2003). Thawing is one of the most delicate processes during semen cryopreservation (Gadani *et al*., 2017). As spermatozoa pass from a dormant metabolic state to sudden awakening in metabolism the rapid utilization of oxygen leads to an overproduction of ROS. When ROS are overproduced spermatozoa cannot easily adapt to this condition, especially without the protective effect of CAT in the SP. Sperm cells are extremely sensitive to H_2O_2 and its toxicity can be prevented by addition of CAT (Medan *et al.,* 2008).

The use of antioxidants during semen cryopreservation has gained popularity, as this may reduce lipid peroxidation. The efficacy of some antioxidants has proved to be better than others. This issue has been addressed by researchers, who evaluated various natural and synthetic antioxidants for their potency in semen cryopreservation.

Some successful results have been obtained with natural antioxidants such as biotin (Kalthur *et al*., 2012), melatonin (Mouse: Chen *et al*., 2016, Human: Karimfar *et al.,* 2015, Bull: Ashrafi *et al.,* 2012, Ram: Succu *et al.,* 2011), epigallocatechin (green tea extract) (Ram: Najafi *et al.,* 2014), rosmarinic acid (Boar: Luño *et al*., 2014), curcumin (Bull: Tvrdá *et al*., 2016), TROLOX (Rabbit: Zhu *et al*., 2015, Boar: Varo-Ghiuru *et al*., 2015, Human: Minaei *et al*., 2012) and catalase (Camel: Medan *et al.,* 2008, Boar: Roca *et al.,* 2005). The beneficial effects of addition of antioxidants in the cryopreservation media on sperm quality parameters suggest that ROS are likely to be important contributors to the reduced viability of spermatozoa following cryopreservation.

Specific objectives

The objective of this study was to determine the effect of adding two natural antioxidants (Catalase and Epigallocatechin) to the thawing media on dromedary camel sperm quality parameters after 1.5 and 3 hours. An indirect measure of ROS production was performed by measuring MDA production (a product of LPO) 3 hours after thawing.

MATERIALS AND METHODS

Unless otherwise indicated, all chemicals were from Sigma-Aldrich Co. (St Louis, MO, USA). Colloid solution was supplied by Prof. Morrell, SLU.

Fresh ejaculates were diluted in Tris-Citrate-Fructose Buffer (TCF, pH = 6.9; 340 mOsm) composed of 300 mM TRIS, 94.7 mM citric acid and 27.8 mM fructose (Evans and Maxwell, 1987). Bovine serum albumin (0.05%) and EDTA (10 mM) were added with 4% (v:v) egg-yolk and the solution filter sterilized (0.22 m). Green Buffer (GB) (IMV: L'Aigle, France), supplemented with 20% (v:v) egg-yolk, was used as freezing extender. Sperm dilution was performed in a two-step procedure, fraction 1 (F1) and fraction 2 (F2), with F2 being similar to F1 but with the addition of 6% (v:v) glycerol.

Animals and semen collection

Six adult dromedary camel males from the Camel Reproduction Centre in Dubai were used in this study. Ejaculates were obtained from each male using an artificial vagina (figure 4) (Skidmore *et al*., 2013) during the breeding season (between February and March). Ejaculates were immediately transported to the laboratory and were placed in a 37 ºC water bath. All animal procedures were approved by the Animal Care and Use Committee (ACUC) of the Camel Reproduction Centre, UAE.

Fig. 4. *Modified artificial bull vagina used for the collection of Dromedary camel semen.*

Semen preparation and cryopreservation

Semen was diluted (1:5) with warm TCF and manually liquefied by gentle pipetting (Malo *et al.*, 2017a). Liquefied samples were evaluated for sperm total motility and concentration. Semen was then subjected to SLC or simple centrifugation (Control) according to Malo *et al.*, (2017a). For the SLC group, 2 mL aliquots of semen were layered over 2 mL of colloid in 15 mL tubes and centrifuged (swing-out rotor) at 300 x g for 20 min. The supernatant was aspirated using a Pasteur pipet and discarded. Pellets were then re-suspended in F1 (GB supplemented with 20% (v/v) egg yolk) and the sperm concentration was determined. The suspension was adjusted to obtain a concentration of 120 x 10^6 sperm/mL, and cooled to 5ºC over 2 hours using a water jacket.

A second extension step (1:1) was performed with F2 (GB extender containing 20% egg yolk and 6% (v/v) glycerol) at 5ºC, resulting in a final cell concentration of 60 x 10^6 sperm/mL. Pre-cooled plastic straws (0.5 mL) were filled with the cooled semen in a cold cabinet and, after 30 min of equilibration, were frozen in liquid nitrogen vapour (1 cm above the liquid) for 15 min and then plunged into liquid nitrogen (-196ºC) for storage.

Experimental design

In this study 3 ejaculates/male were used. Samples were thawed in a circulating water bath (60º C for 10 s). Semen evaluation was performed immediately after thawing and then 3 treatments were prepared: control, catalase (500 units/ml) and epigallocatechin (1 mM). Semen evaluation for the different treatments was performed at 1.5 h and 3 h. Parameters evaluated included: sperm total and progressive motility, kinematics (Computer Assisted Sperm Analysis, CASA), acrosome and membrane integrity. The MDA concentration was measured 3 hours after thawing in the different treatments.

Sperm assessment

To determine sperm total and progressive motility and kinematics, aliquots of sperm samples (2µl) were placed in a disposable chamber (Cytonix, Beltsville, MD; USA) and analysed using CASA (CEROS II®; Hamilton Thorne; MA; USA); images of 300 sperm were captured per sample. Sperm kinematics included average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), amplitude of lateral head displacement (ALH, µm), straightness (STR, %), linearity (LIN, %), and beat cross frequency (BCF, Hz).

Plasma membrane integrity was evaluated using the fluorescent probes SYBR-14 (SY) and propidium iodide (PI) according to the manufacturer's instructions (L-7011, Live/Dead Sperm Viability Kit; Molecular Probes Europe, Leiden, the Netherlands). Briefly, 30 µl of thawed spermatozoa (60 million/ml in GB) was diluted with 6 µl SYBR-14 solution (final concentration: 20 µM) and incubated 10 minutes at 38 ºC and 5% CO2. Then 1 µl of PI solution (final concentration: 12 µM) was added and the mixture was incubated for 10 minutes in the same conditions (Garner & Johnson, 1995). 1 µl of formalin (0.5%) was added to the sample to immobilize the sperm and 30 µl of freezing extender (GB supplemented with 20% (v:v) egg-yolk) was added to obtain a final concentration of 30 million/ml. Spermatozoa were assessed using fluorescence microscopy (Olympus bx53) and allocated to ''intact membrane" or ''dead''

classifications if they exhibited SY+/PI- (green), and SY-/PI+ (red) staining, respectively (figure 5); 400 sperm were evaluated per sample and the proportion of viable sperm was calculated.

Acrosome status was assessed by fluorescein isothiocyanate conjugated with peanut agglutinin (FITC-PNA) and PI staining as described by Nagy *et al* (2003), with slight modifications. Briefly, 30 µl of thawed spermatozoa (60 million/ml in GB) were diluted with 170 µl of GB, 6 µl FITC-PNA solution (2 µg/ml), 2 µl PI solution and 0.5 µl of Paraformaldehyde (4%) and incubated for 10 minutes at 37 ^{o}C. Using fluorescence microscopy (Olympus bx53) spermatozoa were allocated to "damaged acrosome,'' or ''intact acrosome'' classifications if they exhibited PNA+ (green acrosome) and PNA- (absence of colour) staining, respectively (figure 6). Four hundred spermatozoa were evaluated per sample and the percentages of intact acrosome sperm were calculated. The reason for using PI in the staining even though we did not classify the spermatozoa as live/dead was that it was then easier to identify the unstained spermatozoa, because of a faint fluorescence emanating from their surface.

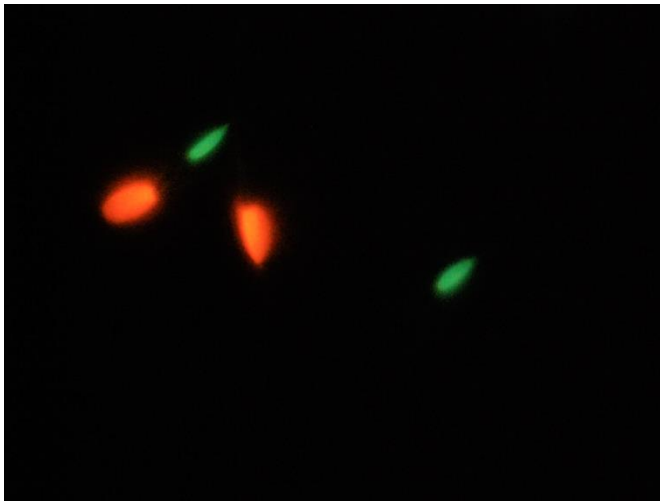

Fig. 5. *Vitality, or assessement of plasma membrane integrity using SYBR-14 and PI staining. Green colour is classified as live and red colour is dead.*

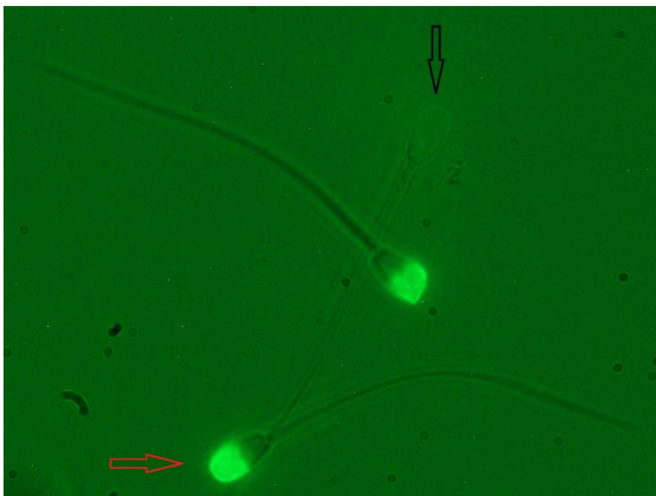

Fig. 6. *Assessement of acrosome status using FITC-PNA and PI staining. Black arrow shows an intact acrosome, red arrow shows a spermatozoa with damaged acrosome.*

Membrane lipid peroxidation was estimated by the end point generation of MDA determined by the thiobarbituric acid (TBA) test (Esterbauer & Cheeseman, 1990). Briefly, diluted sperm samples (60 million/ml) were mixed with 1 mL of cold 20% (wt/vol) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation. (1500 x g for 10 minutes), and 1 mL of the supernatant was incubated with 1 mL of 0.67% (wt/vol) TBA in a boiling water bath at 100 ºC for 10 minutes. After cooling, the absorbance was determined by a spectrophotometer (Shimadzu, UV-visible spectrophotometer; UV-1700, PharmaSpec) at 534 nm. The results were expressed as a concentration of MDA (g per million of sperm).

Statistical analyses

Prior to statistical analysis, data were tested for normality via Shapiro-Wilk tests. All parameters met normality assumptions except for the MDA concentration. To check the male effect, pre-freeze and post-thaw parameters at 0 h were analysed by ANOVA with Duncan as Post-hoc test. Parameters at 1.5 and 3 hours were analysed by generalized linear model (GLM), using male and treatments as fix factors, with means compared by Duncan´s multiple range method. Spearman correlations were calculated between MDA concentration with male and treatment factors. All statistical models were analysed SPSS 11.0 for Windows. The level of significance was set at $P < 0.05$. The data are presented as means ± SEM.

RESULTS

The pre-freezing values for sperm total (TM) and progressive motility (PM) among the different males are presented in Table 1, together with the 0 h post-thaw values for sperm TM and PM, as well as vitality (VIT) and acrosome damage (AD). Results showed that before freezing and at 0 h post-thaw, no significant differences for these parameters among the males of the study could be demonstrated.

Table 1: *Pre-freezing values for total motility (TM) and progressive motility (PM) as well as the 0 h post-thaw quality (TM, PM, VIT, AD) from the 6 males of the study. Data represent means ± SEM*

Male	Fresh semen (% of sperm)		Frozen-thawed semen (% of sperm) at 0 h			
	TM	PM	TM	PM	VIT	AD
A	79.75 ± 6.55	24.45 ± 4.05	46.67 ± 2.51	18.50 ± 4.72	51.17 ± 4.51	22.00 ± 5.53
B	83.20 ± 0.63	38.00 ± 5.30	33.00 ± 1.88	8.07 ± 1.80	39.83 ± 2.33	34.33 ± 3.17
C	79.13 ± 5.59	34.00 ± 6.71	44.50 ± 6.12	17.07 ± 3.95	43.17 ± 8.56	27.33 ±7.10
D	89.05 ± 5.35	38.20 ± 12.10	42.43 ± 2.22	16.83 ± 0.18	42.17 ±1.92	25.67 ± 5.07
E	78.63 ± 7.19	31.10 ± 5.25	44.70 ± 4.31	18.70 ± 1.40	40.50 ± 5.53	25.83 ± 6.93
F	80.10 ±	43.70 ±	39.70 ± 4.96	15.33 ± 2.05	39.17 ± 2.13	38.33 ± 2.45
P-value	0.817	0.663	0.253	0.153	0.541	0.318

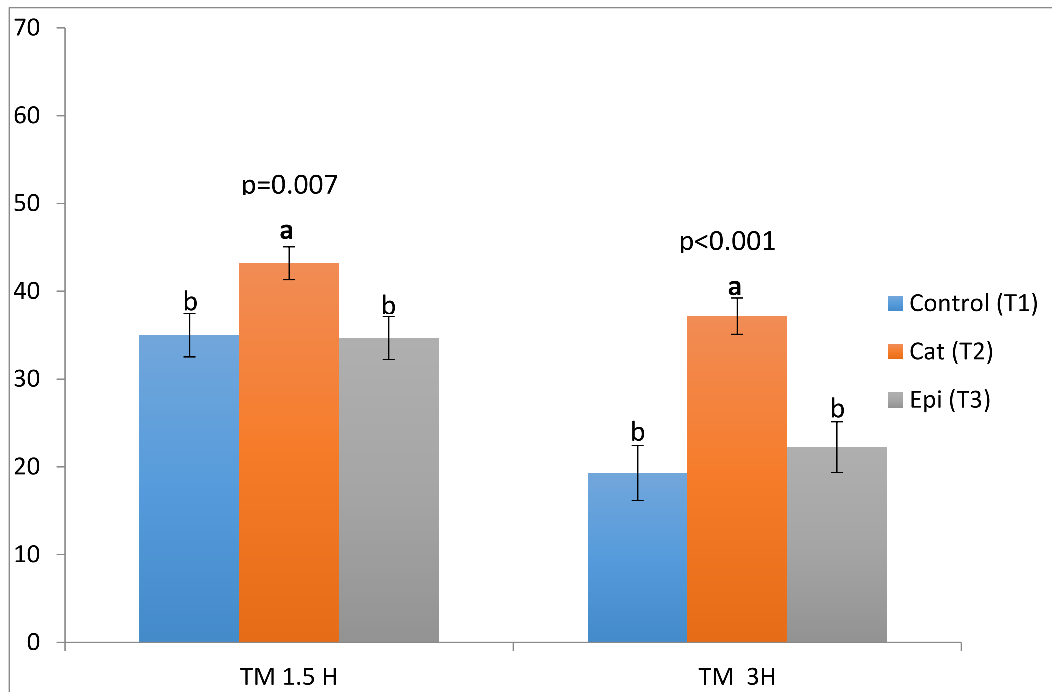

Figure 7. *Effect of the antioxidants (Catalase and Epigallocatechin) added at thawing on sperm total motility (TM) at 1.5 h and 3 h post-thaw. Different letters express the differences among treatments (p<0.05).*

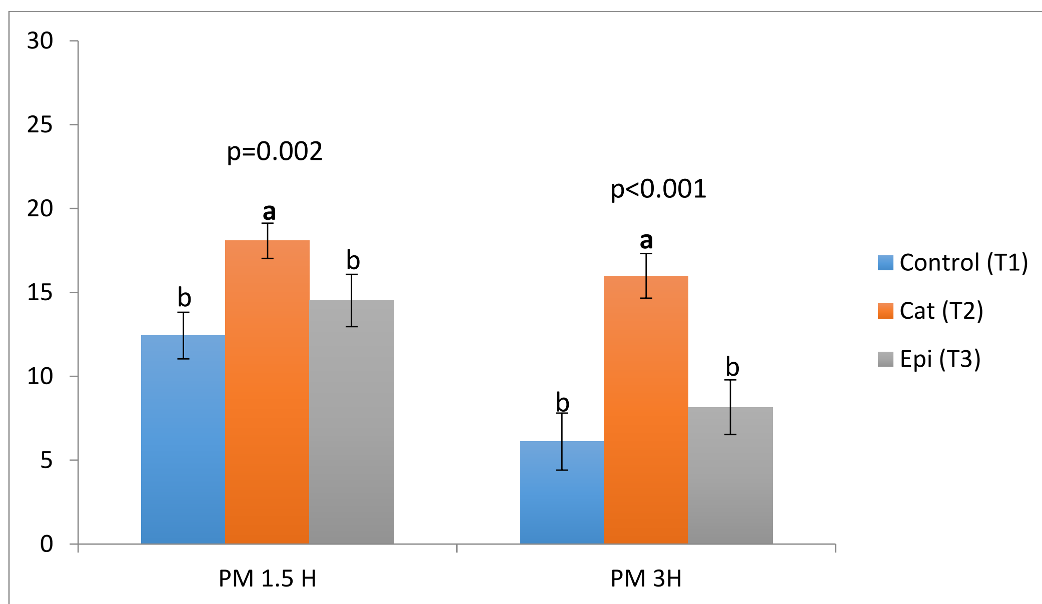

Figure 8. *Effect of the antioxidants (Catalase and Epigallocatechin) added at thawing on sperm progressive motility (PM) at 1.5 h and 3 h post-thaw. Different letters express the differences among treatments (p<0.05).*

Significant differences were found between treatments Control (T1), Catalase (T2) and Epigallocatechin (T3) on sperm TM and PM at 1.5 and 3 h after thawing (Fig 7 and 8). T2 had significantly higher TM and PM values at both 1.5 h and 3h after thawing than T1 and T3; no differences were found between T1 and T3.

Table 2. *Effect of the antioxidants (Catalase and Epigallocatechin) added at thawing on kinematics, vitality (VIT) and acrosome damage (AD) at 1.5 h after thawing. Data represent means ± SEM. Different letters among columns indicate significant differences (p<0. 05)*

	Control (T1)	Catalase (T2)	Epigallocatechin (T3)	*p*-value
ALH	7.64 ± 0.14^b	8.13 ± 0.11^a	7.65 ± 0.15^b	0.008
BCF	19.95 ± 0.28	20.18 ± 0.34	20.43 ± 0.49	0.676
LIN	31.16 ± 0.78	33.22 ± 0.68	31.69 ± 0.69	0.089
STR	64.46 ± 0.86^b	67.26 ± 0.71^a	67.22 ± 1.08^a	0.016
VAP	64.75 ± 2.06^b	73.13 ± 1.58^a	64.62 ± 2.34^b	<0.001
VCL	137.02 ± 3.58^b	151.95 ± 2.94^a	139.39 ± 4.39^b	<0.001
VSL	42.34 ± 1.81^b	50.02 ± 1.52^a	43.72 ± 2.06^b	<0.001
VIT	38.53 ± 1.45	40.81 ± 1.91	40.39 ± 1.94	0.540
AD	31.89 ± 2.65	32.08 ± 2.72	33.72 ± 2.92	0.860

Notes: Average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), amplitude of lateral head displacement (ALH, µm), straightness (STR, %), linearity (LIN, %), and beat cross frequency (BCF, Hz).

When looking at the effect of the different treatments (T1, T2 and T3) on sperm kinematics, VIT and AD at 1.5 h after thawing (Table 2) T2 showed higher values of ALH ($p = 0.008$), VAP ($p < 0.001$), VCL ($p < 0.001$) and VSL ($p < 0.001$) than the other treatments. Both T2 and T3 showed higher STR values compared to T1 ($p = 0.016$). No significant differences for VIT and AD at 1.5 h after thawing could be found among treatments.

Table 3. *Effect of the antioxidants (Catalase and Epigallocatechin) added at thawing on kinematics, vitality (VIT) and acrosome damage (AD) at 3 h after thawing. Data represent means ± SEM. Different letters among columns indicate significant differences (p<0.05)*

	Control (T1)	Catalase (T2)	Epigallocatechin (T3)	*p*-value
ALH	6.96 ± 0.17^b	7.94 ± 0.16^a	7.27 ± 0.20^b	<0.001
BCF	19.20 ± 1.17^b	20.51 ± 0.37^{ab}	22.40 ± 0.83^a	0.049
LIN	33.79 ± 3.88	33.17 ± 0.71	29.65 ± 0.80	0.402
STR	63.67 ± 2.52	67.33 ± 0.58	64.38 ± 1.29	0.299
VAP	55.13 ± 3.11^b	70.19 ± 2.51^a	55.47 ± 2.83^b	<0.001
VCL	112.23 ± 5.87^c	146.24 ± 4.62^a	124.03 ± 5.55^b	<0.001
VSL	36.31 ± 3.53^b	47.96 ± 1.99^a	36.30 ± 2.30^b	0.002
VIT	37.42 ± 1.71	41.36 ± 2.10	39.47 ± 1.91	0.310
AD	32.78 ± 2.54	33.14 ± 2.54	34.81 ± 3.01	0.813

Notes: Average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), amplitude of lateral head displacement (ALH, µm), straightness (STR, %), linearity (LIN, %), and beat cross frequency (BCF, Hz).

Table 3 shows the effect of the different treatments (T1, T2 and T3) on sperm kinematics, VIT and AD at 3 h after thawing: ALH was highest in T2 ($p < 0.001$) compared to T1 and T3. Meanwhile T3 showed higher BCF than T1 ($p = 0.049$), but did not differ from T2. For the

velocity parameters, T2 showed higher values for VAP ($p < 0.001$), VCL ($p < 0.001$) and VSL ($p = 0.002$) than other treatments. No significant differences for VIT and AD at 3 h after thawing could be found among treatments.

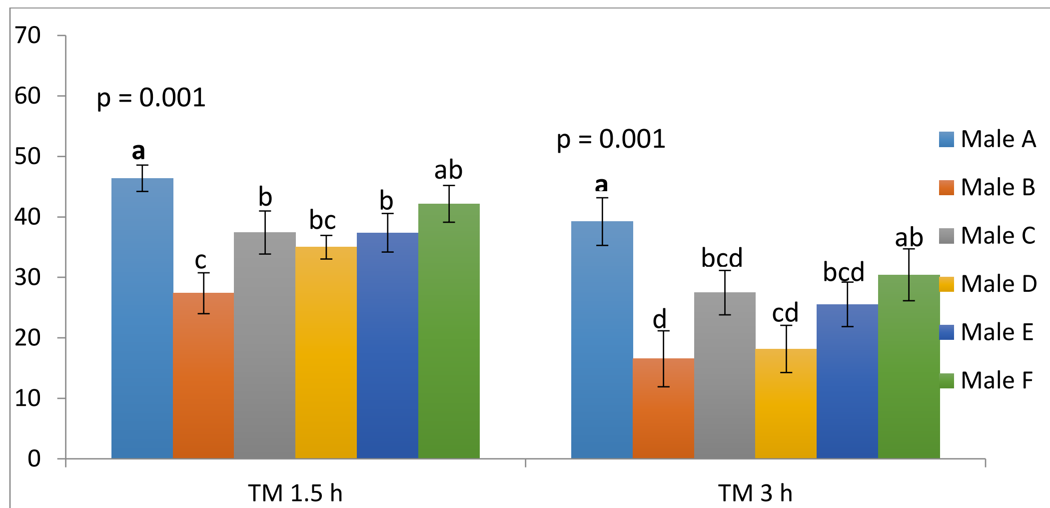

Figure 9. *Male effect on sperm total motility (TM) at 1.5 h and 3 h after thawing. Different letters indicate significant differences (p<0.05). Different letters express the differences among males (p<0.05).*

Figure 9 illustrates the differences among males on sperm TM after thawing, at 1.5 h ($p = 0.001$) and 3 h ($p = 0.001$). Male A and Male F showed the highest values (Male A: 46.39% and 39.23% ; Male F: 42.16 % and 30.43% at 1.5 and 3 h, respectively). Male B showed the lowest values for TM (27.38 % and 16.54% at 1.5 h and 3 h, respectively).

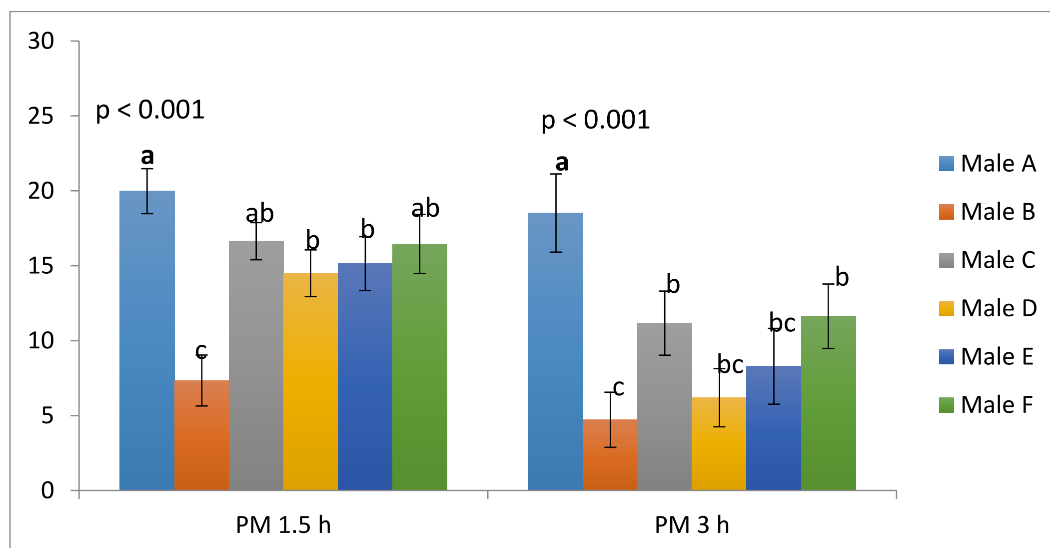

Figure 10. *Male effect on sperm progressive motility (PM) at 1.5 h and 3 h after thawing. Different letters indicate significant differences (p<0.05). Different letters express the differences among males (p<0.05).*

Differences in PM among males at 1.5 and 3 h after thawing are presented in Figure 10. Males A, C and F showed the highest values at 1.5 h post-thaw, but only Male A maintained better PM after 3 h. Male B presented the lowest values for PM at both 1.5 and 3 h after thawing.

Table 4. *Male effect on sperm kinematics, vitality (VIT) and acrosome damage (AD) at 1.5 h after thawing. Data represent means ± SEM. Different letters among columns indicate significant differences (p<0.05)*

	Male A	Male B	Male C	Male D	Male E	Male F	p-value
TM	46.39 ± 2.18^{a}	27.38 ± 3.38^{c}	37.42 ± 3.56^{b}	34.99 ± 1.95b^{c}	37.38 ± 3.19^{b}	42.16 ± 3.04^{ab}	0.001
PM	19.98 ± 1.50^{a}	7.34 ± 1.70^{c}	16.64 ± 1.24^{ab}	14.50 ± 1.56^{b}	15.14 ± 1.80^{b}	16.46 ± 1.97^{ab}	<0.001
ALH	8.16 ± 0.14^{a}	7.29 ± 0.15^{c}	8.23 ± 0.13^{a}	7.65 ± 0.27^{bc}	7.76 ± 0.18^{abc}	7.80 ± 0.16^{ab}	0.002
BCF	20.22 ± 0.47	20.45 ± 0.57	20.96 ± 0.36	19.42 ± 0.46	20.14 ± 0.72	19.86 ± 0.48	0.571
LIN	34.51 ± 0.95^{a}	29.14 ± 0.78^{c}	32.17 ± 0.53^{ab}	33.27 ± 1.27^{ab}	32.85 ± 1.03^{ab}	30.90 ± 0.80^{bc}	0.009
STR	67.78 ± 0.87^{a}	60.97 ± 1.54^{b}	68.29 ± 0.63^{a}	67.46 ± 1.23^{a}	67.14 ± 0.86^{a}	66.70 ± 0.88^{a}	<0.001
VAP	78.93 ± 2.51^{a}	56.82 ± 2.05^{d}	70.75 ± 1.20^{b}	67.89 ± 2.60^{bc}	68.53 ± 2.89^{bc}	64.65 ± 2.29^{c}	<0.001
VCL	158.74 ± 4.68^{a}	121.92 ± 4.17^{c}	153.75 ± 2.76^{a}	140.71 ± 4.82^{b}	142.92 ± 4.63^{b}	142.00 ± 3.46^{b}	<0.001
VSL	54.06 ± 2.34^{a}	35.02 ± 1.72^{c}	48.89 ± 0.89^{b}	46.07 ± 2.54^{b}	46.47 ± 2.53^{b}	43.66 ± 1.96^{b}	<0.001
VIT	49.06 ± 1.86^{a}	33.33 ± 2.59^{c}	39.50 ± 2.16^{bc}	38.67 ± 1.38^{bc}	40.39 ± 2.21^{b}	38.50 ± 1.00^{bc}	0.001
AD	26.94 ± 2.87^{b}	42.50 ± 1.31^{a}	34.67 ± 3.65^{ab}	26.28 ± 2.65^{b}	25.28 ± 3.57^{b}	39.72 ± 2.81^{a}	0.004

Notes: Average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), amplitude of lateral head displacement (ALH, µm), straightness (STR, %), linearity (LIN, %), and beat cross frequency (BCF, Hz).

For kinematics, VIT and AD among males at 1.5 h after thawing (Table 4), Male A and Male C showed higher values for ALH compared to Male B and D (p = 0.002). Male B presented significantly lower values than the other males, except for Male F. Only Male A showed significantly higher LIN than Male F. For STR, Male B was significantly lower than the other males (p < 0.001). For VAP and VSL, Male A presented significantly higher values than the other males (p < 0.001). Meanwhile, both Male A and Male C had higher VCL values compared to the others (p < 0.001).

In terms of VIT at 1.5 h post-thaw, Male A showed the highest value (49.06 %) and Male B the lowest (33.33 %) (p = 0.001). Significantly more AD was found in Males B, C and F (p = 0.004), although Male C did not differ from Males A, D and E.

Table 5. *Male effect on sperm kinematics, vitality (VIT) and acrosome damage (AD) at 3 h after thawing. Data represent means ± SEM. Different letters among columns indicate significant differences (p<0.05)*

	Male A	Male B	Male C	Male D	Male E	Male F	p-value
TM	39.23 ± 3.94[a]	16.54 ± 4.63[d]	27.48 ± 3.67[bcd]	18.17 ± 3.90[cd]	25.54 ± 3.68[bcd]	30.43 ± 4.29[ab]	0.001
PM	18.52 ± 2.61[a]	4.72 ± 1.84[c]	11.17 ± 2.14[b]	6.19 ± 1.94[bc]	8.29 ± 2.53[bc]	11.63 ± 2.15[b]	<0.001
ALH	7.84 ± 0.10[a]	6.73 ± 0.28[b]	8.02 ± 0.17[a]	6.96 ± 0.32[b]	7.09 ± 0.33[b]	7.68 ± 0.19[a]	<0.001
BCF	20.61 ± 0.30	20.33 ± 2.74	21.35 ± 0.77	20.64 ± 0.91	20.47 ± 0.45	20.82 ± 1.12	0.995
LIN	34.17 ± 1.27	37.48 ± 7.64	30.49 ± 0.67	31.26 ± 1.46	30.21 ± 1.36	29.59 ± 1.10	0.511
STR	67.68 ± 1.54	64.97 ± 4.76	66.36 ± 1.20	64.56 ± 1.30	62.49 ± 1.96	64.69 ± 1.80	0.762
VAP	73.68 ± 3.80[a]	51.10 ± 4.59[c]	65.26 ± 2.67[ab]	52.95 ± 4.74[c]	56.90 ± 4.64[bc]	60.79 ± 3.14[bc]	<0.001
VCL	150.27 ± 5.27[a]	101.21 ± 7.65[e]	144.33 ± 4.97[ab]	113.11 ± 8.23[de]	121.38 ± 9.02[cd]	134.70 ± 5.80[bc]	<0.001
VSL	50.48 ± 3.41[a]	35.64 ± 6.06[b]	43.98 ± 2.49[ab]	34.54 ± 3.50[b]	36.39 ± 4.15[b]	40.12 ± 2.76[ab]	0.023
VIT	48.94 ± 2.44[a]	33.39 ± 2.71[b]	39.00 ± 2.79[b]	38.83 ± 0.90[b]	38.56 ± 2.48[b]	37.78 ±2.04[b]	0.005
AD	24.72 ± 2.17[c]	45.33 ± 2.77[a]	33.39 ± 3.24[bc]	28.50 ± 2.67[c]	28.17 ± 4.03[c]	41.33 ± 2.51[ab]	<0.001

Notes: Average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), amplitude of lateral head displacement (ALH, µm), straightness (STR, %), linearity (LIN, %), and beat cross frequency (BCF, Hz).

Table 5 shows kinematics, VIT and AD among males 3h after thawing. Male A, C and F had significantly higher ALH values than other males ($p < 0.001$); VAP and VCL were increased in Male A and Male C ($p < 0.001$). Males A, C and F had the highest values for VSL ($p = 0.023$). For all these velocity parameters (VAP, VCL and VSL) only Male A showed significantly higher values.

VIT was also significantly increased at 3 h post-thawing for Male A compared to other males (48.94 %) ($p = 0.005$), Male B showed the lowest VIT (33.39 %). In terms of AD, Male B showed the highest damage (45.33 %) compared to the other males except for Male F. Male A presented the lowest AD values (24.72 %)

Our evaluation of membrane lipid peroxidation, measured by the end point generation of MDA 3 h after thawing, showed no significant effect of antioxidants added at thawing. Also, no significant male effect on MDA production could be demonstrated.

DISCUSSION

The use of antioxidants during semen cryopreservation has gained popularity, as this may reduce lipid peroxidation, increasing the post-thaw survival of cryopreserved spermatozoa. Several studies have shown a beneficial effect of antioxidants on post-thaw sperm quality parameters implying that oxidative stress occurs during cryopreservation. Successful results have been obtained for several species using natural antioxidants; therefore the aim of this study was to determine the effect of adding natural antioxidants to the thawing media on dromedary camel sperm quality parameters following cryopreservation.

Prior to this study, a pilot study was conducted using 1 ejaculate/male where we compared the effect of 7 natural antioxidants (biotin, melatonin, epigallocatechin, rosmarinic acid, curcumin, TROLOX, and catalase) added at thawing, in order to select two antioxidants for the project. All antioxidants except catalase were added at a final concentration of 1mM per ml at thawing. Catalase was added at a final concentration of 500 units/ml. Total (TM) and progressive motility (PM) were then measured at 2 and 4 hours after thawing. Catalase and Epigallocatechin showed better results than the control and the other antioxidants (data not shown) and were therefore chosen for this study.

Our study showed that the process of freezing and thawing during cryopreservation of dromedary camel sperm decreases sperm total (TM) and progressive motility (PM). This was true for all males of the study and these results are consistent with studies done on the cryopreservation of semen from other species. Furthermore the addition of catalase to the thawing media was better than epigallocatechin in improving the post-thaw quality of cryopreserved dromedary camel spermatozoa. Some of these effects where maintained up to 3 h post-thaw.

None of the treatments showed any significant effect on vitality (VIT), acrosome damage (AD) or LIN after thawing or MDA production. However, a significant difference between males was found for certain post-thaw sperm quality parameters such as TM, PM, VIT, AD and some kinematic parameters indicating that the spermatozoa of some males freeze better than others i.e. that the sperm from some males are more resistant to damage during cryopreservation.

Our results agree with results from similar studies for other species. Roca *et al*., (2005) evaluated the effect of adding CAT to the freezing extender during cryopreservation of boar spermatozoa and obtained significantly increased post-thaw rates for TM and PM compared with control. Maxwell & Stojanov (1996) found that the addition of CAT to the extender improves post-thaw sperm quality parameters, survival and *in vitro* fertility of liquid stored ram spermatozoa.

In the study by Roca *et al*., (2005) authors also evaluated the *in vitro* fertilization ability and measured the MDA production and ROS levels, finding an improved ability of frozen-thawed boar spermatozoa treated with CAT to produce embryos *in vitro* and significantly lower levels

of ROS. The MDA production, however, was unaffected corresponding with the results obtained from our study.

In the dromedary camel there are limited studies to be found evaluating the protective effect of antioxidants in cryopreservation extenders. Malo *et al*., (unpublished data) demonstrated that CAT added to the freezing extender could maintain post-thaw sperm quality significantly longer ($p<0.05$), with increased TM, PM, ALH, VAP, VCL and VSL compared to control. With our experiment we obtained similar results by adding CAT to the thawing extender, although epigallocatechin did not have the same beneficial effect. Medan *et al*., (2008) studied the effect of adding different concentrations of catalase to cooled dromedary camel semen on sperm quality during storage at 5 ^{o}C for up to 5 days. Results showed that addition of catalase at concentrations of 250 and 500 IU/ml significantly increased ($p<0.01$) sperm motility and significantly decreased ($p<0.01$) percentage of dead spermatozoa, sperm abnormalities and acrosomal damage. The conception rate was also found to be significantly higher after AI with cooled liquid stored camel semen supplemented with catalase than after AI with cooled liquid stored camel semen without the addition of catalase.

Studies have also been conducted on other species evaluating Epigallocatechin as a protective additive during semen cryopreservation. Gadani *et al*., (2017) assessed the effect of different concentrations of EGCG (epigallocatechin-gallate) supplementation to the thawing media on sperm quality parameters (viability, acrosome integrity, in-vitro oocyte maturation and in-vitro fertilization) following cryopreservation of boar sperm. Results from this study showed no effect on sperm viability or acrosome integrity after thawing, corresponding with our results. However, in their study no assessment of motility parameters or kinematics (CASA) was performed.

Besides not showing any effects on vitality or acrosome integrity after thawing, the study by Gadani *et al*., (2017) showed a significantly ($p<0.01$) increased penetration rate *in vitro* for EGCG treated sperm compared to control and also a positive effect on the total efficiency of fertilization. However, different results were obtained by Kitaji *et al*., (2015) who observed a higher viability and acrosome integrity of boar sperm incubated prior to freezing in a freezing extender supplemented with green tea polyphenols (i.e. epigallocatechin). Green tea polyphenols added to the semen extender were also used in the study by Kitaji *et al.* (2015) and was found to be effective in increasing the rates of monospermic fertilization and blastocyst formation. Epigallocatechin in the semen extender has also been shown to have beneficial effects on the motility and viability of liquid canine semen stored for 5 weeks at 5°C (Wittayarat *et al., 2013).* These studies indicate that addition of EGCG to spermatozoa during cryopreservation, even if it does not exert any effect on sperm viability and acrosome integrity after thawing, effectively improves *in vitro* oocyte penetration rate and increases the total efficiency of fertilization.

Our positive results using CAT in the thawing media support the theory that H_2O_2 appears to be the key agent in the toxic effects observed in spermatozoa following cryopreservation. Addition of CAT in the cryopreservation extender reduces post-thaw ROS generation and also improves post-thaw motility and in vitro fertilization ability (Roca *et al*., 2005). A study by Baumber *et al*., (2003) showed a similar result for equine spermatozoa; an increase in DNA fragmentation associated with ROS-generating treatment was counteracted by the addition of

CAT but not SOD, suggesting that H_2O_2 and not O_2^- appears to be the ROS responsible for such damage.

To summarize, according to the results of this study, addition of catalase to the thawing media can prove beneficial for the survival of dromedary camel sperm by improving post-thaw sperm quality in terms of motility and kinematics. The beneficial effects of addition of antioxidants in the cryopreservation media suggest that ROS are likely to be important contributors to the reduced viability of spermatozoa following cryopreservation. However, additional studies need to be conducted to fully understand the potential positive impacts of antioxidant treatments in camel sperm cryopreservation. It is important to determine whether improved viability of spermatozoa would be reflected in fertility. As mentioned above, other studies on different species using the same antioxidants, show an improvement in the total efficiency of in vitro fertilization; therefore, further studies regarding the effects of antioxidants in the cryopreservation media on the *in vitro* oocyte maturation and fertilization ability of dromedary camel sperm are important for further evaluation of this technique.

Another interesting subject for further studies is to measure ROS levels following cryopreservation using chemiluminescence methods. Measuring LPO by the end-point generation of MDA can be useful, but LPO is not the only mechanism by which ROS can lead to impaired sperm function. As mentioned above, in the study by Roca *et al.*, (2005) significantly lower levels of ROS were found in spermatozoa treated with CAT in the freezing extender; the MDA production, however, was unaffected, corresponding with our results with addition of CAT in the thawing extender. Previous studies, such as the one by Chatterjee & Gagnon (2001), measured an increase in ROS production following cryopreservation of bovine spermatozoa, together with a decrease in motility parameters. In the same study the authors demonstrated that lipid peroxidation (measured by MDA production) was higher in thawed bovine spermatozoa, a likely consequence of an increase in ROS production, indicating that LPO is a potential cause for reduced viability after cryopreservation. In many studies MDA levels increased in frozen-thawed bull sperm (Bansal & Bilaspuri, 2011). Besides showing an increase in end-products of LPO, studies have also shown a significant decrease in phospholipid content in the sperm plasma membrane following cryopreservation (Alvarez & Storey, 1992), suggesting that one effect of the cryopreservation process is LPO. However, hypothesizing that ROS and LPO are major contributors to cryoinjury, one would expect to see a decrease in MDA production following antioxidant treatment during cryopreservation. Lipid peroxidation, however, is not the only mechanism by which sperm function might be impaired by ROS. Beneficial effects on sperm quality after thawing by adding CAT in the cryopreservation media suggest that ROS are likely to be important contributors to the reduced viability of spermatozoa following cryopreservation. However, alternative beneficial influence of the antioxidants cannot be excluded.

Camel semen cryopreservation is possible and would be useful in the breeding strategy. However, because of the relatively poor quality of the camel ejaculate as well as problems associated with the collection and handling of camel sperm, improvements in the methods used for these steps are needed. The results from this study could be important not only *in vitro*, but also *in vivo* as addition of these antioxidants in commercial thawing media might enhance sperm fertilizing ability and reproductive preformance during AI with frozen-thawed camel semen.

CONCLUSION

According to our results catalase added at thawing improves post-thaw dromedary camel sperm quality and is, therefore, beneficial for the survival of dromedary camel sperm following cryopreservation. These results supports the theory that ROS and LPO contribute to the reduced viability of spermatozoa following cryopreservation and the decrease in pregnancies following AI with cryopreserved spermatozoa compared to fresh semen. However, as our results did not show any effect of catalase in decreasing LPO measured by a decrease of MDA production post-thaw and as ROS levels were not analysed in this study, an alternative beneficial influence of catalase cannot be excluded; thus an evaluation of the effect of antioxidants on ROS levels post-thaw would be an interesting aim for further studies. Since the functionality of the samples could not be tested in this study further studies regarding the effects of antioxidants in the cryopreservation media on the *in vitro/in vivo* oocyte fertilization ability of dromedary camel sperm are important for further evaluation of this technique.

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