Sveriges lantbruksuniversitet
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Effect of different thawing protocols and addition of the antioxidants carnitine, catalase or glutathione prior to freezing on the post-thaw quality of cryopreserved camel spermatozoa

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Effekten av upptiningstemperatur och antioxidanterna karnitin, katalas och glutation på kryopreserverad kamelsperma

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SUMMARY

This study examined whether the addition of antioxidants could improve post-thaw dromedary semen quality, as indicated in studies in other species, and also whether thawing temperature could affect sperm quality. Three antioxidants: catalase (CAT), carnitine (CARN) and glutathione (GSH), were added before freezing, and two thawing temperatures (60 °C for 10 s and 37 °C for 30 s) were evaluated. Semen used in this study came from four fertile dromedary camel males, two ejaculates per male (in total eight ejaculates). One straw from each sample and treatment was thawed at 60 °C for 10 s and the other at 37 °C for 30 s; sperm quality was evaluated at 0, 1 and 2 h post thawing. Parameters evaluated were total and progressive motility (TM and PM), and the kinematics curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and the ratios straightness (STR) and linearity (LIN), using computer-assisted semen analysis (CASA), membrane integrity and acrosome integrity (eosin-nigrosine) and membrane functionality (HOST-test). The results showed a significantly higher ($p<0.05$) TM and VAP 1 h post thawing for CAT and CARN compared to control. At 2 h post thawing were several values significantly higher ($p<0.05$) TM, PM, ALH, VAP, VCL, VSL for CAT, CARN and GSH compared to control. The results suggest that antioxidants exert a protective effect during cryopreservation of camel spermatozoa. Thawing the samples at 60 °C for 10 s gave significantly higher ($p<0.05$) TM and PM at 0 h post thawing, TM, PM, ALH, STR and VCL at 1 h post thawing, and TM and PM at 2 h post thawing compared to 37 °C for 30 s. These results suggest that it is better to thaw camel semen at 60 °C for 10 s compared to 37 °C for 30 s.

SAMMANFATTNING

Syftet med denna studie var att undersöka huruvida tillsats av antioxidanter skulle kunna förbättra kvaliteten hos kryopreserverad kamelsperma efter upptining. Studien undersökte också om upptiningstemperatur kan påverka kvaliteten hos kryopreservarade spermier. Tre olika antioxidanter; Katalas (CAT), karnitin (CARN) och glutation (GSH) tillsattes före frysning, och två olika upptiningstemperaturer (60 °C i 10 s under och 37 °C i 30 s under) utvärderades. Spermierna som användes i studien kom från fyra fertila kamelhanar, två ejakulat från varje hane, totalt 8 olika prover. Från vardera behandlingen tinades sedan ett prov i 60 °C i 10 s och ett i 37 °C i 30 s. Spermiekvaliteten utvärderades sedan vid 0, 1 och 2 h efter upptining. Parametrar som utvärderades var total och progressiv motilitet (TM och PM) och olika kinematik t.ex. curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), tillsammans med straightness (STR) and linearity (LIN) med hjälp av datoriserad spermieanalys (computer-assisted semen analysis, CASA), membranintegritet och akrosomintegritet (med hjälp av eosin-nigrosin färgning) och membranfunktionalitet (HOST-test). Resultaten visade en signifikant högre ($p < 0,05$) TM och VAP 1 h efter upptining för CAT och CARN jämfört med kontroll. Vid 2 h efter upptining var flera värden, TM, PM, ALH, VAP, VCL, VSL för CAT, CARN och GSH, signifikant högre ($p < 0,05$) jämfört med kontroll. Resultaten tyder på att antioxidanter har en skyddande effekt under kryopreservering av kamelspermier. Upptining av proven vid 60 °C i 10 s gav signifikant högre ($p < 0,05$) TM och PM 0 h efter upptining, TM, PM, ALH, STR och VCL 1 h efter upptining och TM och PM 2 h efter upptining jämfört med 37 °C i 30 s. Dessa resultat tyder på att det är bättre att tina kamelsperma vid 60 °C i 10 s jämfört med 37 °C i 30 s.

CONTENT

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INTRODUCTION

Camels have been of substantial importance for people in the dry desert climates for centuries. They possess features that enable them to survive in harsh and dry climates and they have served as an important livestock animal, providing people with milk, meat, transport and security (Skidmore, 2005). Nowadays, camels have also come to play a significant role as a sports animal. The Middle-East racing industry is enormous, and camels can be worth millions of dollars (Spencer et al., 2010). When it comes to reproduction, less work has been done in camels than in other species (Skidmore, 2005). Recently, however, financial incentives from the racing industry have made reproductive- and herd management of greater importance in the Middle East (Spencer *et al.*, 2010).

There are several physiological characteristics in camels that have made optimisation of reproductive technologies challenging compared to other livestock species. The complex reproductive physiology of both male and female contribute to the overall reproductive efficiency being low (Crichton *et al.*, 2015). Partly, the problems can be linked to a short breeding season, long gestation period (13 months), delayed onset of puberty, prolonged period of lactation-induced anoestrus and relatively high rate of embryotic mortality (Skidmore, 2011).

To improve reproductive efficiency, assisted reproduction techniques can be used, such as embryo transfer and artificial insemination. However, as mentioned above, the techniques have not been optimised and, compared to other livestock species, progress in semen preservation has been slow (Bravo *et al.*, 2000). One contributing factor is the problem with long-term storage; camelid spermatozoa are not very tolerant to freezing and thawing using conventional procedures. The use of artificial insemination in camels is mostly limited to fresh semen, and even so, the pregnancy results are relatively low. Cryopreserved camel semen has low post-thaw motility and few, if any, pregnancies have been obtained with frozen-thawed semen (Bravo *et al.*, 2000; Crichton *et al.*, 2015; Morton *et al.*, 2010). To be able to fully benefit from the advantages of AI, methods and specific protocols for cryopreservation of camel semen must be developed. Nowadays, the protocols that have been used are modified from other species, and as already mentioned, published results are few with a relatively low success rate (Deen *et al.*, 2003).

Cryopreservation and artificial insemination is today a fundamental part of reproduction in most species. Cryopreservation is beneficial in many ways, making transportation and storage easier, enabling genetic improvements and preservation of genetic material, preventing transmission of diseases, among others (Sieme and Oldenhof, 2015). Studies in other species have indicated that antioxidants can be of great value to improve the success-rate in cryopreservation, but similar studies are lacking in camels.

The aim of this study was to evaluate if post-thaw camel sperm quality could be influenced by three antioxidants and two thawing temperatures.

LITERATURE REVIEW

Camelus dromedaries

Camelus, or camels, are even-toed ungulate mammals belonging to the family *Camelidae* together with *Lama*. The genera *Camelus* consists of the bactrian camel with two humps and the dromedary camel with one hump. Both bactrian and dromedary camels have 27 chromosomes and can interbreed to produce fertile offspring (Bravo *et al.*, 2000).

Reproduction

Camels are seasonal breeders and reproduce during the cooler months. In Arabia the breeding-season is reported to be between November and April, but warm weather and inadequate feeding can have a negative effect on the season´s length. The gestation period is 13 months and the dromedary camels usually have their first calving at 5 years of age, or more, but the onset of puberty in both male and female camels can be affected by nutritional status. Male camels tend to reach puberty around 3-3.5 years of age (Abdel Rahim, 1997). Camels are induced ovulators, which means that they normally need the stimulus of mating to ovulate. Female camels also have a highly variable oestrus behaviour, both when it comes to expression, intensity and duration. In addition, it can be difficult to relate these behaviours to follicular activity (Skidmore, 2011).

Semen collection

The most common way to collect semen from camels is by using an artificial vagina (AV). The method mimics natural copulation with a sexual receptive female in sternal recumbency (Ziapour *et al.*, 2014). The amount of ejaculate collected varies, but is reported to be between 2-20 ml (Skidmore *et al.*, 2013; Deen *et al.*, 2000). Some problems with the method are reported, including contamination of the samples with e.g. sand and hair (Ziapour *et al.*, 2014), but also incomplete or aspermic ejaculates, and males refusing to serve the AV (Deen *et al.*, 2000). Ziapour and co-authors reported in 2014 an overall good success rate using a phantom instead of a female, and the study also showed that sample contamination could be reduced with this method.

Semen characteristics

Camel semen is highly viscous. The gelatinous attributes are mostly attained by mucopolysaccharides from the bulbourethral gland or the prostate, and individual variations are found. Before liquefaction the spermatozoa are almost immobile and the samples are difficult to handle and evaluate. Liquefaction could be attained by the use of enzymes or by manually breaking the gel, e.g. through pipetting (Bravo *et al.*, 2000). The viscous nature of the ejaculates makes cryopreservation problematic because the seminal plasma prevents penetration of cryoprotectant into the spermatozoa. In addition, as mentioned above, the ejaculates can be of low volume, and also generally have a low sperm count (Skidmore *et al.*, 2013).

Artificial insemination (AI)

As mentioned in the introduction, AI are associated with many benefits. According to Skidmore *et al.* (2013) the first artificial insemination in camelids was performed 1960. Since then there has been little research in the area, with few published reports regarding AI in dromedary camels. Artificial insemination with fresh dromedary semen has been reported to result in a pregnancy rate of approximately 50 % (Crichton *et al.*, 2016). However, fresh semen could only be stored for a certain amount of time. In order to really exploit the benefits of AI, cryopreserved spermatozoa should be used but, to date, there is only one published study regarding AI with cryopreserved spermatozoa. Deen and co-authors (2003) reported a pregnancy rate of 1/13 when using cryopreserved spermatozoa in AI.

Cryopreservation

Cryopreservation is a method for preserving biological material by storing it at very low (cryogenic) temperatures e.g. -196°C. At such low temperatures cell metabolism is reduced, which allows for long-term storage. However, the freezing- and thawing procedures are highly stressful to the cells, and cryopreservation has an overall negative impact on the reproductive performance of the spermatozoa (Yeste, 2016).

When freezing and thawing cells cryoinjuries occur. Cryoinjuries are often associated with the formation and dissolving of ice crystals in the cytoplasm of the cells. To successfully freeze a cell, intracellular water should be drawn out of the cell and ice crystal formation should be reduced as much as possible (Yeste, 2016). Since water has different chemical potentials depending on temperature, water has the ability to flow over cell membranes when the temperature differs within the cell and the surrounding medium. The process of freezing and thawing cannot go too slowly, nor too rapidly; the goal is to find the optimal time- and temperature changes. If the cells are cooled too rapidly, the intracellular water will not have time to flow out of the cell and formation of intracellular ice crystals will damage the cell. In contrast, if the cells are cooled too slowly, too much water will be removed, causing severe damage to the organelles. Hypertonic stress can also lead to electrolyte imbalance which can cause cell lysis due to abnormal isotonic volume when thawed. The most critical temperature span occurs between -15 - -60 °C, and since the cells must pass this critical temperature twice, it is important to try to optimize both the freezing- and the thawing temperature (Gao and Critser, 2000). There are different optimal freezing- and thawing protocols for different species (Yeste, 2016). The freezing protocols used for camel semen nowadays are modified from other species (Deen et al., 2003). Different thawing protocols have been used in different studies (Skidmore *et al.*, 2013), but 37 °C has been stated to be the most commonly used thawing temperature (El-Ahwany *et al.*, 2018). Other studies suggest that faster thawing rates might be beneficial (Yeste, 2016; Elwing, 2016). In this study we chose to study the effect of thawing at 37 °C for 30 s and 60 °C for 10 s.

Optimization of the temperature changes helps to reduce cryoinjury but cannot avoid it completely. Cryopreservation will always be stressful to the cell, but the process can be more successful by diminishing cellular stress. This is obtained by adding cryoprotective agents, such

as egg yolk and glycerol (Yeste, 2016). Studies have showed that one major reason for abnormal sperm function after cryopreservation is associated with lipid peroxidation and excessive amount of reactive oxygen species (ROS) (Partyka *et al.*, 2012). Reactive oxygen species are the natural by-products of cell metabolism, and are normally kept at a low level by the cells´ antioxidant defence. When an imbalance between production and inactivation of ROS occurs, the elevated levels of ROS can damage the cells and eventually lead to cell death (Selvaratnam and Robaire, 2016). The plasma membrane of mammalian spermatozoa contains a high amount of polyunsaturated fatty acids (PUFAs). When the PUFAs in the membrane are damaged by ROS (via lipid peroxidation), membrane fluidity is lost (Agarwal *et al.*, 2014).

Antioxidants

As mentioned above, oxidative stress and imbalance between levels of ROS and antioxidants within the cells are thought to be highly associated with cryo-injuries and low post-thaw sperm fertility. Antioxidants occur naturally in cells and several studies indicate that the addition of antioxidants prior to cryopreservation can prevent cryodamage to some extent and thereby be positive to post-thaw sperm quality. The mechanism of action of antioxidants vary, but in one way or another they are thought to hinder the oxidative chain reaction and thereby reduce the effects of ROS within the cell (Agarwal *et al.*, 2014). Several antioxidative substances have been evaluated in different species with a positive effect on post-thaw sperm motility. Three of these are catalase, glutathione and carnitine.

Catalase (CAT)

Catalase is an enzymatic antioxidant that converts hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2), thereby assisting in removing ROS and preventing lipid peroxidation (Agarwal *et al.*, 2014). Several studies have shown positive results in post-thaw sperm quality parameters after treatment with catalase in several species (Eidan, 2016; Li *et al.*, 2010; Shafiei *et al.*, 2015)

Glutathione (GSH)

Glutathione is also an enzymatic antioxidant that is believed to scavenge free radicals and acts as a reducing agent of ROS in the cell, preventing lipid peroxidation and improving sperm membrane characteristics (Agarwal *et al.*, 2014). Positive results of treatment with glutathione have been seen in several animal studies (Anel-López *et al.*, 2015; Eidan, 2016; Estrada *et al.*, 2014; Ogata *et al.*, 2015).

Carnitine (CARN)

Carnitine is a non-enzymatic antioxidant thought to neutralize free-radicals and act as an energy source, thereby preventing lipid peroxidation and DNA damage (Agarwal *et al.*, 2014). Carnitine has been used for its antioxidant features in some studies in animals and humans (Longobardi *et al.*, 2017; Zhang *et al.*, 2016). Carnitine has also been used as a cryoprotectant in studies involving oocytes (Fathi and El-Shahat, 2017).

MATERIALS AND METHODS

Semen collection

The semen used in this study came from four dromedary camel males. Each male contributed two ejaculates, collected on different occasions using an artificial vagina. All the males were accustomed to the artificial vagina and prior to this study they were proven fertile *in vivo* by natural mating. The semen was collected between March and May 2016 at Camel Reproduction Centre Dubai, which is also where the males were held, and where all the practical work for this study was done. The Animal Care and Use Committee of the Camel Reproduction Centre approved all animal procedures.

Initial treatment

After collection, ejaculates were immediately taken to the laboratory and immersed in a 37 °C water bath. The semen was then extended 1:5 (v:v) with Tris Citrate Fructose buffer (TCF, pH = 6.9). The buffer (Evans and Maxwell, 1987) consisted of TRIS (300 mM), citric acid (94.7 mM) and fructose (27.8 mM) fructose, with Bovine serum albumin (0.05%) and EDTA (10 mM); 4% egg yolk was also added. After extension, the semen was manually liquefied by gentle pipetting.

Single Layer Centrifugation

To separate the spermatozoa from the seminal plasma, the liquefied and extended semen was subject to Single Layer Centrifugation (SLC), using a method modified from Morrell et al. (2008); 2 ml of each semen solution were added on top of 2 ml of a colloid consisting of silane-coated silica particles in a buffered salt solution in 15 ml tubes. After centrifugation at 300g for 20 min, the supernatants (semen extender, seminal plasma and colloids) were discarded by aspiration.

Supplementation of antioxidants

The resulting pellets from the SLC were resuspended in a mixture of 80% Green buffer (IMV Technologies, l'Aigle France) and 20% egg-yolk (v/v) (Fraction A). Each suspension was divided into four for supplementation with the different antioxidants: (T1; catalase, T2; GSH, T3; carnitine, and T4 (CONTROL, without antioxidants), as shown in Table 1. The final concentration of each sample was adjusted to 200 million spermatozoa/ml, counted with a Makler Counting Chamber. Each sample was evaluated for motility by computer-assisted semen analysis (CASA).

Table 1. *The different antioxidants added to treatment (T) 1-4. T4 serves as control with no antioxidant added*

Treatment	Antioxidant
T1	Catalase (CAT)
T2	Glutathione (GSH)
T3	Carnitine (CARN)
T4	Control (CONTROL)

Freezing

The samples were cooled in a water jacket to 5 °C for 2 h and then further diluted with Fraction B (fraction A + 6% glycerol) 1:1 [v:v]), containing the same antioxidants as fraction A. The samples were diluted to a final concentration of 100 million spermatozoa/mL. The samples were then loaded in 0.5 mL semen straws and placed 4 cm above the surface of liquid nitrogen for 15 minutes. Lastly, the straws were plunged into liquid nitrogen and stored.

Thawing of samples

The semen samples were stored in liquid nitrogen for approximately six months. The samples were thawed one by one and evaluated for sperm quality. One straw from each ejaculate was thawed at 37 °C for 30 s and the other at 60 °C for 10 s; sperm quality was assessed after 0 h and 1 h, for all parameters shown in Table 2. Sperm motility was also evaluated after 2 h.

Table 2. *Parameters used to assess sperm quality post-thawing and which tests that were used to evaluate each parameter*

Parameters assessed	Test used
Motility	Computer-assisted semen analysis (CASA)
Membrane integrity	Eosin-nigrosin stain
Acrosome ridge integrity	Eosin-nigrosin stain
Membrane functionality	Hyper osmotic swelling test (HOST)

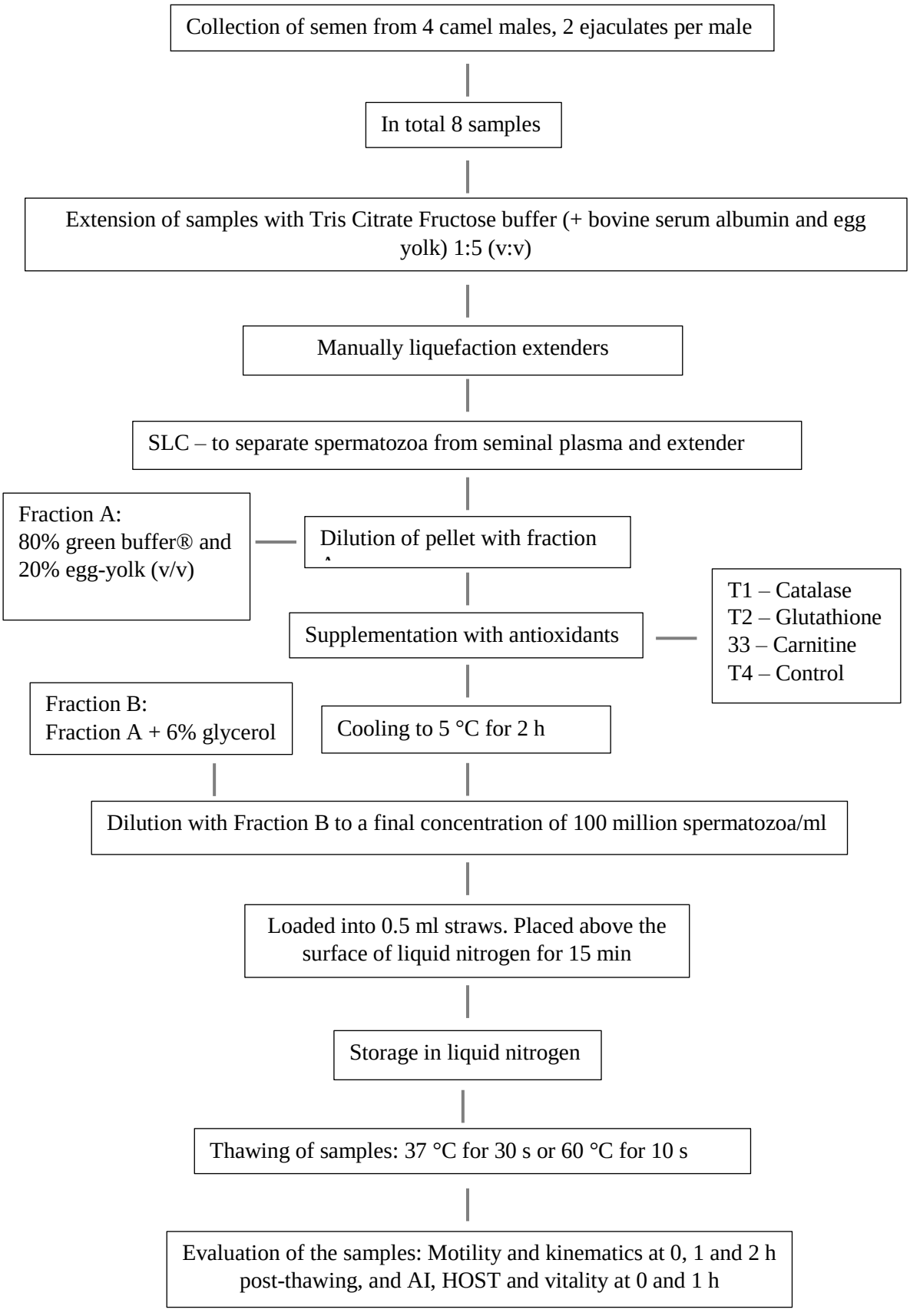

Fig 1. *Flow chart of the process.*

Evaluation of spermatozoa

The parameters used to assess sperm quality were motility, membrane integrity, acrosome integrity and membrane functionality.

Motility

Total motility, progressive motility and kinematics were assessed using computer-assisted semen analysis (CASA). The kinematics were: curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), as well as the ratios straightness (STR) and linearity (LIN). The kinematics were classified as follows: progressive STR 70%, progressive VAP 40 µm/s, slow VAP 20 µm/s, slow VSL 30 µm/s, static VAP 4 µm/s, static VSL 1 µm/s. The CASA system consisted of a CEROS II ® (Hamilton Thorne; MA; US) attached to a Zeiss microscope equipped with a x10 objective and eyepiece, with a warm stage at 37 °C. For each evaluation, a disposable capillary counting chamber (MicroToolTM, Cytonix, USA) was filled with 3 µL aliquot of the sperm sample and approximately 300 spermatozoa were analysed at a frame rate of 30/s. Particles of size 13-101 µm were considered to be spermatozoa. The CASA settings are shown in table 3.

Table 3. *CASA settings (reported by Elwing, 2016)*

Calibration	
Objective	0: 10xZeiss
Objective Magnification X	1.22
Objective Magnification Y	1.22
Cell Detection	
Elongation Max (%)	65
Elongation Min (%)	1
Head Brightness Min	75
Head Size Max (μm^2)	101
Head Size Min (μm^2)	13
Tail Brightness Min	101
Chamber	
Capillary Correction	1.3
Chamber Depth (µm)	20
Chamber Type	Capillary

Kinematics	
Progressive STR (%)	70
Progressive VAP (µm/s)	40
Slow VAP (µm/s)	20
Slow VSL (µm/s)	30
Static VAP (µm/s)	4
Static VSL (µm/s)	1
Morph	
DMR Confidence (%)	50
DMR Droplet to tail end Max (µm)	7
DMR Tail Length Max (µm)	20
Droplet Confidence (%)	50
Droplet Distal Distance Min (µm)	4
Droplet Proximal Head Length (µm)	10.5
Min Tail Length	0
Tail Bend Angle Averaging Length	5
Tail Bending Angle Rate Min (%)	20
Tail Bent Confidence (%)	50
Tail Coiled Angle Min (°)	180
Tail Coiled Confidence (%)	50
Setup	
Name	**CAMEL PROG**
Video Capture	
Frame Capture Speed (Hz)	60
Frame Count	30

Membrane integrity – vitality (VIT)

This parameter was assessed using eosin-nigrosin stain (EN). Ten µL of stain solution (5% eosin, 10% nigrosin in 0.1 M citrate solution) were mixed with an equal volume of the sperm sample and smeared on a glass slide. After drying on a 37 °C plate, the spermatozoa were

examined under a microscope. If the stain had not penetrated the spermatozoon, the membrane was considered to be intact. Spermatozoa with a permeable membrane were coloured red, and therefore counted as non-functional. A total of 100 spermatozoa were counted and the result presented as percentage.

Acrosome ridge integrity (AI)

The integrity of the acrosomal ridge was also evaluated using eosin-nigrosin stain (EN). If the spermatozoa had a clear, even and undamaged apical ridge, the acrosome was considered to be intact. In total, of 100 spermatozoa were counted and the result presented in percentage.

Membrane functionality (HOST)

Membrane functionality was evaluated using the hypo-osmotic swelling test (HOST). 100 µl of hyper-osmotic solution, in this case sucrose (100 mM), was mixed with 30 µL sample and the mixture was incubated for 30 minutes in a 37 °C water bath. After the incubation, the spermatozoa were evaluated under a microscope. Spermatozoa with a functional membrane showed a coiled tip to the tail (see picture), whereas the tail of spermatozoa with a non-functional membrane remained uncoiled. In total, 100 spermatozoa were evaluated, the ones with round coiled tails were counted as having a functional membrane. Incomplete coiling, coiling at the root of the tail instead of the tip, or different types of folding of the tails were counted as non-functional membranes. Any spermatozoa where the tip of the tail could not be evaluated were excluded.

Statistical analyses

Effects of antioxidants and freezing rates were analysed by generalized linear model (GLM); means were compared by Duncan´s multiple range method. All parameters were reported as absolute estimated marginal means ± SEM. The analyses were performed using SPSS 11.0 for Windows, with P-values less than 0.05 being considered significant.

RESULTS

Antioxidants

There were no significant differences in motility between the different treatments and control pre-freezing (Table 4), or immediately post-thawing (Table 5).

Table 4. *Motility values including kinematics, pre-freezing, treatment 1-4. Values shown in means (+SE)*

	Values - pre freezing				
Parameter	CAT	GSH	CARN	CONTROL	P-value
TM (%)	72.04 ± 3.67	73.29 ± 3.24	74.71 ± 3.09	70.40 ± 3.40	0.826
PM (%)	36.59 ± 3.18	37.78 ± 3.47	39.41 ± 3.29	35.98 ± 3.48	0.893
ALH (µm)	10.04 ± 0.42	9.99 ± 0.35	10.11 ± 0.38	10.43 ± 0.41	0.854
BCF (Hz)	20.20 ± 0.76	20.63 ± 0.87	20.44 ± 0.87	20.68 ± 0.58	0.859
LIN (%)	36.69 ± 0.93	36.06 ± 1.21	36.78 ± 1.18	35.46 ± 1.34	0.891
STR (%)	68.78 ± 1.63	68.36 ± 1.59	69.70 ± 1.63	68.53 ± 1.85	0.944
VAP (µm/s)	112.49 ± 4.44	112.51 ± 3.22	111.63 ± 4.47	110.22 ± 3.44	0.895
VCL(µm/s)	216.40 ± 8.69	219.22 ± 9.05	217.46 ± 9.98	218.28 ± 9.42	0.997
VSL (µm/s)	77.25 ± 2.65	77.24 ± 2.50	78.22 ± 2.59	74.72 ± 1.70	0.759

Table 5. *0 h post-thaw sperm quality parameters for different treatments (1-4). Values shown are means (+SE)*

	Values (0 h post-freezing)				
Parameter	CAT	GSH	CARN	CONTROL	P-value
TM (%)	37.46 ± 2.62	34.54 ± 1.93	39.29 ± 2.98	36.06 ± 2.62	0.605
PM (%)	15.09 ± 1.51	14.71 ± 0.80	17.63 ± 2.21	15.53 ± 1.48	0.569
ALH (µm)	8.12 ± 0.25	7.95 ± 0.20	7.96 ± 0.26	7.92 ± 0.19	0.936
BCF (Hz)	19.72 ± 0.35	20.62 ± 0.43	21.06 ± 0.63	20.15 ± 0.40	0.220
LIN (%)	33.33 ± 0.66	33.63 ± 0.37	33.77 ± 0.70	33.57 ± 0.57	0.961
STR (%)	65.16 ± 1.37	67.74 ± 0.48	67.55 ± 1.04	67.45 ± 0.67	0.199
VAP (µm/s)	75.13 ± 2.82	72.50 ± 2.60	72.35 ± 2.49	71.82 ± 2.41	0.805
VCL (µm/s)	150.91 ± 5.19	150.82 ± 4.96	149.26 ± 5.40	149.04 ± 5.29	0.991
VSL (µm/s)	49.28 ± 1.63	49.63 ± 1.61	48.87 ± 1.67	48.71 ± 1.39	0.976
VIT (%)	67.19 ± 2.29	68.88 ± 2.34	67.81 ± 2.50	66.06 ± 2.24	0.860
AI (%)	31.44 ± 1.50	33.25 ± 1.90	33.06 ± 2.03	33.38 ± 2.03	0.873
HOST (%)	17.44 ± 3.09	12.88 ± 2.20	13.19 ± 2.16	14.38 ± 2.32	0.709

However, at 1 h after thawing the CAT and GSH treatments showed a significantly higher ($p<0.05$) total motility and VAP compared to CONTROL (Table 6).

Table 6. *1 h post-thaw sperm quality parameters for different antioxidant treatments (1-4). Values shown are mean (+SE)*[1]

	Values (1h post-thawing)				
Parameter	CAT	GSH	CARN	CONTROL	P-value
TM (%)	36.62 ± 1.84[a]	33.94 ± 2.32[a]	31.64 ± 1.87[ab]	26.05 ± 2.60[b]	**0.009**
PM (%)	14.98 ± 1.24	15.21 ± 1.57	14.25 ± 1.19	11.05 ± 1.54	0.138
ALH (µm)	8.0 ± 0.18	7.91 ± 0.16	7.74 ± 0.17	7.42 ± 0.12	0.065
BCF (Hz)	19.48 ± 0.33	20.66 ± 0.21	20.39 ± 0.41	20.33 ± 0.39	0.098
LIN (%)	33.29 ± 0.43	32.60 ± 0.32	32.84 ± 0.48	32.49 ±0.72	0.691
STR (%)	66.45 ± 0.79	67.32 ± 0.63	68.43 ± 0.57	67.63 ±0.81	0.269
VAP (µm/s)	71.68 ±2.0[a]	69.42 ± 2.0[a]	66.74 ± 2.02[ab]	62.80 ± 1.77[b]	**0.014**
VCL (µm/s)	147.38 ± 3.70	147.27 ± 4.04	143.98 ± 4.18	135.44 ± 3.49	0.108
VSL (µm/s)	48.28 ± 1.40	47.13 ± 1.35	45.81 ± 1.46	42.75 ± 1.56	0.051
VIT (%)	66.38 ± 2.14	69.19 ± 2.39	69.13 ± 2.05	66.25 ± 2.48	0.669
AI (%)	21.13 ± 1.56	21.50 ± 1.69	25.63 ±1.85	21.63 ± 1.06	0.154
HOST (%)	11.94 ± 1.50	10.06 ± 1.55	11.31 ± 1.67	9.56 ±1.38	0.673

[1]*The numbers in bold are statistically significant. Different superscripts within a row indicate statistical difference ($p<0,05$).*

At 2 h after thawing CAT, GSH and CARN showed a significantly higher total-, and progressive motility compared to control (Table 7). The kinematics; ALH, VAP, VCL and VSL also showed significantly higher values in all the three treatments compared to control.

Table 7. *2 h post-thaw sperm quality parameters for different treatments (1-4). Values shown are mean (+SE)*[1]

	Values (2 h post thawing)				
Parameter	CAT	GSH	CARN	CONTROL	P-value
TM (%)	26.91 ± 1.67[a]	25.08 ± 1.86[a]	22.94 ± 2.89[a]	14.66 ± 2.13[b]	**0.001**
PM (%)	9.28 ± 0.98[a]	10.61 ± 1.06[a]	9.72 ± 1.52[a]	4.99 ± 1.05[b]	**0.004**
ALH (µm)	7.60 ± 0.15[a]	7.47 ± 0.23[a]	7.36 ± 0.23[a]	6.64 ±0.25[b]	**0.015**
BCF (Hz)	20.10 ± 0.48	20.74 ± 0.57	20.60 ±0.53	20.67 ± 0.96	0.905
LIN (%)	32.26 ± 0.80	33.60 ± 0.48	32.36 ± 0.65	31.59 ± 1.42	0.585
STR (%)	64.46 ± 1.22	67.54 ± 0.76	66.87 ± 0.96	64.18 ± 1.69	0.175
VAP (µm/s)	65.92 ± 2.28[a]	66.53 ± 2.81[a]	62.60 ± 3.06[a]	54.79 ± 2.59[b]	**0.014**
VCL (µm/s)	136.95 ± 4.33[a]	138.84 ± 5.28[a]	134.33 ± 5.86[a]	116.81 ± 5.53[b]	**0.019**
VSL (µm/s)	43.23 ± 1.98[a]	45.46 ± 1.66[a]	42.36 ± 2.35[a]	35.64 ± 2.42[b]	**0.013**

[1]*The numbers in bold are statistically significant. Different superscripts within a row indicate statistical difference ($p<0.05$).*

Thawing temperatures

As seen in table 8, the total and progressive motility were significantly higher ($p < 0.05$) at 0 h post thawing for the samples thawed at 60 °C for 10 s than the samples thawed in 37 °C for 30 s.

Tabell 8. *0 h post-thaw sperm quality parameters at two thawing temperatures and time (37 °C in 30 s and 60 °C in 10 s). Values shown are mean (+SE)*[1]

	Thawing temperature 0h post-thawing		
Parameter	37 °C	60 °C	P-value
TM (%)	33.50 ± 1.70	40.18 ± 1.58	**0.007**
PM (%)	13.70 ± 1.16	17.79 ± 0.84	**0.002**
ALH (µm)	7.91 ± 0.15	8.06 ± 0.16	0.495
BCF (Hz)	20.78 ± 0.35	20.0 ± 0.30	0.099
LIN (%)	33.28 ± 0.39	33.87 ± 0.42	0.312
STR (%)	66.36 ± 0.73	67.59 ± 0.63	0.165
VAP (µm/s)	72.33 ± 1.67	73.57 ± 1.92	0.631
VCL (µm/s)	148.67 ± 3.16	151.35 ± 3.96	0.659
VSL (µm/s)	48.40 ± 1.13	49.84 ± 1.02	0.352
VIT (%)	66.22 ± 1.40	68.75 ± 1.82	0.275
AI (%)	32.28 ± 1.19	33.28 ± 1.42	0.591
HOST (%)	15.81 ± 1.99	13.13 ± 1.44	0.459

[1]*The numbers in bold are statistically significant. Different superscripts within a row indicate statistical difference ($p<0.05$).*

At 1 h post thawing, total motility, progressive motility, ALH, STR and VCL were significantly higher ($p < 0.05$) for samples thawed at 60 °C in 10 s than for samples thawed at 37 °C for 30 s (see table 9).

Tabell 9. *1 h post-thaw sperm quality parameters for two different thawing temperatures and time (37 °C in 30 s and 60 °C in 10 s). Values shown are mean (+SE)*[1]

	Thawing temperatures 1 h post thawing		
Parameter	37 °C	60 °C	P-value
TM (%)	29.13 ± 1.75	34.99 ± 1.40	**0.011**
PM (%)	11.98 ± 0.98	15.76 ± 0.94	**0.007**
ALH (µm)	7.60 ± 0.12	7.93 ± 0.11	**0.033**
BCF (Hz)	20.02 ± 0.28	20.41 ± 0.22	0.283
LIN (%)	32.97 ± 0.43	32.64 ± 0.26	0.506
STR (%)	66.71 ± 0.58	68.21 ± 0.38	**0.034**
VAP (µm/s)	66.01 ± 1.36	69.31 ± 1.54	0.113
VCL (µm/s)	137.93 ± 2.45	149.10 ± 2.82	**0.004**
VSL (µm/s)	44.56 ± 1.03	47.43 ± 1.05	0.055
VIT (%)	66.75 ± 1.44	68.72 ± 1.73	0.386
AI (%)	21.84 ± 0.93	23.09 ± 1.31	0.439
HOST (%)	10.81 ± 1.16	10.63 ± 0.98	0.904

[1]*The numbers in bold are statistically significant. Different superscripts within a row indicate statistical difference ($p<0.05$).*

As seen in Table 10 total and progressive motility was significantly higher (P-value <0,05) for samples thawed in 60 °C for 10 s then samples thawed at 37 °C for 30 s.

Table 10. *12 h post-thaw sperm quality parameters for two different thawing temperatures and time (37 °C in 30 s and 60 °C in 10 s). Total, and progressive motility (TM, PM) and kinematics (ALH, BCF, LIN, STR, VAP, VCL, VSL). Values shown are mean (+SE)*[1]

	Thawing temperatures 2 h post thawing		
	37	60	P-value
TM (%)	18.73 ± 1.55	25.80 ± 1.70	**0.003**
PM (%)	7.18 ± 0.84	10.06 ± 0.91	**0.013**
ALH (µm)	7.18 ± 0.16	7.36 ± 0.17	0.437
BCF (Hz)	20.78 ± 0.55	20.30 ± 0.36	0.459
LIN (%)	32.14 ± 0.75	32.77 ± 0.50	0.220
STR (%)	65.23 ± 1.03	66.35 ± 0.69	0.481
VAP (µm/s)	60.89 ± 1.82	64.06 ± 2.23	0.279
VCL (µm/s)	128.67 ± 3.42	134.91 ± 4.40	0.272
VSL (µm/s)	40.25 ± 1.48	43.15 ± 1.69	0.202

[1]*The numbers in bold are statistically significant.*

As seen in Table 11, no interactions were found between the different treatments (1-4) and the thawing rates (60 °C and 37 °C). Thus the effect of thawing temperature was independent of the presence of antioxidants.

Tabell 11. *P-values for the interactions between the different treatments (1-4) and thawing rates (60 °C and 37 °C) for 0 h, 1 h and 2 h time points*

Time	Parameters											
	TM	PM	ALH	BCF	LIN	STR	VAP	VCL	VSL	VIT	AI	HOST
0 h	0.991	0.749	0.856	0.336	0.564	0.631	0.830	0.768	0.547	0.879	0.614	0.777
1 h	0.708	0.958	0.879	0.441	0.696	0.889	0.974	0.992	0.933	0.971	0.819	0.503
2 h	0.925	0.986	0.998	0.916	0.949	0.942	0.972	0.997	0.917	-	-	-

DISCUSSION

The aim of this study was to evaluate three different antioxidants; catalase, glutathione and carnitine and their potential positive effect on post-thaw sperm quality. In addition, two different thawing temperatures and times were evaluated: 60 °C for 10 s and 37 °C for 30 s.

The results from this study indicate that catalase can exert a protective role on cryopreserved camel spermatozoa. There are no published studies with cryopreserved camel semen supplemented with catalase, but Medan *et al.* (2008) showed that catalase could exhibit a protective role on short term stored, cooled, camel spermatozoa. Studies in other species such as goats (Shafiei *et al.*, 2015), cows (Eidan, 2016), and human beings (Li *et al.*, 2010), indicated corresponding results with cryopreserved spermatozoa.

After addition of glutathione some of the sperm kinematics were significantly better at 2 h post thawing compared to the control, although no significantly differences was seen immediately after thawing or 1 h post thawing. This suggests that glutathione shows some protective effect towards camel spermatozoa, but possibly only after a longer time-span. There are no published studies regarding glutathione-supplementation and cryopreservation of camel spermatozoa, but Waheed *et al.* (2015) showed that infertile dromedary camel males had a significantly lower amount of glutathione peroxidase compared to fertile males. A study made on boars by Estrada *et al.* (2014) showed that semen supplemented with glutathione had significantly better post-thaw sperm quality compared to control and had a significantly higher success rate in IVF-rate. Corresponding data has also been shown with deer spermatozoa, but no *in vivo* trial was performed (Anel-López *et al*., 2015). Moreover, a study on Holstein bull spermatozoa (Eidan, 2016) and a study with dog spermatozoa (Ogata *et al.*, 2015) showed corresponding results.

Treatment with carnitine produced significantly better ($p<0.05$) TM and VAP 1 h post-thawing, and also significantly better TM, PM, ALH, VAP, VCL and VSL 2 h post-thawing. These results indicate that carnitine could have a positive effect on cryopreserved camel spermatozoa. No trials in camels are published, but a recent study of buffalo spermatozoa showed similar results (Longobardi *et al.*, 2017). Bucak *et al.* (2010) published a trial of bovine cryopreserved spermatozoa that indicated that carnitine could exert a protective effect for sperm DNA, but no differences were seen in TM and PM compared to control. Sarıözkan *et al.* (2014) performed a trial with bovine spermatozoa, but did not find any positive effects of supplementation with carnitine compared to control; there was no difference in fertility rates between treatments. A study with human spermatozoa indicated that carnitine did not have a significant effect in preventing cryo-injuries (Duru *et al.*, 2000).

Regarding the thawing rates, several kinematics were significantly better at various time intervals for samples thawed at 60 °C for 10 s compared to the samples thawed at 37 °C for 30 s. This indicates that the optimal thawing temperature for camel semen is 60 °C for 10 s. These results correspond to results achieved with camel semen (Elwing, 2016). Both Tomás *et al.* (2014) and Eriksson and Rodriguez-Martinez (2000) also showed that faster thawing rates are preferable for cryopreserved boar spermatozoa. Nöthling and Shuttleworth (2005) also showed

that dog semen showed better post thaw parameters when thawed at 70 °C instead of 37 °C. However Muiño *et al.* (2008) did not find any differences among thawing temperature in bovine spermatozoa.

Due to several practical reasons it was not possible to check the functionality of the samples in this study. Some studies indicate that there is a positive correlation between single *in vitro* sperm quality-tests and the prediction of *in vivo* fertilization (Tanghe *et al.*, 2002), whilst other researchers say that isolated tests, such as motility, are not reliable for predicting fertility (Foote, 2003; Mocé and Graham, 2008). Holt (2009) suggests that different combinations of tests should be used for better reliability. Since no functional tests were conducted in this study we cannot make any conclusions about sperm fertility until *in vivo* studies are made, although the results of these laboratory assays indicate that thawing at 37 °C might adversely affect motility and, therefore, possibly fertility.

Since this study only included four males, and in total only 8 ejaculates, the reliability of this study is low. Since there are so few studies on this subject, further studies are needed, including more males and preferably also IVF tests. Also since the HOST and AI was performed manually using a microscope, only 100 spermatozoa per sample were counted. It is debatable if this is a large enough number, but it would have been impractical to count more spermatozoa, because of the number of different assays performed at each time point. The CASA machine assesses motility of a minimum of 300 spermatozoa, which increases the accuracy. Observer error is also another aspect to think about, although since all the microscopic assessments of the spermatozoa were made by one person, there are no inter-observer variations to take into consideration. However, any error should be consistent among samples.

The potential protective effect of antioxidants is complex (Mancilla *et al.*, 2015), and as Agarwal *et al.* (2014) points out, it is hard to measure the effect of one single antioxidant since they are naturally occurring in a complex system. It may be too simplistic to supplement samples with only a single antioxidant. Shafiei *et al.* (2015) showed that the best results of post-thaw parameters on goat spermatozoa were attained when a combination of two antioxidants were used. Further studies could assess the effects of combinations of antioxidants.

CONCLUSION

In conclusion, supplementation of the freezing medium with antioxidants had a positive effect on post-thaw camel sperm kinematics. Thawing semen straws at 60 °C was also beneficial compared to thawing at 37 °C. Both of these interventions could be expected to be useful for the development of cryopreservation protocols for camel spermatozoa.

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