Animal Reproduction Science 209 (2019) 106168

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Individual male dependent improvement in post-thaw dromedary camel sperm quality after addition of catalase

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ARTICLE INFO

Keywords:
Dromedary camel
Sperm
Cryopreservation
Catalase
Epigallocatechin

ABSTRACT

Cryopreservation is stressful to sperm cells inducing an increase in the production of reactive oxygen species and subsequently reducing post-thaw sperm quality. With the present study, there was evaluation of the protective effects of two antioxidants, epigallocatechin (1 mM) and catalase (500 IU/ml), added at thawing, as well as inter-individual variation on quality of cryopreserved dromedary camel spermatozoa. Semen was collected from six males and sperm, selected using single layer centrifugation, were cryopreserved. Post-thaw sperm quality was evaluated by assessing motility variables, viability and acrosome integrity then sperm were co-incubated with or without antioxidant (control) and further assessed at 1.5 and 3 h of the incubation period. Oxidative damage was measured colorimetrically for malondialdehyde production at 3 h of the incubation period. With the use of epigallocatechin there were not promising results, however, with use of catalase there were greater total and progressive motility, and values for some kinematic variables ($P < 0.05$) at both incubation time points, although there were some differences among males. There was no overall effect of antioxidant based on production of malonaldehyde. The capacity of thawed sperm to fertilize, with and without addition of catalase at thawing, was studied using artificial insemination ($n = 10$ per treatment) with no differences between treatments (10% for both). It is concluded that catalase supplementations to semen extender prolong sperm survival, however, there is no improvement of in vivo fertilization as a result of this supplementation. There was an obvious male effect, necessitating further studies to understand the mechanisms of action of catalase.

1. Introduction

Cryopreservation of camelid semen has generally met with little success as camelid sperm have little tolerance for freezing and thawing (Crichton et al., 2016; Skidmore et al., 2018). As interest grows in trying to improve genetic traits, and cryopreservation and artificial insemination (AI), however, these techniques are being used more frequently in camelids such as the dromedary camel (Skidmore et al., 2013). Further research to improve the fertility of cryopreserved camel spermatozoa, therefore, is becoming increasingly important.

The mechanisms behind cryoinjury to spermatozoa are relatively well understood. Cryopreservation is associated with excessive formation of reactive oxygen species (ROS) and injury to spermatozoa occurs due to oxidative stress (OS; Chatterjee and Gagnon, 2001). There is also a decrease in the antioxidant defence mechanisms of sperm following cryopreservation (Bilodeau et al., 2000).

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https://doi.org/10.1016/j.anireprosci.2019.106168
Received 31 December 2018; Received in revised form 4 August 2019; Accepted 19 August 2019
Available online 20 August 2019
0378-4320/

An imbalance between ROS-production and the antioxidant systems of spermatozoa protecting against ROS is now considered to be one of the factors causing sperm damage during sperm processing for artificial insemination (Alvarez and Storey, 1995).

In semen, a variety of enzymatic or non-enzymatic antioxidants maintain a balance between the production and catabolism of ROS to decrease oxidant-induced cell damage, while still allowing for oxidant-dependent cell signaling processes and normal sperm function (Kefer et al., 2009).

Spermatozoa are especially susceptible to oxidative damage during cryopreservation because of the relatively large proportion of polyunsaturated fatty acids in the membranes compared to most other cells. During sperm cryopreservation in some species (e.g., boar and stallion), at least some of the seminal plasma is removed resulting in a loss of antioxidant protection capacity (Roca et al., 2005). Thus the generation of ROS by defective and dead spermatozoa during the freezing-thawing process leads to membrane lipid peroxidation (LPO) and damage in other spermatozoa (Baumber et al., 2003; Roca et al., 2005).

In the preliminary study described in this manuscript the antioxidants biotin, melatonin, epigallocatechin, rosmarining acid curcumin, TROLOX and catalase were tested with dromedary camel sperm samples. The treatments with catalase and epigallocatechin were superior for sustained sperm quality compared with the other antioxidants and thus were the focus of the primary study reported in this manuscript.

The aim of this study was to determine the effect of adding two natural antioxidants, catalase and epigallocatechin, to the thawing media on values for sperm quality variables after 1.5 and 3 h of incubation: motility variables (total and progressive motility), membrane integrity and acrosome integrity. An indirect measure of ROS production was performed by quantifying malondialdehyde (MDA) production (a product of oxidative damage) 3 h after thawing. The effect of individual males on post-thaw sperm quality was also investigated.

2. Materials and methods

Unless otherwise indicated, all chemicals were from Sigma-Aldrich Co. (St Louis, MO, USA). The colloid was supplied by Professor Morrell (patent pending).

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 filter-sterilized (0.22 m). Green Buffer (GB) (IMV: L'Aigle, France), supplemented with 20% (v:v) egg-yolk, was used as a freezing extender. Sperm cryopreservation 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. Antioxidants were prepared in GB solution at 10 mM except catalase, which was prepared at 7000 units/mL.

2.1. Animals and semen collection

Six adult dromedary camel males, 8 to 10 years old, of proven fertility from the Camel Reproduction Centre, Dubai, UAE, were used in this study. Ejaculates were obtained from each male using an artificial vagina (Skidmore et al., 2013), between February and March, which is during the breeding season. Ejaculates were immediately taken to the laboratory and placed in a 37 °C water bath. All animal procedures were approved by the Animal Care and Use Committee (ACUC) of the Camel Reproduction Centre, UAE.

2.2. Semen preparation and cryopreservation

Semen was diluted (1:5) with TCF at 37 °C and manually liquefied by gentle pipetting. Liquefied samples were evaluated for sperm total motility and concentration. Semen was then subjected to centrifugation through a colloid (Single Layer Centrifugation, SLC) according to Malo et al. (2018). In brief, 2 mL aliquots of semen were layered over 2 mL of colloid in 15 mL tubes and centrifuged at 300 x *g* for 20 min at 25 °C using a swing-out rotor. The supernatants were aspirated using a Pasteur pipet and discarded. Sperm concentrations were calculated and pellets were then re-suspended in the required volume of F1 (GB supplemented with 20% (v/v) egg yolk) to obtain a final concentration of 200×10^6 sperm/mL, and cooled to 5 °C during a 2 h period in a water jacket according to Malo et al. (2019).

A second extension step (1:1) was performed with F2 [GB extender containing 20% egg yolk and 6% (v/v) glycerol] at 5 °C, resulting in a final glycerol concentration of 3% and cell concentration of 100×10^6 sperm/mL. Pre-cooled plastic straws (0.5 mL) were filled with cooled semen in a cold cabinet and, after 30 min of equilibration, were frozen in static liquid nitrogen vapour (1 cm above the liquid) for 15 min and then plunged into liquid nitrogen (-196 °C) for storage. Samples were thawed at 60 °C for 10 s, placed at room temperature (25 °C) and immediately evaluated. Antioxidants were added after evaluation, except for the control. Samples were maintained at room temperature for a 1.5 and 3 h incubation period.

2.3. Experimental design

2.3.1. Experiment 1: pilot study

A pilot study was conducted using one ejaculate/male. The effect of seven natural antioxidants (biotin, melatonin, epigallocatechin, rosmarinic acid, curcumin, TROLOX, and catalase) added at room temperature after thawing was assessed to select two antioxidants for the main experiment of this study. All antioxidants except catalase were added at a final concentration of 1 mM during thawing (dilution 1:10 with stock solution). Catalase was added at a final concentration of 500 units/ml (dilution 1:14 with

stock solution). Total and progressive motility were measured at 1.5 and 3 h of an incubation period after thawing, keeping the samples on the bench at room temperature.

2.3.2. Experiment 2: main study

In this study, 18 ejaculates (three ejaculates/male) were used. Samples were thawed in a circulating water bath (60 °C for 10 s). Semen evaluation was performed immediately after thawing and then three aliquots were prepared: control, catalase group (C1345; 500 units/ml) and epigallocatechin (E3768; 1 mM). Semen evaluation for the different treatments was performed at 1.5 h and 3 h of an incubation period. The sperm variables evaluated included total and progressive motility, kinematics (Computer Assisted Sperm Analysis, CASA), acrosome and membrane integrity. The MDA concentration was quantified 3 h after thawing of the different treatment samples. Post-thaw samples for control and catalase treatment were used for heterospermic artificial insemination (ten females per group). Each insemination dose consisted of a mixture of samples from two or three males, in different combinations. Thus, all six males were used for the inseminations.

2.4. Sperm assessment

Sperm motility assessment was performed by CASA as described previously (Malo et al., 2019) using the CEROS II® (Hamilton Thorne; MA; USA) at 10X on the heated stage of a phase contrast microscope (AX10 Zeiss, Gottingen, Germany). 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). Motility was classified as follows: progressive motility: STR 70% and VAP 40 μm/s; slow VAP 20 μm/s, VSL 30 μm/s; static VAP 4 μm/s VSL 1 μm/s.

Plasma membrane integrity was evaluated using the fluorescent probes SYBR-14 (SY) and propidium iodide (PI: L-7011, Live/Dead Sperm Viability Kit; Molecular Probes Europe, Leiden, the Netherlands). The following protocol was adopted: To 30 μL of thawed spermatozoa 6 μL SYBR-14 solution (0.02 mM)(final concentration: 0.55 μM) was added and the solution incubated for 10 min at 38 °C. Then 1 μL of PI (2.4 mM)(final concentration: 64 μM) together with 50 μl GB and 1 μL 4% paraformaldehyde (to immobilize the sperm) were added. Sperm were examined under oil immersion using a fluorescent microscope and a TRICT filter. Spermatozoa (200) were classified as “live (SY + ve: green)" or “dead” (PI + ve: red). The proportion of viable spermatozoa was calculated.

Acrosome status was assessed by fluorescein isothiocyanate conjugated with peanut agglutinin (FITC-PNA) staining as described by Aboagla and Terada (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 mg/ml; final concentration 0.06 ng/ml), and 0.5 μl of paraformaldehyde (4%) and incubated 10 min at 37 °C. Spermatozoa were classified as having a “damaged acrosome,” or an “intact acrosome” if the cells had PNA + (green acrosome) and PNA- (absence of colour) staining, respectively. In total, 400 spermatozoa were assessed per sample at 100X and the proportion of sperm with a damage acrosome was calculated.

Membrane lipid peroxidation was estimated by the generation of malondialdehyde (MDA) determined by using the thiobarbituric acid (TBA) test (Esterbauer and Cheeseman, 1990) 3 h after thawing. Briefly, diluted samples (60 million/ml) were mixed with 1 mL of cold 20% (wt/vol) trichloroacetic acid and centrifuged at 1500 × *g* for 10 min at 25 °C; 1 mL of the supernatant was incubated with 1 mL of 0.67% (wt/vol) TBA in a boiling water bath at 100 °C for 10 min. After cooling, the absorbance was determined by a spectrophotometer (UV-1700; Shimadzu) at 534 nm. The values of MDA aobtained for the samples were compared against a standard curve The results were expressed as the concentration of MDA (g per million of sperm).

For artificial insemination (AI), the ovaries of prospective recipients were examined by trans-rectal ultrasonography using an Aloka Model 500 real-time scanner with a 5-MHz linear array transducer (AI Carmal, Dubai UAE), as described by Skidmore et al. (1996) to monitor follicular development. Ovulation was induced using a single injection of Buserelin (Receptal: 20 μg iv) when the follicle measured 1.3–1.7 cm in diameter. Double inseminations were performed at 26 and 30 h after induction of ovulation. Post-thaw sperm concentration and motility were assessed, and doses of > 300 million motile sperm were loaded into an IMV inseminating pipette and deposited trans-cervically at the utero-tubal junction.

2.5. Statistical analysis

Prior to statistical analysis, data were assessed for normality using the Shapiro-Wilk tests. All variables met normality assumptions except for the MDA concentration. For the normal values, to check the male effect, pre-freeze and post-thaw variables at 0 h were analysed using the ANOVA with the Duncan *post-hoc* test. Improvement in total motility by SLC selection was assessed using the repeated measures ANOVA. Variables at 1.5 and 3 h of the incubation period were analysed using a generalized linear model (GLM), utilising male and treatments as fix factors, with means compared by Duncan *post hoc* test. Male*treatment interactions were calculated using the Univariable General Linear Model. The concentrations of MDA were also assessed for the homogeneity of the variance using the Levene test. The MDA data were logarithmically transformed to conduct a valid repeated measures ANOVA. All statistical analsyses were performed using IBM SPSS 19.0 for Windows. Significance was considered at $P < 0.05$. The data are presented as means + SEM.

3. Results

The results of the pilot study are summarized in Table 1. Both total and progressive motility at 1.5 and 3 h were greater in the

Table 1
Post-thaw values for sperm total motility (TM) and progressive motility (PM) at 1.5 and 3 h after thawing of semen using seven antioxidants and the control; Data represent means (± SEM); Different letters within a column indicate differences among antioxidants ($P < 0.05$).

	TM 1.5 h (%)	PM 1.5 h (%)	TM 3 h (%)	PM 3 h (%)
Control	24.16 ± 13.12 cd	9.10 ± 6.21de	15.10 ± 17.30b	7.26 ± 9.93 cd
Catalase	36.60 ± 10.27a	15.85 ± 6.55a	25.86 ± 19.16a	12.30 ± 11.00a
Epigallocatechin	32.30 ± 11.34ab	14.55 ± 5.91ab	23.90 ± 20.63a	10.36 ± 11.28ab
Biotin	26.88 ± 12.16bc	8.13 ± 4.54de	14.18 ± 20.60bc	4.45 ± 7.35 cd
Melatonin	23.28 ± 12.60 cd	10.66 ± 7.83d	11.06 ± 19.04bc	5.20 ± 9.60 cd
Curcumin	25.96 ± 15.51 cd	11.30 ± 10.37bc	10.05 ± 15.11bc	3.11 ± 5.68d
Rosmarinic	20.40 ± 14.76d	7.31 ± 6.03de	9.08 ± 16.85c	2.91 ± 5.99d
Trolox	23.23 ± 11.83 cd	6.5 ± 4.74e	10.95 ± 18.73bc	2.83 ± 5.5.83d
P-value	0.004	< 0.001	< 0.001	< 0.001

catalase- and epigallocatechin-treated samples ($P < 0.05$) when compared to other treatments. These antioxidants were selected for inclusion in the main experiment.

The results indicate There were differences among males ($P < 0.01$) immediately after collection for total motility (TM), volume (Vol), sperm concentration (Conc) and morphological abnormalities (MA) (Table 2) This intermale-variationwas no longer apparent for total and progressive motility after SLC. Total motility was greater after SLC ($P < 0.001$).

Post-thaw results (Table 3) indicated differences in VAP, VCL, VSL among males, with Male B having lesser values than the other males ($P < 0.05$). Although differences in TM and progressive motility (PM) (TM: 46.67% vs. 33.00; PM: 18.50% vs. 8.07%) were observed between Males A and B, there were no overall differences among the males.

Post-thaw addition of catalase (Figs. 1 and 2), increased TM and PMafter incubation for 1.5 and 3 h after thawing compared with the control samples and epigallocatechin-treated samples ($P < 0.01$). There were no differences between control and epigallocatechin at either 1.5 and 3 h of incubation.

Sperm kinematics were affected by treatment at 1.5 h after thawing (Table 4), with addition of catalase resulting in greater values for ALH ($P = 0.008$), VAP ($P < 0.001$), VCL ($P < 0.001$) and VSL ($P < 0.001$) than the other treatments. Treatment with catalase or epigallocatechin resulted in greater STR compared to the control group ($P = 0.016$). There were no differences for VIT and AD at 1.5 h of the post-thawing incubation period among treatments.

Sperm kinematics, viability, acrosome damage and MDA at 3 h after thawing are provided in Table 5. Catalase treatment resulted in greater ALH, ($P < 0.001$) VAP ($P < 0.001$), VCL ($P < 0.001$) and VSL ($P = 0.002$) than in epigallocatechin-treated or control samples. Although BCF was greater in the epigallocatechin-treated than control samples ($P = 0.049$), there was no difference between catalase and epigallocatechin. There were no differences for VIT and AD at 3 h in any of the samples. Production of MDA was not changed as a result of supplementations with the antioxidants: control 0.029 mg/10^6 cells, catalase 0.022 mg/10^6 cells and epigallocatechin 0.031 mg/10^6 cells ($P = 0.697$).

Total and progressive motility, kinematics, viability and acrosome damage at 1.5 and 3 h after thawing for individual males are shown in Tables 6 and 7. In general, Male A had the greatest values for motility, kinematics, viability and acrosome integrity. There was no significant variation in MDA production among males ($P = 0.424$).

There were no interactions between the treatment and male at 1.5 and 3 h of incubation (results not provided). There was also no improvement in in vivo fertility when samples treated with catalase were used for inseminations, since only one female out of ten was pregnant in each group (10%).

4. Discussion

In the present study, the potential benefits adding catalase or epigallocatechin to dromedary camel sperm immediately after

Table 2
Values for sperm total motility (TM), volume (Vol), concentration (Conc) and morphological abnormalities (MA) after collection as well as total motility (TM) and progressive motility (PM) after SLC selection from six males; Data represent means (± SEM); Different letters within a column indicate differences ($P < 0.05$; $n = 3$).

	Fresh ejaculate				After SLC	
	TM (%)	Vol	Conc	MA (%)	TM (%)	PM (%)
A	62.93 ± 1.23a	3.33 ± 0.33ab	713.33 ± 14.54a	11.33 ± 0.88b	79.75 ± 6.55	24.45 ± 4,05
B	55.26 ± 2.46a	2.33 ± 0.35b	483.33 ± 54.63b	10.66 ± 1.76b	83.20 ± 0.63	38.00 ± 5,30
C	53.43 ± 2.60ab	5.66 ± 1.20a	385.00 ± 20.23b	23.00 ± 1.15c	79.13 ± 5.59	34.00 ± 6,71
D	64.73 ± 1.11a	5.33 ± 1.20a	301.00 ± 58.40c	13.33 ± 0.85b	89.05 ± 5.35	38.20 ± 12,10
E	54.00 ± 3.57a	2.83 ± 0.60b	286.66 ± 8.82c	11.33 ± 1.20b	78.63 ± 7.19	31.10 ± 5,25
F	51.03 ± 4.02b	2.56 ± 0.43b	400.00 ± 102.26b	6 ± 1.52a	80.10 ± 4.56	43.70 ± 7.65
P-value	< 0.001	0.002	0.001	0.003	0.817	0.663

Table 3
Values for sperm total (TM) and progressive (PM) motility, kinematics (ALH,BCF, LIN, STR, VAP, VCL, VSL), viability (VIT) and acrosome damage (AD) at 0 h after thawing from the six males; Data represent means (± SEM); Different letters among columns indicate differences ($P < 0.05$).

	Male A	Male B	Male C	Male D	Male E	Male F	*P*-value
TM	46.67 ± 2.51	33.00 ± 1.88	44.50 ± 6.12	42.43 ± 2.22	44.70 ± 24.31	39.70 ± 4.96	0.253
PM	18.50 ± 4.72	8.07 ± 1.80	17.07 ± 3.95	16.83 ± 0.18	18.70 ± 1.40	15.33 ± 2.05	0.153
ALH	8.30 ± 0.30	7.47 ± 0.29	8.20 ± 0.25	8.41 ± 0.21	7.82 ± 0.26	8.22 ± 0.65	0.331
BCF	19.49 ± 0.56	18.70 ± 0.37	20.06 ± 0.27	18.29 ± 1.03	18.38 ± 0.81	19.94 ± 1.01	0.357
LIN	33.86 ± 2.02	28.72 ± 1.82	31.93 ± 1.76	34.66 ± 2.31	35.87 ± 0.42	30.86 ± 0.09	0.181
STR	65.11 ± 3.59	60.03 ± 2.37	65.15 ± 2.55	66.23 ± 1.63	67.73 ± 1.25	65.38 ± 0.31	0.421
VAP	76.68 ± 3.59[a]	58.25 ± 0.86[b]	74.44 ± 2.24[a]	75.71 ± 3.76[a]	76.93 ± 3.33[a]	72.13 ± 2.73[a]	0.003
VCL	150.6 ± 5.06[a]	123.5 ± 1.22[b]	154.43 ± 7.42[a]	148.92 ± 5.22[a]	148.02 ± 6.38[a]	155.74 ± 7.70[a]	0.018
VSL	51.38 ± 3.58[a]	35.35 ± 2.14[b]	49.42 ± 3.01[a]	50.72 ± 3.61[a]	53.08 ± 2.96[a]	47.58 ± 2.00[a]	0.021
VIT	51.17 ± 4.51	39.83 ± 2.33	43.17 ± 8.56	42.17 ± 1.92	40.50 ± 5.53	39.17 ± 2.13	0.540
AD	22.00 ± 5.53	34.33 ± 3.17	27.33 ± 7.10	25.67 ± 5.07	25.83 ± 6.93	38.33 ± 2.45	0.318

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, %), beat cross frequency (BCF, Hz), vitality (VIT, %) and acrosome damage (AD, %).

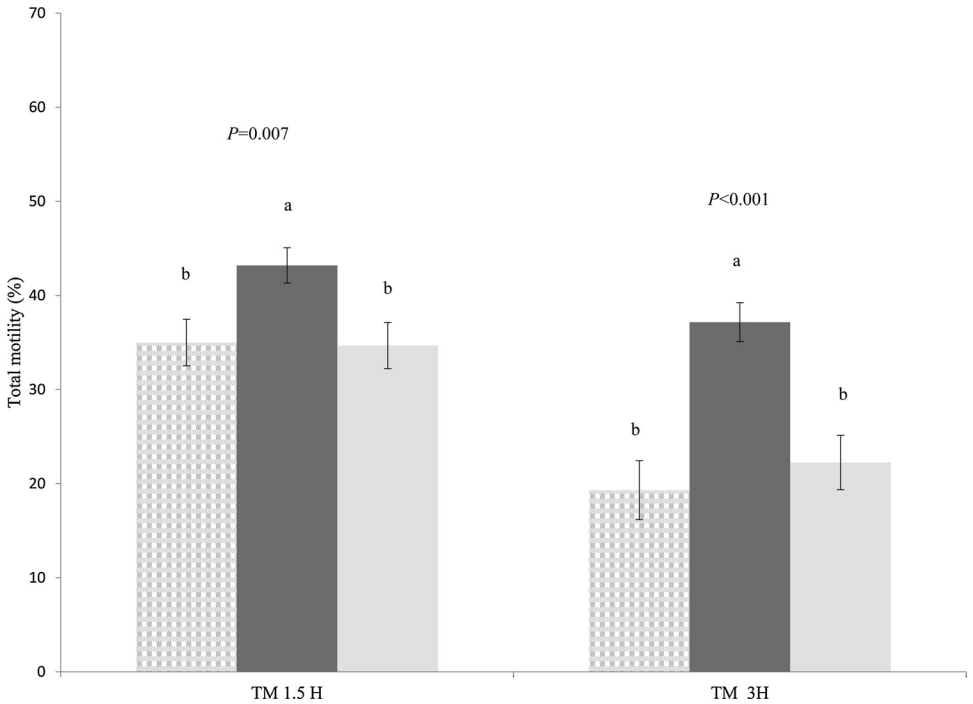

Fig. 1. Effect of the antioxidants Catalase and Epigallocatechin added at thawing on sperm total motility at 1.5 h and 3 h post-thaw. Control (checked bar), catalase (dark grey) and epigallocatechin (light grey). Different letters express the differences among treatments ($P < 0.05$).

thawing were evaluated. Sperm kinematics were improved after 1.5 and 3 hof incubation. However, MDA production was not affected during incubation for 1.5 or 3 h, and addition of catalase did not affect AI outcomes in a preliminary experiment. There was significant variation among males for all sperm variables analysed.

Several previous studies have examined the antioxidant potential of catalase on spermatozoa. The present findings of a positive effect only on kinematics, with no effect on viability or acrosome integrity, confirm results from a previous study (Malo et al., 2017a) in which addition of antioxidants (catalase, carnitine, GSH) to semen prior to freezing resulted in maintenance of total and progressive motility and kinematics, compared to controls. In contrast, Medan et al. (2008) reported that catalase maintained motility, viability and acrosome integrity during storage of dromedary spermatozoa at 5 °C for 5 days. The differences in these results may be due to the differences between liquid storage and cryopreservation or could be due to extender composition. Ermilov et al. (1999) found that most of the components of the culture media (especially zwitterion buffers and EDTA) have antioxidant capacity. Green buffer was used to freze the sperm in the present study whereas Medan et al. (2008) used a fructose-tris buffer extender; the composition of Green buffer (a commercial product) is not stated.

No effect of catalase on viability in dromedary camel sperm was found in the present study. In future studies more specific techniques, such as M540, should be included to determine whether catalase could affect sperm membrane lipid disruption, as

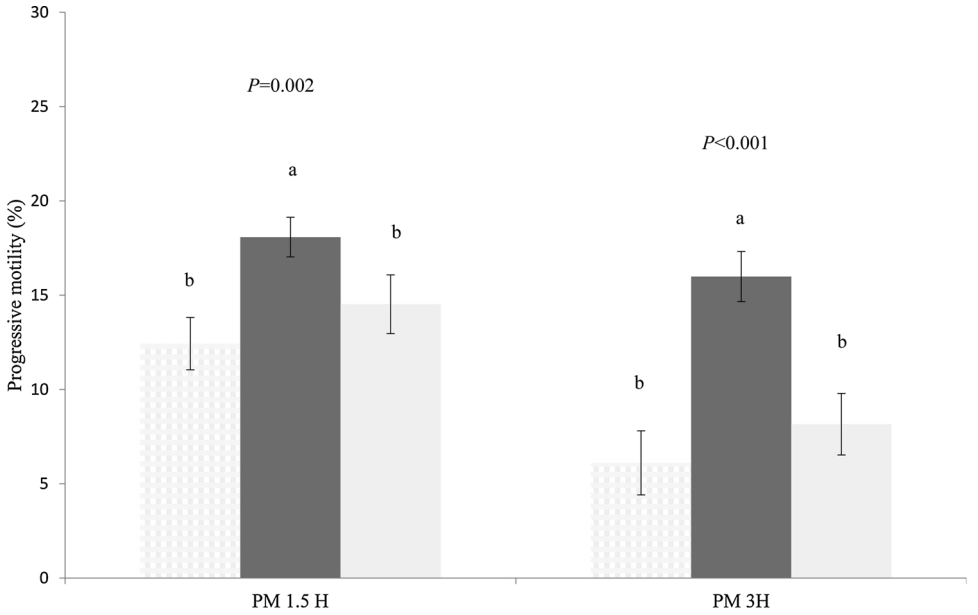

Fig. 2. Effect of the antioxidants (Catalase and Epigallocatechin) added at thawing on sperm progressive motility (PM) at 1.5 h and 3 h post-thaw. Control (checked bar), catalase (dark grey) and epigallocatechin (light grey). Different letters express the differences among treatments ($P < 0.05$).

Table 4
Effect of the antioxidants (Catalase and Epigallocatechin) added at thawing on sperm kinematics (ALH, BCF, LIN, STR, VAP, VCL, VSL), viability (VIT) and acrosome damage (AD) at 1.5 h after thawing; Data represent means (± SEM); Different letters among columns indicate differences ($P < 0.05$).

	Control	Catalase	Epigallocatechin	*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

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, %), beat cross frequency (BCF, Hz), vitality (VIT, %) and acrosome damage (AD, %).

Table 5
Effect of the antioxidants (Catalase and Epigallocatechin) added at thawing on sperm kinematic values (ALH, BCF, LIN, STR, VAP, VCL, VSL), viability (VIT), acrosome damage (AD) and MDA production at 3 h after thawing; Data represent means (± SEM); Different letters within a row indicate differences among treatments ($P < 0.05$).

	Control	Catalase	Epigallocatechin	*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
MDA	0.029 ± 0.005	0.022 ± 0.004	0.031 ± 0.008	0.697

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, %), beat cross frequency (BCF, Hz), vitality (VIT, %), acrosome damage (AD, %) and malondialdehyde (MDA, mg/10^{6} cells).

Table 6
Male effect on sperm total (TM) and progressive (PM) motility, kinematic values (ALH, BCF, LIN, STR, VAP, VCL, VSL), vitality (VIT) and acrosome damage (AD) at 1.5 h after thawing; Data represent means (± SEM); Different letters within a row indicate 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

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, %), beat cross frequency (BCF, Hz), vitality (VIT, %) and acrosome damage (AD, %).

Table 7
Male effect on sperm total (TM) and progressive (PM) motility, kinematic values (ALH, BCF, LIN, STR, VAP, VCL, VSL), viability (VIT), acrosome damage (AD) and MDA production at 3 h after thawing; Data represent means (± SEM); Different letters within a row indicate 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
MDA	0.045 ± 0.013	0.041 ± 0.010	0.014 ± 0.004	0.022 ± 0.004	0.025 ± 0.008	0.013 ± 0.003	0.424

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, %), beat cross frequency (BCF, Hz), vitality (VIT, %), acrosome damage (AD, %) and malondialdehyde (MDA, mg/10^{6} cells).

described by Fernández-Santos et al. (2007) in Iberian deer sperm. In the latter study, catalase did not maintain the stability of the membranes after 2 h, nor did it have an effect on mitochondrial function.

Epigallocatechin supplementation did not improve sperm quality after thawing in the present study, even though there were improvements in both total and progressive motility relative to control in the pilot study. These results are puzzling because the same males and methodology were used in both studies, but may be explained by differences among ejaculates and in the duration of storage. In the pilot study, ejaculates were collected in March with thawing being performed in April, whereas in the main study ejaculates were collected in April and May, with thawing in October. The daytime temperature was higher when the ejaculates for the main study were collected, than when the ejaculates for the preliminary experiment were collected. It is not known whether the natural antioxidant activity of camel semen changes with ambient temperature. The temperature in the laboratory where processing of semen and incubations occurred, however, is maintained at 24 °C throughout the year. In camelids, spermatogenesis has been reported to continue throughout the year, but there are greater rates of spermatogenesis during the months when the temperature is greater such as November and March (El-Kon et al., 2011). In a recent study, Al-Bulushi et al. (2019) indicated that the greatest sperm production in dromedary camels in Oman occurred from December to March, with a peak in the values during December and February.

Bucci et al. (2017) reported that there was no positive effect of green tea extract on stallion semen cooled at 4 °C for 48 h. The lack of an effect was attributed to the presence of seminal plasma in the extended semen, which contains various antioxidants. This, however, was not the situation in the present experiment because the dromedary camel sperm were separated from seminal plasma by colloid centrifugation before freezing. In another experiment in which this antioxidant was added to thawed boar semen, there was a greater in vitro penetration rate of the zona pellucida (Bucci et al., 2018) for treated sperm samples even though there was a lack of a measurable positive effect on sperm quality. Gale et al. (2015) studied the effect of green tea extract (not purified) on boar sperm cryopreservation and found that there was a lesser peroxidation rate (MDA) in the presence of 5% tea but with no improvements for other variables. All of these results indicate that additional sperm characteristics necessary for fertilization, other than those evaluated, are affected positively by these antioxidants.

In the present study, MDA was used to assess ROS scavenger activity of the antioxidants. The evidence from the present study that the addition of catalase at thawing resulted in improved post-thawed motility suggests that oxidative stress occurs during and after thawing but the lack of an effect on MDA concentrations is confusing. Consistent with the results of the present study, Sicherle et al. (2011) reported that catalase did not reduce ROS when oxidation was induced in frozen-thawed ram spermatozoa but the samples were incubated for just 5 min in their study. The lack of information about the catalase content in camel semen should be considered. In an earlier study, it was claimed that there is little of no catalase in most mammalian semen (ram, bull, rabbit, human; Holland et al., 1982; dog, Hatamoto et al., 2006). Because no information is available about catalase in camel semen, the likely consequences of elimination of the seminal plasma are not known. Some important components of seminal plasma (catalase, SOD, Vitamin C, Se, Zn) may have an important function in sperm survival during storage (Bucci et al., 2011).

In the present study, MDA production did not differ among treatments although it varied among males, ranging from 0.013 to 0.045 mg/10^6 cells. All the animals in the present study were of a similar age (8–10 years old), in contrast to a study on Brown Norway rats (Zubkova et al., 2005) where an age-related increase in free radical production was seen. In a study in human patients, men with normozoospermia idiopathic infertility had greater seminal ROS content and less antioxidant capacity than fertile men (Agarwal et al., 2006). In the present study, there were individual variations in all parameters studied, including MDA production. Catalase supplementation reduced the MDA production in males A, D, E and F. Michael et al. (2007) reported the quantification of ROS after catalase supplementation during freezing of dog semen. Consistent with results of the present study, neither SOD nor hydroxyl radicals were reduced post-thawing, although supplementation of extender with catalase increased dog sperm motility and viability. Bucci et al. (2018) measured lipid peroxidation using BODIPY and flow cytometry when freezing boar semen in presence of epigallocatechin, and again there was no improvement in the lipo-oxidation status. In future studies, other methods should be included to assess ROS formation in cells because there may be limitations with the MDA assay used in the present study. Esfandiari et al. (2003) reported that the values obtained with use of the nitroblue tetrazolium (NBT) reduction test to quantify oxidation in sperm were highly correlated with values obtained with use of chemiluminescence which is a direct method of measurement. Chemiluminescence evaluations, however, are not very practical, requiring expensive precision equipment. Gosálvez et al. (2017) confirmed the validity of use of NBT as a marker for oxidation. Tvrda et al. (2016a), however, assessed the antioxidant efficiency of lycopene by assessing the intracellular activity of SOD, CAT, GPx, as well as GSH and MDA concentrations. Lycopene resulted in increased motility and mitochondrial activity and increased antioxidant activity of SOD, CAT, GPx and GSH, although MDA was not affected. Similar results were reported when assessing the antioxidant properties of curcumin (Tvrdá et al., 2016b), indicating a possible lack of specificity of the test.

Preparing dromedary sperm using SLC allowed for the separation of sperm from the viscous seminal plasma that is a feature of camelid semen (Malo et al., 2017b). In another study,post-thaw sperm viability, acrosome integrity and chromatin integrity were improved in SLC-processed samples, as well as heterologous oocyte penetration and pro-nuclear formation (Malo et al., 2018). The results of the present study indicate the use of SLC leads to improvements of sperm quality both pre-freezing and immediately post-thawing, as previously reported, eliminating the differences in freezing capacity among males (Malo et al., 2017b, 2018). Interestingly, however, after the incubation in the present study (1.5 and 3 h), these differences among males were still observed. It, therefore, appears that SLC selects a sub-population of sperm with certain characteristics in different males, but there are differences in how well the sperm survive during incubation post thawing. This observation may be due to incubation in the presence of glycerol, which is believed to be toxic to sperm when temperatures are increased. More research on the effect of the SLC procedure on camel sperm survival after thawing is needed. The existence of individual animals with greater and lesser capacity for cryosurvival in other species has been reported previously (e.g., boar; Medrano et al., 2002) and needs to be taken into account for future research.

There is very limited information about the in vivo fertilising capacity of dromedary camel sperm. Deen et al. (2003) reported the first pregnancy with frozen dromedary semen (1/3). Results of the present study are consistent with those in this previous study indicating there are limitations of AI with frozen semen in camelids. Meanwhile, Akbar et al. (2018) reported a 71% pregnancy rate using frozen semen. These results have not been replicated by others. More information about factors such as timing of AI relative to ovulation, sperm dose and site of deposition, is needed to increase the efficiency of AI with frozen semen in camelids.

5. Conclusions

The results of the present study indicated that there is a beneficial effect of adding catalase to dromedary camel sperm samples at thawing, with a longer maintenance of sperm motility being the primary benefit. This improvement in sperm motility was not reflected in any improvement in in vivo fertility in a small AI experiment. There was a marked variation in values for most sperm variables among males in ejaculates as well as after post-thawing incubation, an important fact that should be taken into consideration when using cryopreserved camelid semen. Future research should be directed at identifying the mechanism of action of this antioxidant, and also in studying the different sensitivities to sperm lipid peroxidation among males. In general, there are many challenges still to be addressed concerning sperm cryopreservation and AI with frozen semen beforecommercial systems can be developed for these purposes.

Acknowledgement

We thank the Manipal University (Dubai; UAE) for allowing us to use the spectrophotometer for MDA determination. This study was sponsored by H.H. General Sheikh Mohammed bin Rashid Al Maktoum, Ruler of Dubai.

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