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Research Article

Effect of Antioxidants on the Stored Dromedary Camel Epididymal Sperm Characteristics

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Abstract

This study was conducted to determine the effect of different type and levels of antioxidant supplementation (0.4 and 0.8 mM from glutathione, GSH or 0.5 and 1.0 g L^{-1} extender from ascorbic acid, AA) as compared to control, on characteristics of camel epididymal spermatozoa stored at 25°C (room temperature) for 0, 2, 4 and 12 h or at 5°C (cool temperature) for 0, 12, 24 and 48 h. Testis of camel were collected after animal slaughtering and placed immediately into plastic bag into ice box at 5°C. Epididymal spermatozoa were collected by aspiration from tail and extended with tris-egg yolk extender. Results of epididymal spermatozoa stored at 25°C showed improvement in livability ($p<0.05$) and abnormality ($p\geq0.05$) with GSH (0.4 mM), while sperm motility and curling spermatozoa improved ($p\geq0.05$) with AA (0.5 g L^{-1}). Storage at 5°C improved ($p<0.05$) motility, livability and curling spermatozoa with GSH (0.4 mM), while sperm abnormality improved ($p<0.05$) with AA (1 g L^{-1}). At different incubation times at 25 or 5°C, percentages of motility, livability and curling spermatozoa decreased ($p<0.05$) and of sperm abnormality increased ($p<0.05$) by increasing storage time. The effect of interaction between antioxidant supplementation and storage time on all sperm characteristics studied was not significant. In conclusion, supplementation of tris-egg yolk extender with GSH (0.4 mM) or AA (0.5 g L^{-1}) has a vital role in maintaining function, morphology and membrane integrity of epididymal camel spermatozoa stored at 25°C for 12 h or at 5°C for 48 h, respectively.

Key words: Camel, epididymis, sperm, dilution, antioxidant, storage, temperature

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Both natural mating and Artificial Insemination (AI) using ejaculated semen are considered to be the best to achieve acceptable fertility rates. However, in certain situations natural mating is not an option and ejaculated semen is unavailable either due to difficulties in handling the animal or its death prior to collection and/or obstructive azoospermia preventing ejaculation (Drouineaud *et al*., 2003). In such instances an alternative source of viable, reproductively capable sperms could be epididymal sperms stored in the cauda epididymis. Research has shown that cauda epididymal sperm can be used effectively to produce viable offspring, using either AI goat (Blash *et al*., 2000), dog (Hori *et al*., 2005) or Intra Cytoplasmic Sperm Injection (ICSI) cattle (Goto *et al*., 1990) and rat (Hirabayashi *et al*., 2002). However, the time period for which epididymal spermatozoa can be stored effectively to produce viable offspring, varies and there is a controversy regarding the effect of storage time for camel epididymal spermatozoa on the rate of blastocyst production. While epididymal spermatozoa stored at 5°C have been reported to show better motility and a lower percentage of abnormalities than those stored at room temperature for 24 or 48 h (Kaabi *et al*., 2003), no differences were observed in the proportion of oocytes that cleaved and/or those that developed to blastocyst stage when camel epididymal spermatozoa stored for up to 8 days in either Tris–test or Tris–lactose egg yolk was used (Wani, 2009). On the other hand, cleavage rate was similar for oocytes inseminated with epididymal spermatozoa stored at 4°C for 0, 4 and 6 days, a higher blastocyst production was observed for epididymal spermatozoa stored for zero compared to six days (16.33 vs. 1.25%, respectively) (Badr and Abdel-Malak, 2010). Reasons for this reduced rate of blastocyst production with epididymal spermatozoa stored for comparatively longer period of time are not clear. Nevertheless, availability of viable and functional spermatozoa during the storage period is a prerequisite for AI and *in vitro* maturation (IVF), thus necessitating the need to determine the proper storage conditions in order to maintain the quality and fertilizing ability of the spermatozoa.

Semen contains appreciable amounts of antioxidants that prevent excessive peroxide formation (Lewis *et al*., 1997). Glutathione (L-glutamyl-L-cysteinylglycine; GSH) is a tri-peptide ubiquitously distributed in living cells. It plays an important role in the intracellular defense mechanism against oxidative stress (Irvine *et al*., 1996). As Reactive Oxygen Species (ROS) generation is a common feature during the process of semen preservation with any extender, therefore, to avoid the toxic effects of these compounds on the sperm, extenders need to be formulated with effective antioxidant properties (Da Silva Maia *et al*., 2010). It is a possibility that spermatozoa of different origins (e.g., species, parts of the reproductive tract) may vary in their endogenous protection from the ROS. For example, a lesser activity of antioxidant enzymes has been reported in buffalo bull spermatozoa due to higher lipid peroxidation, suggesting that there are more prone to oxidative stress when stored at refrigeration temperature (Nair *et al*., 2006). Spermatozoa collected from the epididymis are particularly susceptible to ROS, as they have never been exposed to secretions of the accessory sex glands (seminal plasma) which are recognized as the prime source of endogenous anti-oxidant protection (Chen *et al*., 2003).

In vitro studies have suggested that the addition of glutathione (0.4 mM) to the diluted semen can improve the motility and survival of bull spermatozoa stored at either room temperature (25°C) or at 5°C (El-Sherbieny *et al*., 2006). Ascorbic acid (vitamin C) is an antioxidant substance, which is normally present in the epididymal fluid and seminal plasma of several species including ram (Chinoy, 1972). It also plays a role in protecting sperm from ROS (Buettner, 1993) and in maintaining the genetic integrity of sperm cells by preventing oxidative damage to sperm DNA (Fraga *et al*., 1991). As the endogenous antioxidative capacity of semen is compromised during dilution and/or storage of semen (Maxwell and Salamon, 1993), therefore, it is a possibility that longer storage period may affect the antioxidant ability and therefore, the viability of the semen. The present study was aimed to determine the effect of antioxidant (glutathione and ascorbic acid) supplementation on characteristics of camel epididymal spermatozoa stored at 5°C for a period of 48 h or at room temperature for 12 h.

MATERIALS AND METHODS

Collection and dilution of epididymal spermatozoa: A total of 30 testes from mature camel bulls were collected from the abattoir immediately after slaughtering. They were transported to the laboratory on ice and the maximum time between the collection of the testes and arrival at laboratory was 6 h. Upon arrival in the laboratory, testes were dissected immediately to remove tunica vaginalis and other extraneous tissues, washed with water for 3 times, then rinsed once with 70% ethanol and finally washed with double distilled water. The tail of epididymis was separated and incised several times with a scalpel blade. This was followed by pressing the incised tissues manually to harvest spermatozoa that were aspirated into a sterile 5 mL syringe containing 2 mL semen extender.

The recovered spermatozoa were placed in a 15 mL tube. Tris fructose egg yolk extender supplemented with citrate and caffeine (Table 1) was used to dilute the semen as described previously (Deen *et al*., 2003). Briefly buffer was prepared by dissolving tris, fructose, citrate and caffeine in water making the final volume to 1000 mL. The buffer was autoclaved at 1.1 kg cm^{-2} pressure for 30 min, cooled and refrigerated until used to prepare semen extender by adding 80 mL of buffer to 20 mL of egg yolk along with antibiotics.

Experimental design: Epididymal spermatozoa were collected and diluted with tris-fructose-egg yolk extender containing 0.0, 0.4 and 0.8 mM glutathione (GSH) or 0.0, 0.5 and 1.0 g L^{-1} of ascorbic acid (AA). The diluted spermatozoa were stored at room temperature (25-30°C) for 0, 2, 4 and 12 h or at 5°C for 0, 12, 24 and 48 h.

Semen evaluation: Three diluted semen samples per antioxidant level were evaluated for progressive motility, livability, sperm abnormality and curling (hypo-osmotic swelling test) for each storage time. Progressive sperm motility (%) was determined using microscope supplied with hot stage adjusted to 37°C as described previously (Rao and Hart, 1948). Sperm livability (%) was determined after staining the semen with eosin/nigrosin (Hackett and Macpherson, 1965). Morphological abnormalities (%) were determined according to classification by Blom (1983). Hypo-Osmotic Swelling test (HOS-test) was carried out using solution prepared with fructose (1.25%) and Na-citrate (2.9%) in double distilled water to give osmolarity of 300 m Osm using a freezing-point depression osmometer (Osmett A, Model 5002, Fisher Scientific, Pittsbury, PA, USA). Then, distilled water was added to bring the osmolarity level to 50 m Osm L^{-1}. One drop of diluted semen was added to one ml of hypo-osmotic solution (50 m Osm L^{-1}) and the mixture was immediately incubated for 30 min at 37°C in a water bath. Following the incubation, semen was mixed with eosin/nigrosin stain and dried on a hot stage at 37°C. The number of spermatozoa with curled tails (Fig. 1) was determined using microscope (x 400). One hundred spermatozoa per antioxidant level and per storage time were counted and percentage of spermatozoa with curled tails (Fig. 1) was recorded.

Statistical analysis: As the data were not normally distributed, it were transformed into arc sine before subjecting to the analysis of variance (ANOVA) using general linear model (SAS., 1996). The differences among the treatment means were compared using Duncan multiple range test (Duncan, 1955). For the sake of convenience, the data on mean values were back transformed and are presented as arithmetic values.

RESULTS

Effect of antioxidant supplementation on characteristics of camel epididymal spermatozoa storage at room temperature (25°C): Overall, supplementation with 0.4 mM GSH significantly ($p \leq 0.05$) improved sperm livability and decreased sperm motility but did not affect sperm abnormality or curling percentage (Table 2). Supplementation with 0.8 mM GSH or AA did not affect livability or abnormality compared with controls. However, 0.8 mM GSH significantly

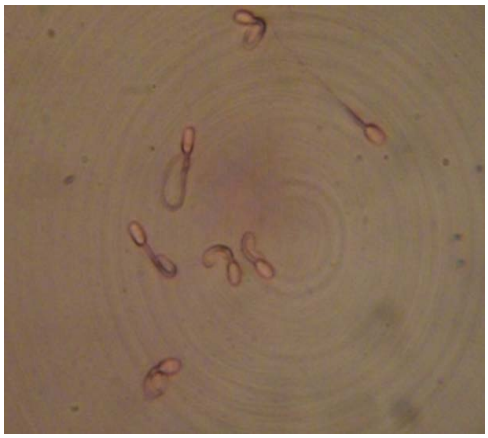

Fig. 1: Camel spermatozoa after subjected to HOS-test, note normal spermatozoa and those with curled tails

Table 1: Composition of buffer and extender

Composition of buffer			
Buffer		Extender	
Tris	30.28 g	Buffer	80 mL
Fructose	12.5 g	Egg yolk	20 mL
Citric acid	16.7 g	Benzyl penicillin	1000 IU mL^{-1}
Caffeine	0.039 g		
Distilled water added to	1000 mL	Streptomycin sulfate	1000 µg mL^{-1}

Table 2: Overall mean and standard error of sperm motility, livability, abnormality and curling percentages when camel epididymal spermatozoa were diluted with tris-fructose egg yolk extender supplemented with different levels of glutathione (GSH) or Ascorbic Acid (AA) and stored at room temperature (25°C)

	Sperm characteristics (Mean±SE)			
Supplements	Motility (%)	Livability (%)	Abnormality (%)	Curling (%)
Control	73.75±0.47^{a}	68.92±0.57^{b}	18.92±0.35	74.75±0.54^{a}
GSH (0.4 mM)	63.33±0.88^{c}	77.33±0.50^{a}	15.92±0.34	70.42±0.70^{ab}
GSH (0.8 mM)	64.17±0.68^{c}	72.83±0.52^{b}	16.67±0.34	67.58±0.71^{b}
AA (0.5 g L^{-1})	68.33±0.62^{b}	69.50±0.73^{b}	17.58±0.44	71.83±0.69^{ab}
AA (1.0 g L^{-1})	74.17±0.73^{a}	72.42±0.50^{b}	18.25±0.44	75.08±0.72^{a}

Means with different superscripts within the same column differ significantly ($p \leq 0.05$)

Table 3: Overall mean and standard error of camel epididymal sperm motility, livability, abnormality and curling percentages, stored at 25°C for different time periods (h) after dilution with tris-fructose egg yolk extender supplemented with antioxidants

	Sperm characteristics (Mean±SE)			
Storage period (h)	Motility (%)	Livability (%)	Abnormality (%)	Curling (%)
0	77.0±0.37^{a}	79.07±0.29^{a}	12.67±0.17^{d}	80.00±0.39^{a}
2	72.0±0.39^{b}	74.27±0.33^{b}	16.00±0.18^{c}	74.47±0.44^{b}
4	67.0±0.43^{c}	70.27±0.36^{c}	19.13±0.21^{b}	68.73±0.35^{c}
12	59.0±0.52^{d}	65.20±0.42^{d}	22.07±0.23^{a}	64.53±0.42^{d}

Means with different superscripts within the same column differ significantly ($p \leq 0.05$)

Table 4: Overall mean and standard error of sperm motility, livability, abnormality and curling percentages when camel epididymal spermatozoa were diluted with tris-fructose egg yolk extender supplemented with different levels of glutathione (GSH) or Ascorbic Acid (AA) and stored at 5°C

	Sperm characteristics (Mean±SE)			
Supplement	Motility (%)	Livability (%)	Abnormality (%)	Curling(%)
Control	71.67±0.72^{c}	72.33±0.43^{b}	18.50±0.30^{a}	76.17±0.45^{ab}
GSH (0.4 mM)	76.67±0.57^{a}	77.42±0.53^{a}	17.58±0.37^{a}	77.17±0.54^{a}
GSH (0.8 mM)	72.92±0.76^{bc}	76.25±0.61^{a}	14.83±0.30^{b}	76.42±0.40^{ab}
AA (0.5 g L^{-1})	75.83±0.53^{ab}	78.42±0.40^{a}	15.50±0.30^{b}	78.83±0.29^{a}
AA (1.0 g L^{-1})	70.42±0.70^{c}	71.67±0.62^{b}	12.92±0.26^{c}	73.67±0.56^{b}

Means with different superscripts within the same column differ significantly ($p \leq 0.05$)

($p<0.05$) decreased motility and curling percentage. The AA at (0.5 g L^{-1}) also significantly ($p \leq 0.05$) decreased motility (Table 2).

Irrespective of antioxidant supplementation, increasing incubation period significantly ($p \leq 0.05$) decreased the sperm motility, livability and curling percentage and increased ($p \leq 0.05$) the sperm abnormality (Table 3). A non significant interaction was observed between antioxidants levels and storage periods for any of the sperm characteristics. However, it is worth to note that the highest values for sperm livability and abnormality (72 and 20%, respectively) were recorded when spermatozoa were diluted with extender containing 0.4 mM GSH and stored for 12 h.

Storage at cool temperature (5°C): Supplementation of the semen extender with GSH at a level of 0.4 mM and AA at a level of 0.5 g L^{-1} significantly ($p \leq 0.05$) increased sperm motility and livability percentages compared to the control extender. Moreover, supplementation of the semen extender with 0.8 mM GSH significantly ($p \leq 0.05$) increased the sperm livability and decreased sperm abnormality. Supplementation of extender with AA at a level of 0.5 g L^{-1} significantly ($p \leq 0.05$) increased percentages of curled spermatozoa and decreased sperm abnormality compared with the control extender (Table 4).

The overall effect of storage period regardless antioxidant addition showed that sperm motility, livability and curling spermatozoa percentage significantly ($p \leq 0.05$) decreased and sperm abnormality percentage significantly ($p \leq 0.05$) increased by increasing storage period. However, acceptable sperm characteristics were obtained after 48 h storage (Table 5).

A non significant interaction between antioxidants and storage periods for any of the semen characteristics suggested that sperm characteristics in extenders supplemented with different levels of antioxidants showed similar trend of change with increasing storage time at 5°C. However, a different rate of changes in sperm characteristics was observed at different levels of antioxidants and storage period after 48 h storage period, the highest percentages of motility, livability and curling of spermatozoa (68.3, 71.6 and 75.0%, respectively)

Table 5: Overall mean and standard error of camel epididymal sperm motility, livability, abnormality and curling percentages, stored at 5°C for different times (h) after dilution with tris-fructose egg yolk extender supplemented with antioxidants

	Sperm characteristics (Mean±SE)			
Storage period (h)	Motility (%)	Livability (%)	Abnormality (%)	Curling (%)
0	82.00±0.28[a]	82.07±0.26[a]	11.87±0.18[d]	82.20±0.21[a]
12	76.67±0.27[b]	77.60±0.25[b]	14.47±0.18[c]	78.07±0.20[b]
24	71.33±0.27[c]	73.27±0.29[c]	17.20±0.19[b]	74.73±0.26[c]
48	64.00±0.38[d]	67.93±0.32[d]	19.93±0.20[a]	70.80±0.31[d]

Means with different superscripts within the same column differ significantly ($p \leq 0.05$)

were recorded with supplementation of AA at a level of 0.5 g L^{-1} and the lowest percentage of sperm abnormality 19.0% was recorded with supplementation of AA at a level of 1.0 g L^{-1}.

DISCUSSION

The results of the present study have shown that camel epididymal spermatozoa can be stored at room temperature (25°C) for 12 h or at 5°C for 48 h with acceptable percentage of sperm livability and abnormality if the semen extender is supplemented with antioxidants (GSH or AA). The GSH is a widespread molecule found in sperm cells and is able to react with many POS directly and is also a co-factor for glutathione peroxidase that catalyses the reduction of toxic H_2O_2 and hydro-peroxides (Bilodeau *et al*., 2001) and improves DNA integrity when added to bull semen extender (Tuncer *et al*., 2010). The relative amounts of glutathione peroxidase, glutathione reductase and glutathione transferase activities in the cauda epididymidal spermatozoa were negligible (Koziorowska-Gilun *et al*., 2011). The use of glutathione as semen additive may be used for improving post-thaw semen quality and overall augmentation of pregnancy in cows. The addition of GSH suggestive of reducing lipid peroxide levels (Perumal *et al*., 2011). The addition of GSH (0.5, 1.0 or 2.0 mM), did not affect the total antioxidant capacity of the tris-egg yolk extender throughout refrigeration and freezing. However, maintaining ovine semen at 5°C for 12 h prior to cryopreservation reduced cell membrane damage (Camara *et al*., 2011). The addition of GSH (2 and 5 mM) preserve the integrity of the acrosome of frozen ram spermatozoa (Silva *et al*., 2012).

In accordance with the results obtained on camel spermatozoa in the present study, supplementation of bull spermatozoa with 0.4 mM GSH and stored at room temperature resulted in the highest sperm livability and lowest sperm abnormality percentages. Moreover, supplementation of 0.4 mM GSH to Friesian bull semen stored at 5°C showed the highest ($p<0.05$) percentages of motility and livability of bull spermatozoa as compared to 0.8 or 1.2 mM GSH (El-Sherbieny *et al*., 2006). Similarly, addition of 1.0 and 1.5 mM of GSH in TALP-extender to bull semen stored at room temperature (25°C) has been reported to significantly increase the sperm motility (Foote *et al*., 2002).

Ascorbic acid is considered to be a very efficient antioxidant and its addition to semen extender is reported to reduce the oxidative stress provoked by thawing. It protects sperm membrane integrity and sperm chromatin (Fernandez-Santos *et al*., 2009; Hu *et al*., 2010) and its supplementation to the extender has shown to improve the refrigerated storage of red deer epididymal spermatozoa (Fernandez-Santos *et al*., 2009). Sperm viability was higher ($p = 0.001$) and Superoxide production was significantly lower when vitamin C were added in semen extenders (Michael *et al*., 2009).

In similar trend with the present results, (El-Sherbieny *et al*., 2006) found that sperm motility and livability percentages significantly ($p<0.05$) decreased, however, percentage of sperm abnormality was not affected significantly by increasing storage time of Friesian semen diluted with Tris-extender supplemented with different GSH levels and stored for 96 h at 4°C.

Storage of semen at 4°C, increasing ROS production (Wang *et al*., 1997) has been shown to decrease motility in mouse, human, bull, and rabbit spermatozoa. Hydrogen peroxide (H_2O_2) as ROS has been shown to decrease sperm motility and livability (Alvarez and Storey, 1989). Under these conditions, adding several types of antioxidants could help to maintain survival and motility of spermatozoa (Beconi *et al*., 1993; Bilodeau *et al*., 2001; Foote *et al*., 2002).

Lipid peroxidation does not increase substantially during semen storage at 5°C. The natural antioxidative systems in whole semen apparently are sufficient to prevent ROS production. The addition of semen extender further increases the antioxidative activity of stallion semen. Both the basal antioxidative activity in native semen and the increased activity in extended semen are maintained over 24 h at 5°C as storage period. If peroxidative damage occurs during semen storage, this is not the result of a reduced or insufficient antioxidative capacity (Kankofer *et al*., 2005).

CONCLUSION

In conclusion, the results of the present study suggest beneficial effects of GSH and ascorbic acid, on membrane integrity, function and morphology of camel epididymal spermatozoa, when used as antioxidant supplement in the semen extender.

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