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Assessment of Freezability and Functional Integrity of Dromedary Camel Spermatozoa Harvested from Caput, Corpus and Cauda Epididymides

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camel – epididymis – spermatozoa – cryopreservation – IVF – comet

ABSTRACT:

The present study aimed to assess the freezability and functional integrity of dromedary camel spermatozoa harvested from three epididymal regions. Twenty five epididymes were obtained from slaughtered adult camels. The cauda, corpus and caput epididymides were isolated, incised and rinsed for obtaining the sperm rich fluid. Portion of this fluid was processed for cryopreservation. Fresh and frozen-thawed spermatozoa collected from different epididymal regions were evaluated for motility, livability, morphological abnormalities, membrane and acrosomal integrities as well as mitochondrial activity. Also, in vitro fertilization using camel mature oocytes and measuring DNA integrity using Comet assay were performed for these spermatozoa. The results showed that, there were no significant differences in livability among spermatozoa freshly collected from cauda, corpus and caput epididymides (81.16 ± 1.43, 80.20 ± 0.90 and 76.76 ± 1.95%, respectively). Total sperm motility increased dramatically from the caput (22.60 ± 0.96%) to the cauda (67.92 ± 1.14%) of the epididymis. Viability index of cauda frozen-thawed spermatozoa (96.50 ± 2.36%) was significantly higher than those of corpus (53.20 ± 3.11%) and caput epididymides (12.10 ± 1.10%). Fresh and frozen thawed spermatozoa of the cauda epididymides had significantly higher percentages of membrane integrity, cytoplasmic droplets and MTT reduction rate than the corresponding parameters of corpus and caput spermatozoa. Fresh and frozen thawed spermatozoa of the cauda epididymides had significantly higher fertilization rates (50.88 ± 1.10 and 38.64 ± 0.77%, respectively) than those of corpus (36.92 ± 0.79 and 22.16 ± 0.79%, respectively) and caput epididymides (12.48 ± 1.09 and 4.36 ± 0.59%, respectively). Only oocytes fertilized with fresh and frozen-thawed cauda epididymal spermatozoa developed to blastocysts (10.92 ± 0.52 and 8.12 ± 0.81%, respectively). The percentage of fresh cauda epididymal spermatozoa with non-fragmented DNA was higher than those of corpus and caput of epididymis (90.88 ± 1.55 vs. 78.28 ± 0.72 and 76.24 ± 1.02%, respectively). In conclusion, obtaining spermatozoa of good quality and freezability from dromedary camel cauda epididymides is possible, and these fresh and frozen-thawed spermatozoa may have the potential uses in IVF and AI for improving breeding potentials in this species.

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1. INTRODUCTION

The interest in developing assisted reproductive technologies and cryobanking for the conservation of camel genetic resources has recently increased (Turri et al., 2013). In these cases epididymal sperm from slaughtered or recently died animals will increase the opportunities to create semen storages. The function of the epididymis is to bring testicular spermatozoa (which are non-motile and incapable of fertilization) to functional as well as morphologically mature. The epididymis also protects the spermatozoa as they become mature and provides an environment for storage following the maturation process (Hafez et al., 2011). The regions of epididymis have different functions. The head and corpus are considered to be the districts in which sperm maturation take place, whereas the tail region is thought to be primarily involved in sperm storage prior to ejaculation (Glover and Nicander, 1971).

In dromedaries, there are no reports on the use of stored ejaculated semen for IVF mainly because of the difficulties in semen collection, the gelatinous nature of ejaculated semen and the lack of suitable extenders for its storage (Bravo et al., 2000; Deen and Sahani, 2000). Keeping in view these problems, the use of epididymal spermatozoa could be an

alternative. However, availability of viable and functional spermatozoa during the storage period is a prerequisite for AI and IVF, thus necessitating the need for optimal diluents and proper storage conditions to maintain the quality and fertilizing ability of the spermatozoa for longer periods (Wani, 2009).

There are many successful reports of collection and cryopreservation of epididymal spermatozoa in buffalo: Lambrechts et al., 1999; horses: Morris et al., 2002; dog: Hewitt et al., 2001; ram: Kaabi et al., 2003; boar: Suzuki and Nagai, 2003; red deer: Soler et al., 2005; Spanish ibex: Santiago-Moreno et al., 2006; brown bear: Anel et al., 2011. Although all previous reports of successful production of living animals using epididymal sperm in different species (white-tailed deer: Jacobson et al., 1989, chimpanzee: Kusunoki et al., 2001, Spanish ibex: Santiago-Moreno et al., 2006), there are few reports on IVF with epididymal sperm that is typically used frozen-thawed (Katska et al., 1996).

Sperm DNA fragmentation is an important parameter to assess sperm quality and can be putative fertility predictor (Evenson et al., 1980). The evaluation of DNA status is not included in the standard semen analysis, but the frequency of spermatozoa containing fragmented DNA may be an important parameter of semen quality, and a useful index of fertility potential (Vernocchi et al., 2014).

For AI and IVF, availability of viable and functional camel epididymal spermatozoa during the storage period is a prerequisite, thus necessitating the need for optimal harvesting and proper processing conditions to maintain the quality and fertilizing ability of the spermatozoa for longer periods. So, the aim of this study was to assess the freezability and functional integrity of dromedary camel spermatozoa harvested from caput, corpus and cauda epididymides.

2. Materials and methods

2.1. Chemicals:

Chemicals and media were purchased from Sigma (St. Louis, MO) unless otherwise stated.

2.2. Collection of epididymal spermatozoa:

A total of twenty five apparently healthy male dromedary camels, aged between 6 to 12 years, were enrolled in this study during the rutting season (December to April, Shalash, 1980). Camel testes (N = 50) were transported from a local abattoir (Kerdasa abattoir, Giza) to the laboratory in normal saline solution (NSS). Testes were washed with sterile NSS. The cauda, corpus and caput epididymides were isolated (Fig. 1), incised longitudinally (Fig. 2) and rinsed 3-4 times with 2 ml of Brackett and Oliphant (BO) medium (Fig.3) in 60 mm petri dishes (Bacto Laboratories, Liverpool, Australia) placed on heated stages (37 °C).

2.3. Cryopreservation and thawing of epididymal sperm:

Fluid rich in spermatozoa that collected from cauda, corpus and caput epididymides was diluted with Shotor (Tris-based egg yolk extender, Niasari-Naslaji et al., 2007) at 37oC in incubator in an appropriate dilution rate to obtain a final concentration of 40 x 106 sperm cell/ml. Diluted samples were then cooled slowly to 5oC in a cold cabinet for a period of 1.5 h, loaded in 0.25 ml straws (IMV, France) and placed 4 cm above liquid nitrogen in the vapor phase in a foam box for 15 min before being plunged into the liquid phase (Khalifa, 2001). Straws were stored in liquid nitrogen until thawing at 37oC in a water bath for 30 sec.

2.4. Evaluation of the epididymal spermatozoa:

Freshly harvested and frozen-thawed spermatozoa were evaluated for total and progressive motilities under phase-contrast microscope. Sperm viability, abnormalities and acrosomal status was evaluated by a dual staining procedure (Didion et al., 1989). Briefly, spermatozoa were incubated with an equal volume of 0.2% trypan blue for 10 min and washed twice (centrifugation at 700g for 6 min) with BO medium. Smears were made on glass slides and dried quickly on a warm stage. Slides were stained with 10% giemsa stain for 40 min. They were rinsed under a stream of distilled water, air-dried, and covered with coverslips. Spermatozoa were classified as live [unstained post acrosomal region], dead [stained blue in the post acrosomal region], acrosome intact [light purple - dark pink acrosome] and damaged/lost acrosome [unstained or blue acrosome]. The procedure described by Jeyendran et al. (1984) was used to determine the percentage of HOS positive sperm cells in each semen sample. A 100 µl aliquot of each semen sample was mixed in 1.0 ml of a pre-warmed hypo-osmotic solution (0.735 g of sodium citrate dihydrate and 1.351 g of fructose in 100 ml of de-ionized water). The mixture was incubated at 37°C for 30 min in a 1.5 ml micro-centrifuge tube. Following incubation, a small drop of sample was placed on a clean microscope slide and cover-slipped for examination using phase contrast microscopy (400X) to evaluate 100 spermatozoa for evidence of swelling and curling changes of the sperm tail (Fig.4).

2.5. MTT reduction assay:

The MTT assay was performed according to the method of Mosmann (1983). For each fresh or frozen-thawed sample, six wells of the 96-well microplate were used. The 100 µl of semen sample plus 10 µl of MTT stock solution (5 mg MTT/ml of PBS) was placed in each well. The rates of MTT reduction were determined using an ELISA reader at a wavelength of 550 nm. The optical density of fresh and frozen-thawed samples was measured 2 times (immediately and after 1 h of incubation at 37 ◦C). MTT reduction rates (optical density) for each semen sample was calculated by concurring the difference between the first and second reading of the ELISA reader.

2.6. Comet (Single cell gel electrophoresis assay):

The alkaline comet assay for freshly harvested and frozen-thawed spermatozoa was carried out according to Hughes et al. (1996). Fully frosted glass slides were covered with 100 µl of 0.5% normal melting point agarose, a coverslip was added and the agarose was allowed to solidify. The coverslips were removed and 1x105 sperm cells in 50 µl PBS (7.2 pH) were mixed with 50 µl of 1.2% low melting point agarose and used to form the second layer. The slides with coverslips removed were then placed in lysis buffer for 1 h (2.5 M NaCl, 100 mM Na EDTA, 10 mM Tris, 1% Triton X at a pH of 10). The slides were then incubated at 37°C in 100 µl/ml of proteinase K in lysis buffer overnight. After draining the proteinase K solution from the slides, they were placed in a horizontal electrophoresis unit filled with freshly prepared alkaline electrophoresis solution containing 300 mM NaOH and 1 mM EDTA for 20 min to allow the DNA to denature. Electrophoresis was performed at room temperature, at 25 V (0.714 V/cm) and 300 mA, obtained by adjusting the buffer level, for 10 min. The slides were then washed with a neutralizing solution of 0.4 M Tris at pH 7 to remove alkali and detergents. After neutralization, the slides were stained with 50 µl of 20 µg/ml ethidium bromide and mounted with a coverslip. A total of 200 sperm cells were examined under fluorescent microscope (400X). The intensity of the stain in the comet tail region is presumed to be related to the DNA content, and DNA damage is estimated from measurements of the percent DNA in tail, tail length and Olive tail moment, using an image analysis system (Comet-Score program). Spermatozoa with fragmented DNA (damaged) display increased migration of the DNA from the nucleus towards the anode (Fig. 5), while spermatozoa with non-fragmented DNA (undamaged) do not form a "comet" (Fraser, 2004).

2.7. In vitro fertilization:

2.7.1. Collection and maturation of oocytes: camel ovaries were transported from a local abattoir to the laboratory in a thermo container containing PBS at 30°C. Cumulus oocyte complexes (COCs) were aspirated from follicles 2–8 mm in diameter using 18-gauge needle attached to 10 ml syringe and placed into 50 ml conical tubes in a warming box at 39 oC. After being washed 3 times in PBS, COCs with at least 2–3 compact layers of cumulus cells and a homogeneous cytoplasm were selected and washed 3 times in maturation medium. For maturation, cumulus-enclosed oocytes were cultured in 100 µl droplets of maturation medium (10–15 oocytes per droplet) covered with mineral oil for 30 h (Khatir and Anouassi, 2006) at 38.5°C in 5% CO2 and humidified air. The basic maturation medium consisted of TCM-199 enriched with 10% v/v fetal calf serum (FCS), 1 mg/ml FSH, 1 mg/ml estadiol and 50 mg/ml gentamicin.

2.7.2. In vitro fertilization:

Five replicates of both freshly harvested and frozen-thawed spermatozoa that collected from cauda, corpus and caput epididymides were prepared for IVF (each replicate included pooled semen harvested from the epididymides of five animals) as described by Niwa and Ohgoda (1988). Briefly, the spermatozoa were washed by centrifugation (800 g for 10 min) in BO medium (NaCl 6.550 g, CaCl2.2H2O, 0.300 g, NaH2PO4 0.113 g, MgCL2.6 H2O 0.106 g, NaHCO2 3.014 g, glucose 2.500, pyruvic acid 0.110, Pencillin G. sodium 0.031 g, dist. Water to 1000 ml, pH 7.8, osmolarity: 305 mOsm/Kg) without BSA and containing 10 mg/ml heparin and 2.5 mM caffeine (Brackett and Oliphant, 1975). The sperm pellets were diluted with BO medium containing 20 mg/ml bovine serum albumin to adjust the concentration of spermatozoa to 2.5 x 106 sperm/ml. Matured oocytes were washed 3 times in BO medium containing 10 mg/ml BSA and were introduced into 100 µl droplets of sperm suspension (about 10–15 oocytes/droplet, a total of 3 droplets were used for each treatment) under paraffin oil, the spermatozoa and oocytes were co-cultured for 5 h under the same culture conditions (5% CO2, 38.5°C, 95% humidity). After that the oocytes were washed in TCM-199 to remove attached spermatozoa. Groups of 10–20 oocytes were again replaced with previously prepared co-culture 100 µl droplet consisting of TCM-199 + 10% FCS. Oocytes

of all groups were in vitro cultured for 8 days. After that, the morphological appearance of embryos was evaluated under an inverted microscope (Fig. 6-7).

2.8. Statistical analysis:

Two way analysis of variance and Duncan's multiple range tests were done for the obtained data after angular transformation of percentages to their corresponding arcsin values (Snedecor and Cochran, 1989). Data were analyzed using the 1984-version of Costat (Ecosoft, inc, USA), and the level of statistical significance was set at $P \leq 0.05$.

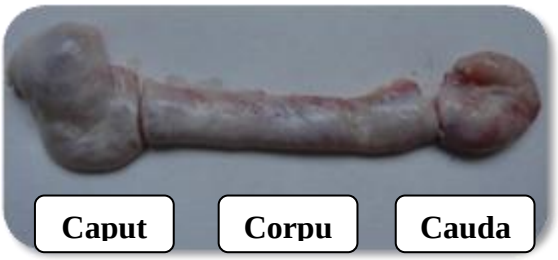

Fig.1: Separation of epididymal regions

Fig. 2: Longitudinal incision in cauda Epididymidis

Fig.3: Obtaining of fluid rich spermatozoa from cauda epididymedis by repeated rinsing with BO medium.

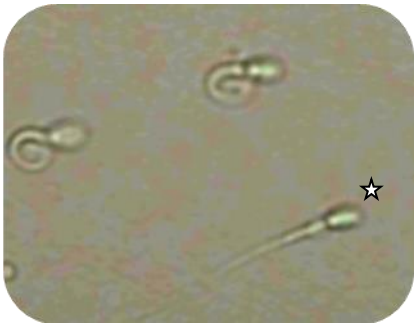

Fig.4: HOS test; one –ve (☆) and two +ve spermatozoa to HOST.

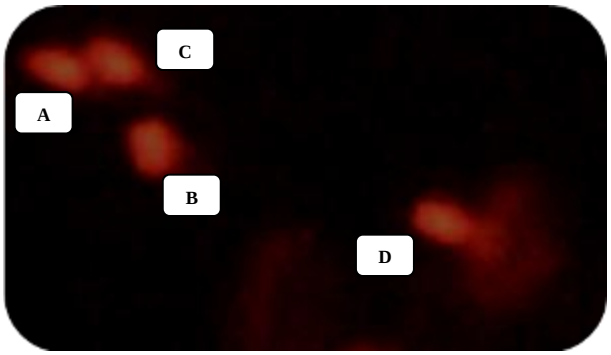

Fig.5: Comet picture of camel cauda epididymal spermatozoa without (A) or with (B, C and D) DNA fragmentation in different degrees.

Fig.6: Two cell stage produced by IVF of camel oocyte by frozen-thawed cauda epidydimal sperm

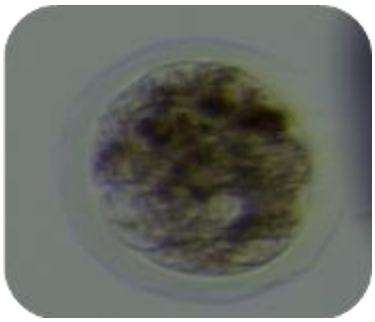

Fig.7: Morula stage produced by IVF of camel oocyte by frozen-thawed cauda epidydimal sperm

3. RESULTS

As presented in table 1, there were no significant differences in livability among spermatozoa freshly collected from cauda, corpus and caput epididymides (81.16 ± 1.43, 80.20 ± 0.90 and 76.76 ± 1.95%, respectively). Concerning the frozen thawed spermatozoa, the sperm cells obtained from the cauda epididymides had significantly higher ($P \leq 0.05$) livability as compared with those of corpus and caput epididymides (67.60 ± 1.25 vs. 61.12 ± 0.80 and 59.20 ± 0.55%, respectively).

Freshly collected spermatozoa from cauda epididymides had significantly higher ($P \leq 0.05$) total and progressive motility (67.92 ± 1.14 and 49.80 ± 1.50%, respectively) than those collected from corpus (49.80 ± 1.10 and 32.80 ± 1.53%, respectively) and caput epididymides (22.60 ± 0.96 and 14.20 ± 0.99%, respectively).

The post-thaw total and progressive motility for all the studied post-thaw times (0. 1, 2 and 3 hours) was highest ($P \leq 0.05$) in cauda epididymal spermatozoa, intermediate ($P \leq 0.05$) in corpus epididymal spermatozoa and lowest ($P \leq 0.05$) in caput epididymal spermatozoa. As for spermatozoa post-thaw motility, the viability index was highest ($P \leq 0.05$) in cauda epididymal spermatozoa (96.50 ± 2.36), intermediate ($P \leq 0.05$) in corpus epididymal spermatozoa (53.20 ± 3.11) and lowest ($P \leq 0.05$) in caput epididymal spermatozoa (12.10 ± 1.10).

As shown in table 2, the percentages of normal acrosome and morphologically abnormal spermatozoa did not differ among cauda, corpus and caput epididymal fresh and frozen-thawed spermatozoa.

The percentages of swollen spermatozoa (HOS +ve) and cytoplasmic droplets of fresh and frozen-thawed spermatozoa were highest ($P \leq 0.05$) in cauda epididymal spermatozoa as compared with corpus and caput ones.

The mitochondrial activity (MTT reduction rate) of fresh and frozen-thawed spermatozoa was highest ($P \leq 0.05$) in cauda epididymal spermatozoa (1.18 ± 0.02 and 0.85 ± 0.01, respectively), intermediate ($P \leq 0.05$) in those of corpus epididymides (0.78 ± 0.01 and 0.22 ± 0.01, respectively) and lowest ($P \leq 0.05$) among the corresponding ones of caput epididymides (0.30 ± 0.01 and 0.12 ± 0.01, respectively).

Table 1 Pre-freeze and post-thaw Livability, total and progressive motility of spermatozoa collected from cauda, corpus and caput epididymides of dromedary camels.

Parameters		Semen type	Epididymal region		
			Cauda	Corpus	Caput
Livability		Fresh	81.16 ± 1.43 [a]	80.20 ± 0.90 [a]	76.76 ± 1.95 [a]
		Frozen	67.60 ± 1.25 [a]	61.12 ± 0.80 [b]	59.20 ± 0.55 [b]
Total motility (%)		Fresh	67.92 ± 1.14 [a]	49.80 ± 1.10 [b]	22.60 ± 0.96 [c]
Progressive motility (%)		Fresh	49.80 ± 1.50 [a]	32.80 ± 1.53 [b]	14.20 ± 0.99 [c]
Post-thaw total motility (%)	0 h	Frozen	47.40 ± 1.29 [a]	29.20 ± 1.46 [b]	13.80 ± 1.20 [c]
	1 h	Frozen	32.80 ± 1.16 [a]	18.00 ± 1.20 [b]	5.20 ± 0.79 [c]
	2 h	Frozen	25.80 ± 1.25 [a]	13.40 ± 1.03 [b]	0.00 ± 0.00 [c]
	3 h	Frozen	14.20 ± 1.03 [a]	7.20 ± 1.08 [b]	0.00 ± 0.00
Post-thaw Progressive motility	0 h	Frozen	32.80 ± 1.25 [a]	17.80 ± 1.00 [b]	8.00 ± 1.12 [c]
(%)	1 h	Frozen	25.60 ± 1.24 [a]	12.80 ± 1.12 [b]	1.80 ± 0.57 [c]
	2 h	Frozen	17.80 ± 0.92 [a]	8.00 ± 0.76 [b]	0.00 ± 0.00 [c]
	3 h	Frozen	11.20 ± 0.93 [a]	5.80 ± 1.11 [b]	0.00 ± 0.00 [c]
Viability index		Frozen	96.50 ± 2.36 [a]	53.20 ± 3.11 [b]	12.10 ± 1.10 [c]

Means with different alphabetical superscripts within row are significantly different at $P \leq 0.05$

Table 2 Pre-freeze and post-thaw acrosome and membrane integrities, morphological abnormalities as well as percentage of cytoplasmic droplets of spermatozoa collected from cauda, corpus and caput epididymides of dromedary camels.

Parameters	Semen type	Epididymal region		
		Cauda	Corpus	Caput
Normal acrosomes (%)	Fresh	86.64 ± 1.47 [a]	89.16 ± 0.82 [a]	85.72 ± 1.34 [a]
	Frozen	71.24 ± 0.80 [a]	68.32 ± 1.01 [a]	68.28 ± 1.35 [a]
Swollen spermatozoa (HOS +ve %)	Fresh	58.00 ± 1.00 [a]	51.68 ± 0.86 [b]	49.60 ± 0.80 [b]
	Frozen	44.84 ± 0.82 [a]	41.08 ± 0.81 [b]	39.80 ± 0.35 [b]
Morphologically abnormal spermatozoa (%)	Fresh	13.04 ± 0.41 [a]	12.08 ± 0.78 [a]	12.48 ± 0.73 [a]
	Frozen	17.04 ± 0.50 [a]	16.72 ± 1.07 [a]	19.00 ± 0.75 [a]
Cytoplasmic droplet (%)	Fresh	68.28 ± 1.05 [a]	56.84 ± 0.96 [b]	55.00 ± 1.40 [b]
	Frozen	38.36 ± 0.74 [a]	30.20 ± 0.92 [b]	28.00 ± 1.12 [b]
MTT reduction rate	Fresh	1.18 ± 0.02 [a]	0.78 ± 0.01 [b]	0.30 ± 0.01 [c]
	Frozen	0.85 ± 0.01 [a]	0.22 ± 0.01 [b]	0.12 ± 0.01 [c]

Means with different alphabetical superscripts within row are significantly different at $P \leq 0.05$

Table 3 The fertilization and developmental rates of dromedary camel oocytes in vitro fertilized with fresh and frozen-thawed spermatozoa collected from cauda, corpus and caput epididymides of dromedary camels.

Parameters	Semen type	Epididymal region		
		Cauda	Corpus	Caput
Fertilization rate (%)	Fresh	50.88 ± 1.10 [a]	36.92 ± 0.79 [b]	12.48 ± 1.09 [c]
	Frozen	38.64 ± 0.77 [a]	22.16 ± 0.79 [b]	4.36 ± 0.59 [c]
Cleavage rate (%)	Fresh	36.28 ± 0.82 [a]	18.24 ± 0.66 [b]	7.44 ± 1.03 [c]
	Frozen	28.04 ± 0.75 [a]	11.24 ± 0.67 [b]	2.24 ± 0.46 [c]
Morula rate (%)	Fresh	19.76 ± 0.66 [a]	1. 32 ± 0.48 [b]	0.00 ± 0.00 [c]
	Frozen	12.44 ± 0.61 [a]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]
Blastocyst rate (%)	Fresh	10.92 ± 0.52 [a]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]
	Frozen	8.12 ± 0.81 [a]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]

Means with different alphabetical superscripts within row are significantly different at $P \leq 0.05$

The fertilization and developmental rates of dromedary camel oocytes in vitro fertilized with fresh and frozen-thawed spermatozoa collected from cauda, corpus and caput epididymides of dromedary camels are summarized in table 3. The fertilization, cleavage and morula rates were highest ($P \leq 0.05$) among oocytes in vitro fertilized by fresh (50.88 ± 1.10, 36.28 ± 0.82 and 19.76 ± 0.66%, respectively) and frozen-thawed (38.64 ± 0.77, 28.04 ± 0.75 and 12.44 ± 0.61%, respectively) cauda epididymal spermatozoa, intermediate ($P \leq 0.05$) among those fertilized by fresh (36.92 ± 0.70, 18.24 ± 0.66 and 1.32 ± 0.48%, respectively) and frozen-thawed (22.16 ± 0.79, 11.24 ± 0.67 and 0.00 ± 0.00%, respectively) corpus epididymal spermatozoa and lowest ($P \leq 0.05$) among those fertilized by fresh (12.48 ± 1.09, 7.44 ± 1.03 and 0.00 ± 0.00%, respectively) and frozen-thawed (4.36 ± 0.59, 2.24 ± 0.46 and 0.00 ± 0.00%, respectively).

Fertilization of camel oocytes with fresh and frozen-thawed cauda epididymal spermatozoa yielded blastocyst rates of 10.92 ± 0.52 and 8.12 ± 0.81%, respectively. While none of the oocytes fertilized by fresh and frozen-thawed corpus and caput epididymal spermatozoa had developed to blastocyst stage.

The percentage of spermatozoa with non-fragmented DNA freshly collected from the cauda epididymides was significantly higher ($P \leq 0.05$) than those collected from corpus and caput epididymides (90.88 ± 1.55, 78.28 ± 0.72 and 76.24 ± 1.02%, respectively). The percentage of frozen-thawed spermatozoa with non-fragmented DNA was highest ($P \leq 0.05$) for spermatozoa collected from the cauda epididymides, intermediate ($P \leq 0.05$) for those collected from corpus epididymides and lowest ($P \leq 0.05$) for caput epididymal ones (82.32 ± 0.69, 72.00 ± 0.54 and 68.08 ± 0.46%, respectively).

Table 4 DNA integrity (Comet assay) parameters of pre-freeze and post-thaw spermatozoa obtained from cauda, corpus and caput epididymides of dromedary camels.

Parameters	Semen type	Epididymal region		
		Cauda	Corpus	Caput
Spermatozoa with non-fragmented DNA (%)	Fresh	90.88 ± 1.55 [a]	78.28 ± 0.72 [b]	76.24 ± 1.02 [b]
	Frozen	82.32 ± 0.69 [a]	72.00 ± 0.54 [b]	68.08 ± 0.46 [c]
DNA in head of comet (%)	Fresh	92.88 ± 0.57 [a]	87.52 ± 0.88 [b]	81.92 ± 0.81 [c]
	Frozen	86.36 ± 0.53 [a]	81.16 ± 1.00 [b]	74.84 ± 0.87 [c]
DNA in tail of comet (%)	Fresh	7.12 ± 0.57 [c]	12.48 ± 0.88 [b]	18.08 ± 0.81 [a]
	Frozen	13.64 ± 0.53 [c]	18.84 ± 1.00 [b]	25.16 ± 0.81 [a]
Tail length (pixel)	Fresh	10.96 ± 0.41 [a]	11.88 ± 0.76 [b]	17.84 ± 0.43 [b]
	Frozen	12.00 ± 0.55 [c]	14.56 ± 0.42 [b]	19.32 ± 0.54 [a]
Olive tail moment	Fresh	0.45 ± 0.05 [c]	0.84 ± 0.08 [b]	1.88 ± 0.10 [a]
	Frozen	0.93 ± 0.06 [c]	1.58 ± 0.08 [b]	2.85 ± 0.15 [a]

Means with different alphabetical superscripts within row are significantly different at $P \leq 0.05$

The percentage of DNA in head of Comet in fresh and frozen-thawed spermatozoa was highest ($P \leq 0.05$) in spermatozoa obtained from cauda epididymides, intermediate ($P \leq 0.05$) for those obtained from corpus epididymides and lowest ($P \leq 0.05$) for caput epididymal ones. On the other hand, the percentage of DNA in tail of comet, comet tail length and Olive tail moment were lowest ($P \leq 0.05$) in cauda epididymal fresh and frozen-thawed spermatozoa, intermediate ($P \leq 0.05$) in those of corpus epididymides and highest ($P \leq 0.05$) for caput epididymides ones.

4. DISCUSSION

Over the years, different techniques for semen collection and cryopreservation have been developed, but few researches have focused on obtaining and freezing epididymal sperm cells from domestic animals (Papa et al., 2008). Although, the first pregnancy using frozen–thawed stallion spermatozoa was reported in a mare inseminated with epididymal spermatozoa since 1957 (Barker and Gandier, 1957), there is no available data regarding survival and fertility of frozen–thawed epididymal camel sperm.

The livability of freshly collected cauda epididymal spermatozoa in the present study (81.16 ± 1.43%) was slightly less than that recorded for camel epididymal spermatozoa (88.80%) by Tajik et al. (2007 and higher than that of camel ejaculated spermatozoa (72.20%, Ziapour et al., 2014). Our finding that, there was no significant difference in live sperm percentage in different parts of camel epididymis came in accordance with the results of Tajik et al. (2007).

The total motility of freshly collected cauda epididymal spermatozoa reported herein (67.92 ± 1.14%) was higher than most reported results such as for dromedary (36.25%, Waheed et al., 2011 and 52.80%; Turri et al., 2013) and alpaca epididymal spermatozoa (53.00%; Morton et al., 2010). However, this value was less than that recorded for dromedary epididymal spermatozoa by Wani, 2009 (85.50%).

Our data reflect the trend that total and progressive sperm motility increased dramatically from the caput to the cauda of the epididymidis, as previously reported for epididymal spermatozoa of camel (Waheed et al., 2011), donkey (Contri et al., 2012), horse (Johnson et al., 1980), bull and ram (Amann, 1987). This trend could be explained partially by our finding that sperm mitochondrial activity (MTT reduction rate, table 2) was related with those parameters, suggesting the involvement of mitochondrial energy production in sperm motility and velocity.

The percentage of viable spermatozoa in the present study was higher than that of motile spermatozoa, suggesting that some of the immotile spermatozoa were alive. These findings are in agreement with that recorded for dromedary (Wani et al., 2008) and boar (Zou and Yang., 2000) ejaculated spermatozoa. Similarly, Goovaerts et al. (2006) recorded the presence of a high percentage of non-moving spermatozoa (38.2%) collected from the cauda epididymides of bulls. Moreover, Guimarãesa et al. (2012) suggested that the tail of the epididymis is a reservoir of mature spermatozoa, where they are kept in a metabolic quiescent state to prevent premature activation.

Based on the present data, there was about 30% decline in total motile spermatozoa of camel cauda epididymides as a consequence of freezing-thawing procedures. Higher percentage of such decline (more

than 50%) was reported for alpaca (Morton et al., 2010) and buffalo (Lambrechts et al., 1999) epididymal spermatozoa. On the other hand, lower decline (less than 20%) was reported for epididymal spermatozoa of rams (Kaabi et al., 2003) and stallions (Neuhauser et al., 2013).

In the current study, there were no significant differences between the percentages of spermatozoa with intact acrosome among the different regions of the epididymis which ranged from 85.72 to 89.16%. Similar values were reported for alpaca epididymal spermatozoa (Morton et al., 2010) and camel ejaculated spermatozoa (Deen et al., 2003; Wani et al., 2008).

Cryopreservation processes are known as being damaging to the sperm cells, and can have an effect on fertilization rate due to compromising the integrity of acrosomal structures (Critser et al., 1987; Wakayama and Yanagimachi, 1998). However, cryopreservation of dromedary epididymal spermatozoa in the current work was associated with more than 80% of spermatozoa retained their acrosomal integrity post-thaw. This finding came in accordance with the results of Morton et al. (2010) for alpaca epididymal spermatozoa.

The percentage of cauda epididymal spermatozoa shown to be with intact membranes in the present study (58.0%) was similar to that of dromedary camel ejaculated spermatozoa (58.9%, Ziapour et al., 2014) and lower than that of ram (84.2%, Kaabi et al., 2003) and dog (78.80%, Filho et al., 2014) epididymal spermatozoa. The assessment of membrane integrity by the hypo-osmotic test is simple (Quintela et al., 2010), and some studies consider it an indication of fertility in species such as cattle (Revell and Mrode, 1994) and boar (Perez-Llano et al., 2001).

In the present study, the percentage of morphologically abnormal spermatozoa was not differ among the three regions of the epididymis which ranged from 12.08 to 13.04%. Similar values were reported for camel ejaculated spermatozoa (13.95%, Wani et al., 2008).

These cytoplasmic droplets are known to appear in high amounts in epididymal spermatozoa of many animal species such as camel (Wani et al., 2005), bull (Amann and Almquist, 1962; Goovaerts et al., 2006; Martins et al., 2007), ram (Kaabi et al., 2003) and boar (Briz et al., 2005). The percentage of spermatozoa with cytoplasmic droplets in the present study was significantly higher in the cauda than in the corpus or caput epididymis (68.28 vs. 56.84 and 55.00%, respectively). Similar findings of higher percentage in cauda spermatozoa, but without significant difference, (74.0 vs. 70.0 and 66.0%, respectively) were recorded for camel epididymes by Tajik et al. (2007).

Despite the fact that a high occurrence of cytoplasmic droplets in epididymal sperm, mainly distally located, is considered to be normal by many authors (Kishikawa et al., 1999; Cooper et al., 2003; Goovaerts et al., 2006), in ejaculated bovine sperm, Thundathil et al. (2001) found a negative correlation between the cleavage rates after IVF and the percentage of cytoplasmic droplets.

Based on the present data, there was about 44% of cytoplasmic droplets were lost as a result of cryopreservation. Similarly, Wani et al. (2005) reported that about 34–67% of cytoplasmic droplets are lost on liquid storage of dromedary epididymal spermatozoa. Also, Perez-Garnelo et al. (2001) found decreased percentages of cytoplasmic droplets of gazelle's epididymal spermatozoa after freezing, when compared to a fresh sample.

The ability of spermatozoa to be motile develops during maturation (Amann, 1987), and the propulsive efficacy of spermatozoa is primarily dependent on mitochondrial function. In our study the results suggested that mitochondrial activity, as measured by MTT reduction rate, of cauda epididymal spermatozoa was higher than those collected from corpus and caput epididymides (1.18 vs. 0.78 and 0.30, respectively). Similar findings in donkey epididymis were reported by Contri et al. (2012), who found a significant correlation between sperm mitochondrial activity and sperm motility parameters. In the present study, there were decreased MTT reduction rates of camel's epididymal spermatozoa after freezing, when compared to fresh samples. This finding came in accordance with the results of El-Badry et al. (2008) for buffalo ejaculated spermatozoa.

Using the freshly collected cauda epididymal spermatozoa, the in vitro fertilization rate reported herein (50.88%) was higher than results after IVF of dromedary (47.78%, Badr and Abdel-Malak, 2010) and llama oocytes (29.20%, Del Campo et al., 1994) that fertilized by epididymal spermatozoa. However, the values were lower than other reports (52-67%, Wani, 2009 and 58.98%, Fathi et al., 2014) following IVF of dromedary camel oocytes by epididymal sperm. The difference may reveal the variations of maturation conditions and the method of harvesting epididymal sperm.

As compared with previous studies in dromedary camel using freshly collected cauda epididymal spermatozoa, the cleavage rate recorded herein (36.28%) was similar to 37.68% (Badr and Abdel-Malak, 2010), higher than 15-32% (Nowshari and Wani, 2005; Moawad et al., 2011) and lower than 43-60% (Wani, 2009).

Using the freshly collected cauda epididymal spermatozoa, the morula production rate reported herein (19.76%) was more or less similar to the reported results after IVF of dromedary (21.32%, Badr and Abdel-Malak, 2010; 20.0%, Fathi et al., 2014).

As reported in our study, the blastocyst production rate was 10.92% when fresh cauda epididymal spermatozoa were used for IVF of dromedary oocytes. Fresh epididymal spermatozoa have been previously used for IVF of dromedary oocytes with a blastocyst production rate ranging from 6 to 24% (Nowshari and Wani, 2005; Wani, 2009; Badr and Abdel-Malak, 2010).

To the best of our knowledge, there are no reports on the evaluation of in vitro fertilizing ability of frozen-thawed spermatozoa (epididymal or ejaculated) in camelids. The blastocyst production rate recorded herein was 8.12% when frozen-thawed cauda epididymal spermatozoa were used for IVF of dromedary oocytes.

Based on the present data, none of the oocytes fertilized by fresh and frozen-thawed corpus and caput camel epididymal spermatozoa had developed to blastocyst stage. Similarly, delayed fertilization and cleavage arrest were reported in one case of IVF with human spermatozoa from the corpus epididymidis (Mahadevan and Trounson., 1985). Moreover, in mice, Wazzan et al. (1990) reported that only 8% of oocytes fertilized by caput epididymal spermatozoa were capable of developing into blastocysts in vitro, compared with 48% of oocytes fertilized by cauda spermatozoa.

As compared with corpus and caput epididymal spermatozoa, fresh and frozen-thawed cauda epididymal sperm had higher DNA integrity presented as decreased percentage of sperm with non-fragmented DNA and the percentage of DNA in tail of the comet and increased percentage of DNA in tail of comet, comet tail length as well as Olive tail moment. Similarly, in rams, Garcia-Macias et al. (2006) reported that caput and corpus epididymal ram spermatozoa had less chromatin maturity. Consequently, the low capacity for fertilization and embryo development by corpus and caput epididymal spermatozoa herein may be attributed to higher levels of DNA fragmentation. Moreover, Virro et al. (2004) reported that spermatozoa with high levels of DNA fragmentation, showed a significant decrease in blastocyst and ongoing pregnancy rates, with a trend towards a lower rate of pregnancies and a higher rate of spontaneous abortions.

In conclusion, it is possible to obtain spermatozoa of good quality and freezability from dromedary camel cauda epididymides, and these fresh and frozen-thawed spermatozoa may have the potential uses in laboratory studies in IVF as well as in the means of AI as a useful tool in animal breeding programs.

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