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CRYOPRESERVATION OF DROMEDARYCAMEL SEMEN SUPPLEMENTED WITH α-AMYLASE ENZYME

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ABSTRACT

This study was conducted to investigate the effect of α-amylase on the high viscous nature of dromedary camel semen used for processing of cryopreserved doses. Five different concentrations (0, 2.5, 5, 10, 15 µl/ml) were used as a mucolytic agent for seminal plasma glutinosity liquefaction prior freezing (during 4 hours of equilibration period). The enzyme was added in Tris-Lactose 3% glycerolated extender (TLG) supplemented with 20% fresh egg-yolk. Semen was collected from 3 adult bulls (12 years old); semen ejaculates were assessed immediately for motility. Samples with mass motility higher than 60% and individual forward motility more than 20% were directly splitted on the α-amylase – TLG mix and equilibrated on 5°C for 4 hours before freezing. The results showed a significant ($P<0.05$) increase in the sperm motility at the high concentrations of amylase treatments 5, 10, 15 µl/ml being 55%, 56.6% and 55% respectively, compared to 40% for both of the control sample (0 µl/ml) and that with added 2.5 µl/ml of amylase. No significant effect was reported due to the treatment on either the detached acrosome or the abnormalities. Computer system for semen assessment (Cell motion analyser CMA) was used to investigate some parameters of the cryopreserved treated semen sperms after thawing. Most movements and velocities parameters, namely; distance along tract (µm), straight distance start finish (µm), distance along average track (µm), VCL(µm/S), VSL (µm/S), VAL (µm/S), linearity%, wobble% and straightness% showed increased values ($P < 0.05$) at concentration of 15 µl of α-amylase as compared with the control sample or at other α-amylase concentrations.

In conclusion, α-amylase at concentrations of 5, 10, 15 µl/ml under slow thawing conditions is a proper mucolytic agent for camel seminal plasma viscosity elimination prior cryopreservation. This enhanced the post-thaw forward motility of camel sperm with no significant detectable effect on both of the acrosomal integrity and the abnormalities.

Key words: Amylase, camel, mucolytic, semen, viscosity

Low sperm motility and the viscous nature of seminal plasma have been reported in old and new world camels (Agarwal *et al,* 1995; Neely and Bravo, 1997; Aminu *et al,* 2003). Skidmore (2003) and El-Zanaty *et al* (2004) reported that the gelatinous nature of the dromedary camel semen is a major constraint in the development of artificial insemination, as a reason for giving a failure assessment of sperm forward motility. There appears no homogeneity in sperm concentration after dilution (Adler *et al,* 1997) and during straws packing. Recently, Aminu (2008) mentioned that although thick gel camel ejaculate can be considered satisfactory but the problem that it does not mix with semen extenders leading to errors in physical assessments.

Different methods for elimination of semen viscosity were studied by Bravo *et al* (1999) and Mendeluk *et al* (2000). The effect of different extenders on camel semen liquefaction was studied by Wani *et al* (2008), while Medan *et al* (2008) investigated some mucolytics as they supplemented camel semen extender with catalase. Recently, El-Bahrawy and El-Hassanien (2009) used different mucolytic agents for the elimination of semen viscosity in Tris-Lactose extender.

Motility is commonly believed to be one of the most important characteristics associated with the fertilizing ability of sperm. Significant differences have been observed in motility parameters between sperm that achieved a high percentage of fertilisation and those that failed in the achievement of pregnancy (Donnelly *et al,* 1998). Computer-aided sperm motility analysis systems (CMA) are able to determine sperm proportions of motile (and progressively motile)

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spermatozoa (Mortimer, 1990; Davis and Katz, 1992). CMA allows an objective assessment of different sperm characteristics as motion and velocity. It provides precise and useful information on various sperm motion characteristics like progressive motility, path velocity, progressive velocity, track speed, amplitude of lateral head displacement, and beat cross frequency (Verstegen *et al,* 2002). For camel semen evaluation, Al-Qarawi *et al* (2002) reported that CMA is a reliable system for determination of semen characteristics and considered as an accurate, rapid method for evaluating and predicting fertility in the one humped camel bulls.

Accordingly, this study was conducted to investigate the ability of using different concentrations of a-amylase enzyme to eliminate viscosity of one-humped dromedary camel semen. The effect on physical and kinetics of sperm motility as well as post-thawing of cryopreserved doses was studied.

Materials and Methods

Animals, Location and Semen Collection

Three adult dromedary camel males (12 years old) with an average weight of 400 kg were used for semen collection in the artificial insemination laboratory in Maryout Research Station, Desert Research Centre, located 34 km North West of Alexandria, Egypt.

Semen collection from trained males by artificial vagina (with a disposable plastic inner liner to avoid the expected lethal effect of rubber on sperm viability) (Musa *et al,* 1992) placed in El-Hassanien camel dummy (El-Hassanien, 2003). Semen was collected twice weekly during the breeding season. Collecting tubes with a modified plastic water jacket were used to maintain semen samples at 37°C during the long lasting mating durations. Semen samples were transmitted immediately to a 37°C adjusted water bath for semen quality assessment.

Semen assessment

Collected semen was immediately assessed for mass motility using a portion of 20µl sample on a glass slide covered with a clear clean cover and placed under a magnification of 40 x using a hot stage Lica microscope adjusted at 37°C. The local sperm motility due to the high viscosity of the seminal plasma was detected as a mass motility. An aliquot of 50 µl of the raw semen was dropped on 200 µl of glutarylaldehyde 0.02% in 5 ml weatherman test tubes for assessment of acrosomal integrity and abnormalities just before the addition of the extender, a magnification 400X oil lens microscope was used for counting the samples, (Johnson *et al,* 1976).

Sperm motility parameters were assessed using a computer-assisted cell motion analyser (CMA) system (SM-CMA^{TM}; MTM Medical Technologies, Montreaux, Switzerland). This system has a specific set-up for cattle sperm evaluation and was equilibrated for camel sperm evaluation. Briefly, a 5 µl drop of sperm was placed onto a slide and covered with a cover slip (10 mm X 10 mm). Sperm motility was assessed at 37°C using a phase contrast microscope. For each sample 10 microscopic fields were analysed and a minimum of 100 sperm were evaluated. The average path velocity (VAP, mm/s; the average velocity of the smoothed cell path), curvilinear velocity (VCL, mm/s; the average velocity measured over the actual point to point track followed by the cell), straight-line velocity (VSL, mm/s; the average velocity measured in a straight line from the beginning to the end of the track), linearity index (LIN,%; the average value of the ratio VSL/VCL), and wobble (WOB = VAP/VCL)%; a measure straightness%.

Viscosity assessment

Subjective visual eye observation was used to evaluate the seminal plasma viscosity through monitoring the suction and deposition of semen samples through 200µl automatic pipette. A scale of fair for the high viscosity samples, followed by good, very good and excellent for assessing the total elimination of viscosity. That was accompanied with objective monitoring the individual motility of sperms.

Semen dilution, amylase addition and cryopreservation

A tris-lactose 3% glucerolated extender, supplemented with 20% fresh egg yolk as described by El-Bhrawi (2005) and El–Bhrawy *et al* (2006) with pH 7.4 and an osmotic pressure 0.351 osmol/kg was used a freezing extender. Semen extension was carried out using a split sample technique in one-step freezing method on five different concentrations with a final dilution rate of 1 semen portion to 3 portions of extender.

TERMAMYL SUPRA enzyme, (a trademarked amylase available from Termamyl Supra) by Novozymes (Novo Nordisk), Denmark extracted from *Bacillus licheniformis*, with five different concentrations of 0, 2.5, 5, 10, 15 µl/ml. were added to the previously prepared extender, as soon as semen samples were diluted, semen was transported to a mini-tube

cooled handle cabinet for four hours equilibration time, before packing in 0.5 ml French straws using a 133 model type mini-tübe filling and sealing machine. A mini-tübe biological freezer (-140°C nitrogen vapor) was used for doses cryopreservation before transporting to the storage tanks for further investigations.

Thawing method

A programmable mini-tube thawing device was used for slow thawing; the device was programmed for the desired time and temperatures which were 40°C for 40 seconds

Statistical analysis

Data analysis was performed using SPSS software (SPSS version 11.5 for Windows; SPSS Inc., Chicago, IL, USA) computer programme. Results are quoted as arithmetic mean ± standard error of mean (S.E.M.) and significance was attributed at $p < 0.01$.

Results and Discussion

Observations in the present work revealed that a positive correlation was demonstrated between the concentrations of the sample and its viscosity. The addition of 5, 10, 15 µl/ml of alpha-amylase had a rapid and irreversible effect on liquefying the gelatinous nature of the semen samples, however, 2.5 µl, or 0 µl/ml of alpha-amylase had no observable effect in the elimination of viscosity after an equilibration period for four hours at 5°C. Garnica *et al* (1993) and Fuentes (1990) incubated semen samples at 37°C from 8 to 22 hours for viscosity elimination of new world camel semen.

The mean values obtained from sperm physical parameters are presented in Table 1. Several significant parameters were obtained for sperm motility under different concentrations of amylase. The results showed that addition of 5, 10, 15 µl/ml of alpha-amylase had a significant positive effect in increasing the motility of semen samples post-thawing. No significant differences were detected between 2.5 µl/ml concentration and the amylase free extender (the control) as they both showed a significant decrease in motility percentages, a reliable change in the individual motility of sperms were detected. Movement of spermatozoa was recognised clearly by the naked eyes as a progressive motility and not as a local standing motility; this is obviously attributed to the elimination of seminal plasma viscosity, these results totally agreed with Bobak *et al* (2009) studying semen viscosity as they reported that, increased viscosity correlated with lower motility. Contrarily, these findings disagreed with Bravo *et al* (2000) who reported that even though semen samples were viscous after enzyme addition, there was no significant change in motility.

Table 1. Effect of different concentrations of α-amylase enzyme on physical characteristics of camel semen.

Treat	Mot.%	DA	1st ab.	2nd ab.
0 µl/ml	40^{b}±2.8	7.0±2.1	10.3±2.6	9.6±3.5
2.5 µl/ml	40^{b}±2.8	12.3±1.3	10.6±0.3	10.6±1.3
5 µl/ml	55^{a}±0.01	9.67±2.6	9.6±1.2	14.0±1.7
10 µl/ml	56.6^{a}±4.4	12.6±2.08	10.0±1.15	8.6±0.3
15 µl/ml	55^{a}±5.7	10.0±3.8	8.66±1.2	9.3±1.4

a,b Means with different superscripts in the same column are significantly different at P<0.05

Only the sperm that maintain an intact acrosome can take part in the fertilisation of an oocyte. Therefore the percentage of sperm with damage acrosome should be low in order to maintain high fertility levels. Saacke and White (1972) in their early investigation reported a significant correlation between the fertility of bull sperms and the percentage of spermatozoa with intact acrosome. The same observations were remarked by Zhang *et al* (1990) on stallion spermatozoa, while, Cumming (1995) failed to prove these results in his study on bulls. Sperm morphology assays are simple to do and permit ejaculates with high percentage of detached acrosome or abnormal sperm cells to be culled prior to semen samples cryopreservation process avoiding low fertility after artificial insemination. Percentage of detached acrosome and abnormalities were not affected by the addition of different alpha-amylase concentrations, even the observed acrosomal damage and abnormalities as compared to the control results had no significant difference. These results were in agreement with Bravo *et al* (1999 and 2000). On the other hand, Tibary and Anouassi (1997) noted that all enzymes had been seen to cause acrosomal damage in spermatozoa. The mean ± SE of different sperm motion characteristics assessed by CMA results are presented in Table 2. Most motility and velocity parameters: Distance along tract (µm), Straight distance start finish (µm), Distance along average track (µm), VCL (µm/S), VSL (µm/S), VAL (µm/S), Linearity%, Wobble% and Straightness% showed an increase values ($P < 0.05$) with 15 ul of a-amylase as compared to fresh samples or other a-amylase concentrations, in contrast, (0, 2.5, 5, 10, 15 µl/ml) a-amylase supplemented cryopreserved samples did not differ significantly after thawing.

Table 2. Mean±S.E. of different sperm parameters assessed by computer system for semen assessment (Cell motion analyser CMA).

	0 µl/ml	2.5 µl/ml	5 µl/ml	10 µl/ml	15 µl/ml
Distance along tract (µm)	35.05^{b}±2.85	$55.48^{ab\pm}$7.64	48.52^{ab}±6.87	41.67^{ab}±4.11	64.01^{a}±6.36
Straight distance start finish (µm)	9.95^{b}±1.57	17.45^{ab}±2.84	18.48^{ab}±3.01	18.97^{ab}±1.41	23.85^{a}±3.09
Distance along average track (µm)	16.72^{b}±1.49	25.0^{ab}±4.20	26.4^{ab}±3.08	24.56^{ab}±1.66	34.0^{a}±2.74
Velocity curve line (µm/S)	75.61^{b}±6.4	102^{ab}±33.2	93.9^{ab}±15.7	90.5^{ab}±26.8	129.4^{a}±12.8
Velocity straight line (µm/S)	23.27^{b}±4.2	28.7^{ab}±5.2	35.9^{ab}±6.4	43.04^{ab}±3.8	46.9^{a}±5.4
Velocity average line (µm/S)	37.59^{b}±4.29	49.02^{ab}±7.75	50.7^{ab}±6.3	54.21^{ab}±3.63	67.8^{a}±4.41
Linearity (%)	30.92^{ab}±5.22	25.87^{b}±3.49	39.54^{ab}±4.52	49.5^{a}±4.44	40.0^{ab}±4.8
Wobble (%)	51.5^{ab}±4.13	44.34^{b}±4.19	57.03^{ab}±2.74	61.73^{a}±3.12	56.9^{ab}±3.73
Straightness (%)	59.47±7.35	58.14±6.82	69.31±6.89	78.46±4.03	69.16±5.7

a,b Means with different superscripts in the same raw are significantly different at P<0.01

The subjective assessment is considered to be practical in the elimination of processed semen depicting spermatozoa characteristics, as a promising clinical indicator for potential sub-fertility or infertility.

Sperm motility is the parameter that is most frequently used to measure camel sperm viability in the ejaculate during and after the process of storage or cryopreservation. Indirectly, sperm motility is expected to provide clues on the potential fertility of the spermatozoa. Since subjective motility assessment is considered less objective than computer-assisted motility analyses, there has been an interest in including these instruments in practice especially after elimination of seminal plasma viscosity as a major constrain for the application of this instruments in commercial practice. However, lake of data of sperm motion characteristics from camels is not available to confirm it. The current data obtained in the present work are below the range previously reported by Qarawi *et al* (2002) and Qarawi *et al* (2004).

In addition, CMA system gives information about the kinetics of sperm motility. Some of these parameters (VCL, VSL, LIN or ALH) have been correlated to the hyperactivity of the sperm in several species, in humans; (Mortimer, 2000); in rams (Mortimer and Maxwell, 1999). However, data of sperm motion characteristics from camels are not available to confirm it, except for Qarawi *et al* (2002) mentioning that linearity percentage is strongly correlated (P<0.05) with fertility in dromedary bulls.

Since hyper-activation is a motility pattern observed in spermatozoa undergoing capacitation, samples showing this motility pattern may remain viable for a shorter time and therefore have a low in vivo fertilizing ability. On the other hand, CMA analyses permit subpopulations of motile sperm in a whole ejaculate to be delineated (Quintero *et al,* 2004). The sub-population structure of the ejaculates may be important in determining the fertilising potential of the sample, especially those results of this study revealed that CMA is likely to be more accurate in the analysis of sperm track movement and velocity detection than using ordinary microscopes.

Results of the present study indicate good conception rate of artificially inseminated she-camels with cryopreserved semen doses supplemented with alpha-amylase as compared to cryopreserved doses with normal gelatinous nature of semen. Medan *et al* (2008) reported a higher conception rate when used a catalase supplemented extended cooled semen for artificial insemination rather than using enzyme free cooled extenders.

Prior cryopreservation with 5, 10, 15 µl/ml of alpha-amylase concentrations were very effective in overcoming seminal plasma viscosity, enhancing the post-thaw forward motility of camel sperm with no significant detectable effect on both acrosomal integrity and abnormalities.

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Monophyletic origin of domestic bactrian camel (*Camelus bactrianus*) and its evolutionary relationship with the extant wild camel (*Camelus bactrianus ferus*)

The evolutionary relationship between the domestic bactrian camel and the extant wild two-humped camel and the factual origin of the domestic bactrian camel remain elusive. We determined the sequence of mitochondrial *cytb* gene from 21 camel samples, including 18 domestic camels (three *Camelus bactrianus xinjiang*, three *Camelus bactrianus sunite*, three *Camelus bactrianus alashan*, three *Camelus bactrianus red*, three *Camelus bactrianus brown* and three *Camelus bactrianus normal*) and three wild camels (*Camelus bactrianus ferus*). Our phylogenetic analyses revealed that the extant wild two-humped camel may not share a common ancestor with the domestic bactrian camel and they are not the same subspecies at least in their maternal origins. Molecular clock analysis based on complete mitochondrial genome sequences indicated that the sub-speciation of the two lineages had begun in the early Pleistocene, about 0.7 million years ago. According to the archaeological dating of the earliest known two-humped camel domestication (5000–6000 years ago), we could conclude that the extant wild camel is a separate lineage but not the direct progenitor of the domestic bactrian camel. Further phylogenetic analysis suggested that the bactrian camel appeared monophyletic in evolutionary origin and that the domestic bactrian camel could originate from a single wild population. The data presented here show how conservation strategies should be implemented to protect the critically endangered wild camel, as it is the last extant form of the wild tribe Camelina.

(Source: Ji, R, Cui, P, Ding, F, Geng, J, Gao, H, Zhang, H, Yu, J, Hu, S, & Meng, H (2009, August). Monophyletic origin of domestic bactrian camel (*Camelus bactrianus*) and its evolutionary relationship with the extant wild camel (*Camelus bactrianus ferus*). Animal Genetics, 40(4), 377-382.)