Effect of Seminal Plasma Centrifugation for Viscosity Elimination on Cryopreservation of Dromedary Camel Semen

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Abstract: Dromedary camel semen is characterized by bad freezability and low post-thaw motility. The highly viscous nature of camel semen is one of the major constrains facing semen packing and freezing process. A primary experiment was conducted on fresh semen obtained from five dromedary bulls (n=5) to compare two methods of semen cryopreservation (pellets vs. 0.5 ml French straws). The results showed significant (P<0.05) decrease in post thaw motility of pellets (20.8%) compared to straws (43.3%). Meanwhile, the sperm survival rates were 68.7 and 33.1% for both French straws and pellet post cryopreservation, respectively. Centrifugation of viscous seminal plasma and re-suspension of sperm rich fraction in a Tris-Lactose extender under two glycerol levels (3 and 6%) was carried out in 2nd experiment. High glycerol level (6 %) had a significant deleterious effect (P<0.05) on semen freezability and showed low post-thaw motility (19.2%) for the un-centrifuged semen compared to the centrifuged semen (38.3%). However, no significant effect was observed using the 3% glycerol level for neither the un-centrifuged (40.3%) nor the centrifuged (43.3%) raw samples. The survival rates of the centrifuged samples were 62.2 and 72.2 % compared to the un-centrifuged samples 39.7 and 68.8%, for both 6 and 3% glycerol levels, respectively. Percentage of intact acrosome decreased significantly (P<0.05) for the centrifuged semen samples regardless of glycerol level. In conclusion, French straws technique is preferable for camel semen cryopreservation than pellets technique. In addition, using Tris-Lactose extender of 3% glycerol level after eliminating seminal plasma viscosity by centrifugation is an efficient method to overcome constraints of dromedary camel semen freezability. [Nature and Science 2010;8(9):196-202]. (ISSN: 1545-0740).

Key Words: Camel; Semen; Cryopreservation; Straws; Pellets; Glycerol.

1. Introduction:

Artificial insemination (AI) is considered one of the most important, and fastest way in the modern technology for the application of genetic improvement, through the breeding programs of farm animals (Durrant, 2009). However, Deen, (2008) reported that progress in AI, semen preservation and related techniques in camelids is slow in comparison to other livestock species. Freezing methodology, camel semen nature and extenders constituents are some of the constraints affecting camel semen cryopreservation with acceptable post-thaw motility. Freezing bull spermatozoa in the form of pellets was primarily carried out by Nagase and Niwa (1964). The cost of the equipment required for pelleting semen was found to be much less than other types of semen packaging. Accordingly, pelleting semen for further storage in liquid nitrogen is useful and recommended in the developing countries and harsh areas.

Results of post-thaw motility and fertilization rate varied greatly between different reports. Recently, pellet-freezing for Damascus goat semen and bovine bulls gave promising post-thaw motility (Awad and Graham, 2004 and Khalifa and El-Saidy, 2006). Deen (2008) and Skidmore (2003) noted that one of the most important facts affecting camel freezability is the gelatinous nature of the semen. As seminal plasma is generally removed from equine spermatozoa prior to cryopreservation. Moore *et al.* (2005) noted that the re-addition of seminal plasma prior to freezing for short term had no significant effect on sperm motility, while prolonged exposure to seminal plasma prior to cryopreservation was deleterious. Sabine, *et al.* (2006) assessed the effects of semen centrifugation on post-thaw semen quality in canines semen. They achieved acceptable post-thaw motility and viability using different extenders and different freezing methods for centrifuged semen.

As for boars semen, Matas, *et al*. (2007) reported that under different semen centrifugation regime, the raw sperm quality was not significantly affected by centrifugation, especially when using a cushioned method for centrifugation for post-freezed semen quality. Watson (1990) reported that among the permeable cryoprotectants used for spermatozoa, glycerol has been selected for most domestic animals, Fahy, *et al*. (1990) noted that the range of glycerol concentration for semen cryopreservation was between 0.25M (2.25%) and 1M (9%), in many studies demonstrating toxicity beyond this concentration. Final glycerol concentrations of 2% and 3% have also been successfully used for semen cryopreservation of Bactrian and Dromedary camel (Sieme, *et al*., 1990; Deen, *et al*., 2003 and El-Bhrawi, 2005). Recently, Niasari, *et al*. (2007) reported that semen cryopreservation in Bactrian camel is feasible when it is extended in SHOTOR (Tris-Citrate) diluent, using glycerol level at a final concentration of 6%. Therefore, the aim of this study was to identify the best method for camel semen cryopreservation (pellets vs. 0.5 ml French straws). In addition to investigate the effect of centrifugation as a method for elimination of seminal viscosity.

2. Materials and methods:

2.1 Experimental animals:

The present study was carried out in Maryout Research Station, in a semi-arid area. Five dromedary camel bulls aged 10 years (average weight 550 kg) were used. The camel bulls were fed daily at 9 a.m. on a pelleted concentrate feed mixture with a crude protein content of 14%. The concentrates were supplemented with barley as a source of energy. Berseem hay as a roughage was also offered all round the year .The animals were allowed to drink twice a day. Duration of 3 to 4 hours free grazing period was alternatively allowed twice a week.

2.2 Semen collection:

Semen was collected during the breeding season twice a week at 8 p.m. using an artificial vagina (AV), the collection procedure was carried out in a clean barn adjacent to the artificial insemination laboratory. A camel dummy like the shape and size of a female camel was used for semen collection instead of using a teaser female (El-Hassanien, 2003). Beneath the dummy a well-equipped laboratory for primary semen examination was established. Also the artificial vagina was modified by an additional disposable polyethylene inner sleeve inserted into the AV to avoid the contact of the ejaculate with the rubber inner sleeve. The semen glass collecting tube was coated with a larger plastic tube filled with warm water (40^0C) to keep the ejaculate in an adequate temperature during the long time of the ejaculation process. The male was left to seek the entrance of the fixed artificial vagina alone, with a little help from beneath to direct the penis into the AV entrance.

2.3 Semen assessment:

Motility: A phase-contrast microscope (Leica), with warm stage adjusted at a temperature of 37^0C was used to examine sperm motility at a magnification of 40X and an average of 5 fields was obtained to the nearest 5%. The intact acrosome was examined in a sample of 10µl semen added to 200µl of a solution of 0.2 % glutaraldehyde using a phase contrast microscope at 800X magnification according to Johnson *et al*. (1976).

2.4 Semen centrifugation and dilution:

Semen samples in the collecting tubes were transferred directly into a water bath adjusted at 37^0C and split into two portions. The first portion was immediately divided into two further portions and diluted with Tris -Lactose diluents containing either 3 or 6 % glycerol. The second portion of raw semen was centrifuged at 18000 X g for 15 min. The centrifuged pellet (sperm fraction) was re-suspended and divided into two portions of Tris –Lactose diluents containing 3 and 6 % glycerol.

2.5 Extender preparation:

Tris-Lactose base extender is mainly composed of a mixture of 50% buffer base (Tris buffer, 0.25 Mol) and 50% sugar base (lactose, 11) according to the method of (El-Bahrawy *et al*., 2006). The extenders were supplemented with two glycerol concentrations 3 and 6 % in one step method and supplemented with 20% egg-yolk with a dilution of 1:3. Re-suspension of the centrifuged semen samples was dissolved in diluents with a volume of three times of the initial whole semen sample before centrifugation to achieve an approximate 1:3 dilution rate after centrifugation.

2.6 Semen processing:

2.6.1 Pellets

Cooled extended semen was taken out of the cooling chamber, after equilibration period of 4 hr. Small depressions were made on a block of solid carbon dioxide (dry ice, -79^0C) to accommodate the droplets of semen (0.1 ml.) for freezing. These depressions were made by a round heated end of a metal rod. The droplets of the extended semen were

made by the use of 0.1 ml. automatic micro-dispenser. A clean tip of the micro dispenser was used for each sample to avoid contamination of the extenders. Four minutes were enough time to prevent pellets from fragmentation. The frozen pellets were stored in liquid nitrogen in small aluminum vials fixed to aluminum cassettes dipped in the storing tanks containing (LN2, -196^{0}C). Every cassette was labeled with the sample code and the treatment number (El-Bhrawi, 2000).

Two pellets were thawed for 60 seconds (slow thawing) in a water bath at a temperature of 37^{0}C in a clean 5 ml glass test tube with slight shacking.

2.6.2 Straws

Semen assigned to be frozen in straws was packed in 0.5 ml French straws using an automatic mini-tube filling and sealing machine (Type MPP133). After the completion of the equilibration period, the straws racks were delivered into a programmable freezing unit adjusted at -140^{0}C using liquid nitrogen vapor. The straws were kept for 15 minutes at this temperature until frozen and then finally dipped in liquid nitrogen storage tanks. A mini-tube thawing device was used for slow thawing for the straws. The device was programmed for the desired time (40seconds) and temperature (37^{0}C) for post-thaw motility and intacted acrosome assessing (El-Bhrawi, 2005).

2.7 Statistical analysis:

Analysis of variance was detected using GLM procedure by SPSS (SPSS version 11.5 for Windows; SPSS Inc., Chicago, IL, USA).The differences between means were detected using Duncan's Multiple Range Test (DMRT) according to Snedecor and Cochran, 1967. Results were quoted as arithmetic mean ± standard error of mean (S.E.M.) and significance was attributed at $p < 0.05$.

3. Results:

In the first experiment, raw fresh semen was diluted in Tris-lactose extender and after equilibration, processed either in a pellets form or packed in 0.5ml French straws. The results in Table (1) showed that pellet freezing of camel semen gave more than 50% significant reduction in post-thaw motility (p<0.05) as compared for French straws freezing technique by using programmable liquid nitrogen cryo-freezer. Post thaw motility percent for pellets was 20.8 ± 8.5 % as compared with cryopreserved straws post thawing motility (43.3 ± 4.9 %) with a survival rate of 33.1 and 68.7 % for pellets and straws, respectively, as illustrated in Figure (1).

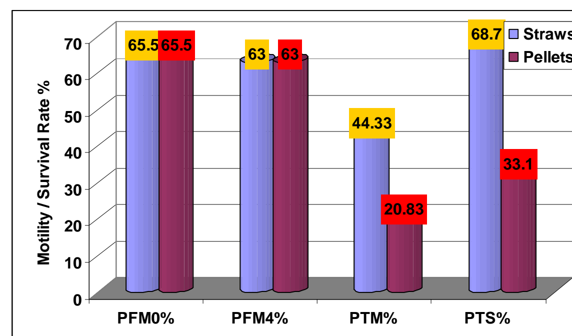


Fig (1) : Effect of freezing method of camel semen on motility percentage after 0h. of dilution (PFM0%), 4h. of equilibration (PFM4%), after thawing (PTM%), and Post-thaw survival rate (PTS%)

Table(1). Effect of cryopreservation methods on camel semen freezability (M±SE):

Treat.	PFM$_0$%	PFM$_4$%	PTM %	PTS %
Straws	65.5± 2.8	63.0± 3.4	3.33± 4.9^a	68.7 %
Pellets	65.5± 2.8	63.0± 3.4	20.83±8.5^b	33.1 %

- Different letters within a column indicates significant different (P<0.05).
- PFM0%: Pre-freezing motility % after 0h. of dilution.
- PFM4%: Pre-freezing motility % after 4h. of dilution.
- PTM%: Post-thaw motility %.
- PTS%: Post-thaw survival rate %.
- Survival rate estimated by =Post-thawing motility/ Pre-freeze (4h.) motility X 100

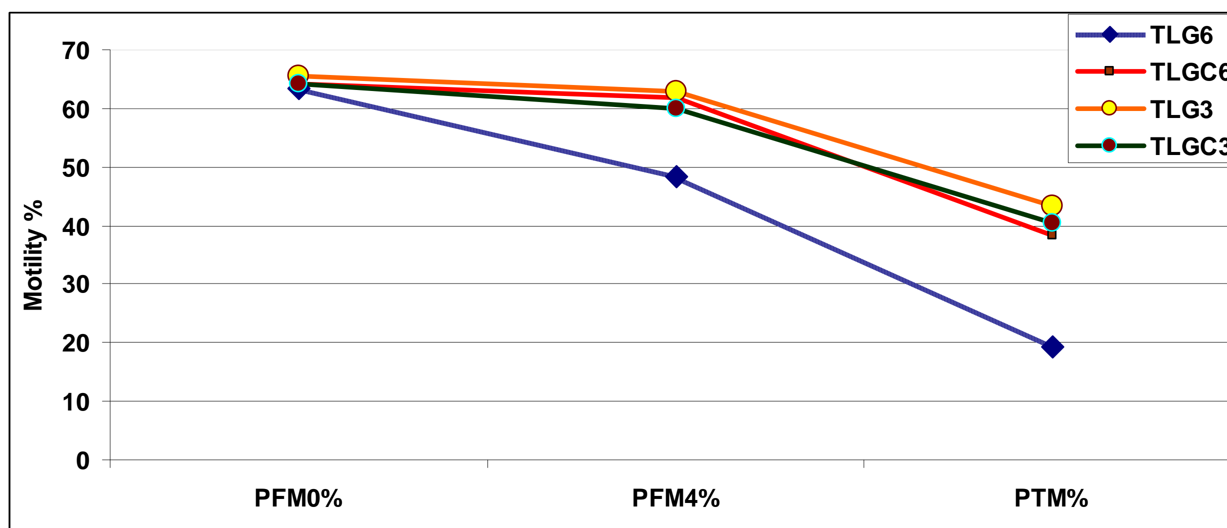
It was suggested to use 0.5 ml straws for camel semen packing using liquid nitrogen in the next experiment. In this experiment, raw semen was divided into two portions, the first one was directly dissolved in two concentrations of 3 and 6% glycerol levels extenders, the other portion was centrifuged. The centrifuged precipitate (rich sperm fraction) were re-suspended and divided into two portions of Tris –Lactose diluents containing 3 and 6 % glycerol.
The effect of semen centrifugation and glycerol levels at 6 and 3% on the quality of cryo-preserved dromedary camel semen is presented in Table 2.

Table (2). Effect of seminal plasma centrifugation and glycerol levels on pre-freezing motility, post-thaw motility, survival rate and detached acrosome percentage:

	PFM0%	PFM4%	PTM%	PTS%	PTD4%
Fresh semen	72.5 ± 2.8[a]	72.5 ± 2.1[a]	72.5 ± .8[a]	------	3.2 ± 0.8[c]
TLG6	63.3 ± 6.1[a]	48.3 ± 9.5[b]	19.2± 3.9[c]	39.7	9.2 ± 0.7[a]
TLGC6	64.2 ± 4.2[a]	61.7± 6. 7[ab]	38.3±7.2[b]	62.2	7.8 ± 0.1[ab]
TLG3	65.5 ± 2.1[a]	63.0± 3.4[ab]	43.3±4.9[b]	68.8	9.8 ± 0.3[a]
TLGC3	64.2 ± 5.1[a]	60.0± 6.3[ab]	40.3±9.4[b]	72.2	6.5 ± 0.8[b]

- Different letters within a column indicates significant different ($P<0.05$).
- PFM0%: Pre-freezing motility % after 0 hours of dilution.
- PFM4%: Pre-freezing motility % after 4 hours of dilution.
- PTM%: Post-thaw motility %.
- PTS%: Post-thaw survival rate %.
- Survival rate estimated by =Post-thawing motility/ Pre-freeze (4hrs.) motility X 100
- PTD4%: Post-thaw detached acrosome after 4 hours.
- TLG6: Tris-lactose extender with 6% glycerol level
- TLGC6: Tris-lactose extender with 6% glycerol without seminal plasma.
- TLG3: Tris-lactose extender with 3% glycerol level
- TLGC3: Tris-lactose extender with 3% glycerol without seminal plasma

The post-thaw progressive motility was significantly decreased by the addition of 6% glycerol for un-centrifuged samples (19.2± 3.9) as compared with the centrifuged samples with the same glycerol concentration (38.3± 7.2%). On the other hand 3% glycerol had no significant effect on post-thaw motility of both of the un-centrifuged and the centrifuged samples (40.3± 9.4 and 43.3± 4.9 % respectively), as illustrated in Figure (2).



- TLG6: Tris-lactose extender with 6% glycerol level
- TLGC6: Tris-lactose extender with 6% glycerol without seminal plasma.
- TLG3: Tris-lactose extender with 3% glycerol level
- TLGC3: Tris-lactose extender with 3% glycerol without seminal plasma

Fig. (2). Effect of seminal plasma centrifugation (c) cand glycerol (g) levels on pre-freezing motility at 0 hrs ((PFM0%), 4hrs. (PFM4%) and post-thaw motility (PTM %).

Seminal plasma removal by centrifugation significantly improved progressive post-thaw motility at 6% glycerol (38.3± 7.2%) and 3% glycerol (43.3± 4.9%) with a superiority survival rate for the centrifuged being, 62.2 and 72.2% , respectively as illustrated in Figure (3).

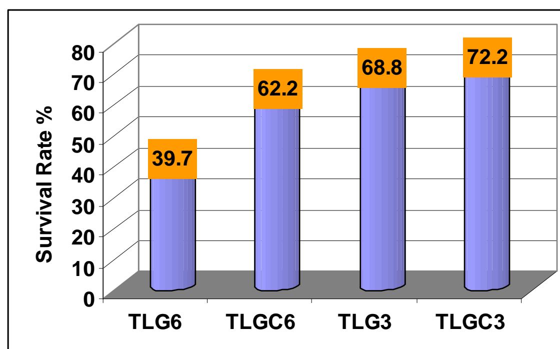


Fig (3): Effect of seminal plasma centrifugation and glycerol levels on post-thaw survival rate (%).

The un-removed seminal plasma samples had lower survival rate of 39.7 % in 6% glycerol level compared to 68.8 % upon using glycerol level at 3%. Intacted acrosome was significantly ($P<0.05$) improved by removing seminal plasma by centrifugation under different glycerol concentrations as seen in Table (2) and Figure (4).

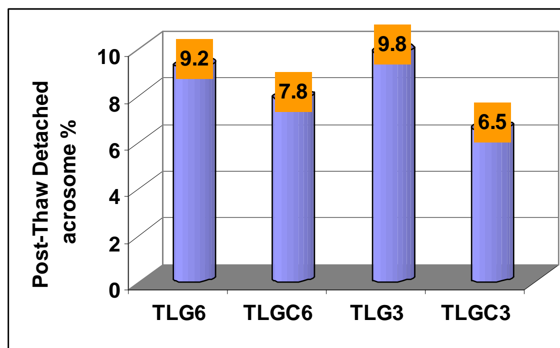


Fig (4). Effect of seminal plasma centrifugation and glycerol levels on detached acrosome percent (PTD %).

It is worth to mention that due to the high viscosity nature of the seminal plasma and in-spite of the high speed of centrifugation, there is still some observed loss of the sperms in the supernatant seminal plasma. This loss can be detected either by re-examination of the diseard seminal plasma or by observation of less concentration for centrifuged doses as compared to the un-centrifuged raw semen doses. The dilution rate of the centrifuged samples should be considered for further investigations to demonstrate better sperms recovery.

4 Discussion

The results for pellet form cryopreservation are the same as those obtained by Awad (1989) and Awad (1997), who compared various freezing techniques, namely; pellets on dry ice, pellets on cold paraffin wax and plastic straws of 0.25 ml capacity for cryopreservation of ram semen. Post-thaw motility and sperm recovery were the highest in the semen frozen in plastic straws followed by pellet / paraffin wax and the least was pellet / dry ice. On the other hand, Morton, *et al.* (2007) recommended the use of pellet form for freezing of male alpacas after achieving promising post-thaw motility. This may be due to the different techniques used in preparing semen samples and the pelleting process in addition to the less gelatinous characteristics of alpaca semen as compared to the dromedary male semen.

In the present study, significant increase of post-thaw progressive motility was recorded when using low concentration of glycerol (3%) versus the higher concentration (6%). That is true especially in the free seminal plasma cryo-preserved semen diluted in Tris-lactose diluent. Seminal plasma removal improved survivor rate of cryo-preserved spermatozoa under both of high and low glycerol levels and also improved the intacted acrosome. This is possibly is due to the fact that lower concentrations of glycerol may provide an adequate protective effect during cryopreservation; while, higher concentrations may be toxic for sperms. The toxic effect of glycerol on spermatozoa has been reported by Fahy, *et al.* (1990). Hammerstedt and Graham (1992) they reported cellular effects caused by glycerol which included changes in cytoplasmic events due to increased viscosity by intracellular glycerol, altered polymerization of tubulin, alteration of microtubule associations, effects on bioenergetics' balances, and direct alteration of the plasma membrane and glycocalyx. Moore *et al.* (2005) noted that the prolonged exposure to seminal plasma prior to cryopreservation was deleterious. Sabine *et al.* (2006), achieved acceptable post-thaw motility and viability for centrifuged semen. As for boars' semen, Matas *et al.* (2007) reported that the raw sperm quality was not significantly affected by centrifugation.

In conclusion, French straws technique is more practical and promising for camel semen cryopreservation than the pellets technique. In addition, using Tris-Lactose extender of 3% glycerol

level after eliminating semen viscosity by centrifugation is an efficient way to overcome constraints of dromedary camel semen freezability.

Acknowledgment

The author is deeply thankful to Mr. Ibrahim El Hareiry and Mr. Demian Gali, technicians of AI lab in Maryout Station, Desert Research Center, for their help, patience and valuable opinions during this work. **The study was kindly sponsored by Agriculture Research Developing Fund (ARDF).**

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7/25/2010