



## Preservability and conception of chilled and cryopreserved semen with Triladyl egg yolk extender in dromedary camels (*Camelus dromedarius*)

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### ABSTRACT

Artificial insemination (AI) technology has not shown much advancements in camels, attributable to various difficulties of semen preservation and insemination in the species. The present study intended to explore the preservability of camel semen under chilled and frozen states, improve proficiency of AI steps and assess conception to AI. Over the two years of study, 383 ejaculates collected from five adult male camels were preserved in Triladyl egg yolk extender using refrigeration or liquid nitrogen and used for AI trials in camels subjected to induction of ovulation. Under chilled storage, even though few samples retained satisfactory motility even beyond 96 h, a majority of the samples suffered significant drop in motility within 24 h. Out of 239 and 739 freezing trials (FT) respectively in the first and second seasons, 18.83% and 23.00% FT achieved a post-thaw motility (PTM) of at least 40%. Insemination studies showed progressive decrease in conception rate with the duration of chilled storage since fresh extended and 24 h chilled semen samples produced 32% and 3.7% conception respectively. Cryopreserved semen achieved two pregnancies (1.82%) out of 110 inseminations in the second season. It is concluded that camel spermatozoa suffers rapid depletion of conception chance upon preservation and underlying reasons needs to be investigated. However, the achievement of two pregnancies indicates the possibility of conception and the scope for cryopreservation based AI technology in dromedary camels.

**Keywords:** Camel, Conception, Cryopreservation, Insemination, Semen

Artificial Insemination (AI) has become the routine breeding tool for faster and extensive dissemination of germplasm from superior males among farm animal species. However, AI is not being practiced in camels due to the lack of a refined technology in this species. Even though there are reports of successful AI using fresh extended (Skidmore and Billah 2006) and chilled semen (Medan *et al.* 2008) in camels, satisfactory cryopreservation and successful AI using frozen semen is yet to be developed among dromedary camels.

Dromedary camels, constitute one of the major domestic animal group in the arid regions of Asian and African continents and there exists enormous demand for productivity enhancement of the species (Faye and Bonnet 2012). Faster propagation of superior camels necessitate breeding technologies especially AI (Skidmore *et al.* 2013). However, practical difficulties in collection (Deen and Sahani 2000), processing and preservation of semen (Deen 2007, Wani *et al.* 2008) and poor conception rate to AI (Skidmore *et al.* 2013), especially with frozen semen

(Skidmore 2002) form major hurdles contributing to the non-availability of AI technology for dromedary camels.

In this context, the present study was carried out in continuation of the previous studies on semen collection, evaluation and processing (Kutty and Koroth 2012, Cholakkal *et al.* 2016), preparation of the female for AI (Skidmore *et al.* 2013) and insemination with fresh extended semen (Cholakkal 2019). Preliminary trials revealed the superiority of Triladyl based extender for the preservation of camel semen (Cholakkal *et al.* 2015). Hence, the objective of the present study was to assess the preservability and explore the possibility of conception using dromedary camel semen stored by refrigeration as well as cryopreservation using Triladyl egg yolk extender.

### MATERIALS AND METHODS

The study was carried out at the Artificial Insemination and Embryo Transfer Section of Abu Dhabi Food Control Authority located at Al Wafea-Abu Dhabi in UAE. Five adult male and 50 female camels, including six heifers, belonging to Omani breed were utilized for the study. The male camels were managed in individual pens while females were housed in two groups. Feeding, general management and healthcare measures were provided as per standard practices.

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*Semen collection and processing:* The male camels were trained for semen collection using modified bovine artificial vagina (Kutty and Koroth 2012, Cholakkal *et al.* 2016) making use of female camels as the mount. Routine semen collection started from December was carried out at 2–3 days interval until the end of breeding season. The study was continued in the subsequent season using the same male camels and replacing those conceived with breedable females.

The semen ejaculates collected were subjected to evaluation of preliminary attributes and vitality parameters and those having optimum quality (Kutty and Koroth 2012) were extended using Triladyl egg yolk extender (Readymade Triladyl Medium [Minitube, Germany] –20 ml, distilled water 60 ml and egg yolk 20 ml) (Vyas *et al.* 1998, Cholakkal 2019). Extension rates of 1:2 to 1:10 were used depending upon the sperm concentration and initial oscillatory movement to achieve a final sperm concentration of 100 million motile sperms per ml of extended semen (Skidmore *et al.* 2013, Cholakkal *et al.* 2015).

*Preservation studies:* The semen was extended to the final desired concentration over multiple steps. The neat semen was extended two to three times its volume by addition of extender warmed to 37°C and subjected to gentle physical agitation by plastic straws to minimize gel formation (Cholakkal 2019). The partially extended semen was placed in a water bath at 37°C along with the remaining volume of the extender and subjected to slow cooling in a cold handling cabinet maintained at 5–7°C. Further extensions were made at one hour intervals, adding installments of the extender (1–2 times the initial volume of semen together with gentle physical agitation), until the required sperm concentration was achieved.

Extended semen were checked for motility after 6 h of chilling and those having at least 60% progressive motility (PM) were randomly selected for prolonged storage under refrigeration (4–5°C) or cryopreservation trials. The semen samples stored under refrigeration were checked for motility at 24, 48, 72 and 96 h intervals.

Freezing trials (FT) for cryopreservation were carried out as per Cholakkal *et al.* (2015). In brief, the extended and cooled semen samples were filled in French medium straws and frozen manually using liquid nitrogen taken in a Styrofoam box. Various freezing protocols were tried based on the variations of factors such as initial sperm concentration (subjective grading as high, moderately high and low), extension ratio (1:2 to 1:10), extension speed (single step or multiple steps), additional glycerol in the medium (0% to 3% extra) and equilibration time (2 to 10 h). Freezing trials also varied with respect to height above the liquid nitrogen level (fixed at 3, 5 and 7 cm or stepwise declining heights), duration of freezing (7 to 15 min), method of dipping the straws (with or without opening the box), intensity of nitrogen vapour (made by agitation or not) and thawing temperature-time combination (such as 34°C for 3 min, 37°C for 1 min and 40°C for 0.5 min). Post-thaw motilities (PTM) of each of the trial variations were recorded.

*Insemination trials:* The female camels were examined by per rectal palpation and trans-rectal B-mode ultrasound scanning (Aloka-prosound) from mid-November at weekly or shorter intervals, for assessing the functional status of the reproductive organs and ovarian follicular dynamics. Hormonal preparations such as GnRH (Cystorelin – 100 µg or Receptal – 200 µg) or hCG (Chorulon – 3000 IU) were injected intramuscularly for induction of ovulation in camels possessing at least one mature follicle of 1.2 to 2.0 cm diameter (El-Hassanein *et al.* 2010).

Animals were inseminated 24 h after the hormonal injection, using semen doses such as fresh extended or 6 h chilled samples with at least 60% PM, 24 h chilled samples of at least 50% PM and frozen-thawed semen of at least 40% PM. The semen doses (2–3 ml) were loaded in a sterile intrauterine catheter (Bovivet pipet – Kruuse, UK) covered with a sterile plastic sleeve (Minitube) and connected to a 5 ml syringe. Per vaginal insertion of the hand followed by recto-vaginal manipulation of the catheter was used for insemination in parous females while recto vaginal method alone was used in maiden camels (Cholakkal 2019). At least 100 million actively motile spermatozoa were deposited into the uterine horn ipsilateral to the ovary with mature follicle as suggested by Skidmore and Billah (2006).

Inseminated animals were checked with male camel for behavioural signs of conception at 14<sup>th</sup> and 19<sup>th</sup> days of insemination as per Tibary and Anouassi (1997). Absence of the typical behavioral signs was considered as the indication of conception failure. Such camels were subjected for trans-rectal B mode ultrasonography and those confirmed non-pregnant were enrolled for further AI trials. Camels that showed behavioural signs or internal changes of conception were checked again at day 25 for confirmation of pregnancy based on behavioral features and ultrasonography. Details of semen collection and processing, preservability, female side interventions and outcome of AI were analyzed for making conclusion.

## RESULTS AND DISCUSSION

*Semen quality:* A total of 538 ejaculates were collected during the two seasons and subjected to preliminary evaluation of semen quality parameters. The mean volume of the ejaculates was 2.7 ml with 96% of the ejaculates having a volume ranging from 1 to 6 ml. On the basis of preliminary evaluation (poor initial quality, very low volume and/or gross contamination), 155 ejaculates (28.81%) were readily discarded and the remaining 383 ejaculates were further evaluated and processed. The selected ejaculates had an initial oscillatory movement (IOM) grading of ‘++’, ‘+++’ and ‘++++’ in 65 (16.97%), 206 (53.79%) and 112 (29.24%) collections respectively. Depending upon the available volume, IOM and subjective assessment of sperm concentration, ejaculates were extended 1:2 to 1:10, divided into suitable aliquots and utilized for further studies such as chilled storage, cryopreservation, and insemination trials. Details of semen samples collected and processed during the two seasons are shown in Table 1.

Table 1. Dromedary camel semen ejaculates collected and processed for chilled storage

Parameter	Season 1	Season 2	Total
Ejaculates collected	268 (49.81%)	270 (50.18%)	538 (100%)
Ejaculates discarded	87 (56.13%)	68 (43.87%)	155 (100%)
Ejaculates processed	181 (47.26%)	202 (52.74%)	383 (100%)
Samples fit after cooling	150 (45.73%)	178 (54.27%)	328 (100%)
Samples stored chilled	128 (57.66%)	94 (42.34%)	222 (100%)

Out of the 383 selected ejaculates, 29% were extended two to three folds in a single step and 71% (having higher sperm concentration) were extended more than 3 folds in multiple steps together with physical mixing at each addition of the extender. Progressive motility of the samples was up to 50%, 51–60%, 61–70%, 71–80% and more than 80% respectively in 53 (13.84%), 64 (16.71%), 97 (25.33%), 86 (22.45%) and 83 (21.67%) ejaculates extended. Thus 69.45% of the extended ejaculates had an initial motility exceeding 60% and were subjected to initial cooling for preservation trials or used for insemination as fresh extended semen within 1 h of extension.

**Chilled storage:** During the first season, 150 (82.87%) out of 181 extended samples maintained PM of at least 60% at 6 h of chilled storage. Out of these 150 extended samples, 22 were used for AI or for FT. The remaining 128 were stored further under refrigeration. Among the 128, 51 (39.84%) and 23 (17.97%) had a PM of not less than 50% at 24 h at 48 h respectively (Table 2). In the second season, 178 (88.12%) out of 202 extended samples showed PM of at least 60% at 6 h of chilled storage. Of these 178 samples, 94 were stored further under refrigeration and 39 (41.49%) had a PM of at least 50% at 24 h.

Out of 39 samples continued under chilled storage beyond 24 h, 18, 11 and 3 samples showed a PM of at least 50% at 48, 72 and 96 h, respectively (Table 2), which was in agreement with the report of Deen and Sahani (2006). It was noticed that even though motility reduction at 6 h of refrigeration was not very marked, majority of samples suffered a major drop in motility within 24 h of chilled storage ( $P < 0.01$ ) making them unfit for insemination (Deen

*et al.* 2004, Wani *et al.* 2008). Wani *et al.* (2008) also had reported similar reduction in motility proportion at 24 and 96 h of chilled storage. However, the motility figures recorded during both the seasons were better than those observed by Morton *et al.* (2009).

Though satisfactory PM of few samples was maintained beyond 96 h, in many instances semen samples of almost similar initial quality and processing, and even different aliquots of the same ejaculates failed to preserve satisfactory motility even at 24 h. Thus an inconsistency with respect to quality of preserved semen was evident concurring to the report of Deen and Sahani (2006). Variations in motility of chilled semen samples observed between ejaculates of similar initial quality make it difficult to predict preservability of camel semen based on the routine quality assessments. Hence, there is a need for more detailed studies to develop better extenders and/or processing techniques so that satisfactory preservability and fertility of camel semen can be ensured.

Triladyl being an extender containing glycerol, intended for cryopreservation, utilization of the same for chilled preservation was based on the previous finding that Triladyl and glycerolated fractions of freshly constituted Tris extenders, preserved motility better than non-glycerolated fractions of the same extenders under refrigeration (Kutty and Koroth 2012). Even though cold shock protectants such as egg yolk and buffers added in the media suffice chilled preservation (Skidmore *et al.* 2013), enhancement of preservability of camel semen under refrigeration (4–5°C) by glycerol containing extenders needs further investigation. However, it appears that camel spermatozoa are highly vulnerable to the lowered temperature of preservation and could be the reason for the poor fertility.

**Cryopreservation:** Freezing protocols involved many combinations with respect to variables such as semen donor, initial quality of the ejaculate, extender used, proportion of extension, glycerol level, method of glycerolation, equilibration time, packaging material used, time allowed for freezing, freezing rate, height from nitrogen liquid and time-temperature combinations used for thawing. During the first season, 45 FT out of 239 carried out (18.83%) yielded a PTM adequate for insemination (40% or more). Only 5 of these 45 FT yielded a PTM of at least 50%, even though PTM of 60–80% is usually obtained for semen of other livestock species. Further, semen straws prepared from

Table 2. Dromedary camel semen samples with at least 50% motility at different hours of chilled storage during the two seasons

Samples preserved		Samples with at least 50% progressive motility			
		24 h	48 h	72 h	96 h
Season 1	128	51 (39.84%)	23 (17.97%)	-	-
Season 2	94	39 (41.49%)	18 (46.15%)	11 (23.07%)	3 (7.69%)

Table 3. Freezing trials and post-thaw motility of semen samples compared between seasons

Parameter	Season 1		Season 2		Total	
	#	%	#	%	#	%
Ejaculates used	92	34.33	156	57.78	248	46.1
Freezing trials	239	100	739	100	978	100
At least 40% PTM	45	18.83	170	23	215	21.98
At least 50% PTM	5	2.09	96	13	101	10.33
At least 60% PTM	0	0	44	5.95	44	5.95

Table 4. AI and conception with preserved dromedary camel semen

	Female camels inseminated	AI and Conception	Type of semen used			
			Fresh extended	6 h chilled	24 h chilled	Frozen–thawed
Season 1	27	AI done	25	29	28	19
		Conceived	8 (32.00%)	3 (10.34%)	1 (3.57%)	0
Season 2	44	AI done	–	–	26	110
		Conceived	–	–	1 (3.85%)	2 (1.82%)

the same samples under same batches of freezing and thawing also showed wide variation with respect to PTM, indicating inconsistency and unpredictability of freezing outcome as reported earlier (Deen and Sahani 2006, Marai and Zeidan 2007). Details of FT carried out and the outcomes are shown in Table 3.

During the second season, a total of 739 FT were carried out using variations of freezing-thawing protocols and PTM adequate for insemination was achieved in 23% trials. At least 50% PTM was obtained in 13% of the trials, of which 6% trials had PTM of 60–80%. A major hike in PTM ( $P < 0.05$ ) was recorded for FT in the month of April and was corresponding to a significantly higher pre-freeze motility ( $P < 0.01$ ) observed for ejaculates processed in the month of April.

The proportion of FT with at least 40% PTM was 44.84% during April as against 23.18% for the entire season. The probable contributory factors for better PTM in the month of April appeared to be due to the use of increased proportion of Triladyl in the extender in 71% of such trials, extension ratio exceeding 1:4 and equilibration time exceeding 4 h in most of the trials. Further, one step freezing at a height of 4–4.5 cm above liquid nitrogen for 7.5 min and thawing at 34°C for 3 min were also involved in majority of the trials which had at least 40% PTM. However, the influence of each of those factors needs separate investigation for confirmation.

*Insemination outcome:* Details of different types of semen doses used for AI and conception rate are shown in Table 4. In the first season, 101 AI were carried out in 27 female camels, which included 25, 29, 28 and 19 inseminations respectively using fresh extended, 6 h chilled, 24 h chilled and cryopreserved semen samples respectively. Previous studies had advocated the use for AI of 24 h chilled and frozen semen doses with at least 35–40% PM (Skidmore *et al.* 2013) and 30% PTM respectively. But, in the present study, fresh extended and 6 h chilled semen doses with at least 60% PM, 24 h chilled semen with at least 50% PM and frozen doses with at least 40% PTM, respectively were used.

A total of 12 conceptions were obtained which included 8 from fresh extended, 3 from 4–6 h chilled, 1 from 24 h chilled, and 0 from cryopreserved doses inseminated. Total AI done in the second season were 136, which included 26 and 110 respectively using 24 h chilled and cryopreserved semen and resulted in 3 conceptions, 1 from chilled and 2 from cryopreserved semen doses.

Even though insemination of at least 100 million actively

motile sperms was ensured in chilled/frozen semen doses, the conception rate of preserved semen was very low. In the 2 seasons of study, 54 inseminations using 24 h chilled semen resulted in only 2 (3.70%) conceptions, as against 10.34% from 4–6 h chilled and 32.00% from fresh extended semen, which indicated a continuous depletion of semen fertility upon storage. Bravo *et al.* (2000) and Morton *et al.* (2010) have reported a conception rate of 25–30% and 17.6% in bactrian and dromedary camels, respectively using chilled semen. Whereas, Deen *et al.* (2003 and 2005) failed to obtain any conception when AI was done using chilled or cryopreserved semen. Likewise, cryopreserved semen failed to yield any conception in the first season, while 2 conceptions were obtained in the second season out of 110 inseminations.

Many AI performed using chilled or cryopreserved semen having adequate number of sperms together with PM exceeding 50% failed to achieve conception. This indicated that there were other factors, which influenced the conception of preserved semen in camels. However, the 2 pregnancies obtained with cryopreserved semen in this study, even though very low in proportion (1.82%), has proved the possibility of conception with cryopreserved semen in dromedary camels and signifies the chances of developing AI technology using cryo preserved semen in dromedary camels as well.

It is concluded that camel spermatozoa suffer rapid depletion of fertilizing capacity upon preservation. The inconsistency of the preservability irrespective of initial quality, makes the prediction difficult and necessitates in-depth studies to identify the underlying factors affecting preservability under low temperature. Nonetheless, two pregnancies achieved from cryopreserved semen and increase in freezability of semen samples achieved in the study gives hope for better conception rates in the coming years. However, together with intensive efforts for refining the cryopreservation protocols and conducting more AI trials, there is a need for micro-level investigations to understand and manage the factors hindering conception of preserved semen in dromedary camels.

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