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CRYOPRESERVATION OF CAMEL SEMEN

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ABSTRACT

The study was conducted on cryopreservation of 70 semen samples artificially collected from 11 adult male camels of Jaisalmeri breed (Dromedary) of this centre. The semen samples were diluted at the rate of 1: 3 with Tris Egg Yolk glycerol extender, cooled in a refrigeratory unit and evaluated for progressive sperm motility in Inverted Phase Contrast Microscope (Nikon, 400X magnification) with attached video monitor 4-6 hr after collection of semen. Those semen samples, which exhibited good sperm motility were transferred to cryo-vials, each sample in duplicate, labelled and frozen in automated liquid nitrogen based cryo-freezer (Planner KRYO 10-1.3), where the vials were cooled to -100 ° C followed by their immersion in liquid nitrogen container. Pre-freeze motility varied from 47 to 70% in different males. Post-thaw motility declined from 23.5 to 47.5% in individual semen samples with an overall estimated loss of 62.5% of the progressively motile spermatozoa due to freeze thaw process. Based on criteria adopted by several workers in camel AI, to approve semen of 30% or greater post-thaw motility, only 37% of semen samples processed in present study qualified for approval to be of use for AI. The rejection rate of more than 50% was greater than those of dairy bulls of 5-15%. Post thaw motility of same semen sample cryopreserved in duplicate vials in same batch differ significantly. Post-thaw duration of survival of thawed spermatozoa was studied by incubating at 37 and 4°C. At 37°C, the reduction in motility was about 50% than at 0 hr. At 2,3,4 and 24 hr after incubation, almost 93, 99 and 100 % spermatozoa lost motility. At 4°C incubation, the per cent decline was 17, 30, 35.8, 44.1 and 65.5%, at 1, 2, 3, 4 and 24 hr of incubation.

Key words: camel, cropreservation, semen

Artificial insemination (AI) using cryopreserved semen is extensively used in cattle to avoid inbreeding and facilitate genetic heterogeneity. But, in dromedary camels, despite presence of vigorously motile spermatozoa (Billah and Skidmore, 1992) in frozen-thawed semen, no successful pregnancy could be obtained with frozen thawed semen (Skidmore, 2003). Technique like AI can be maximally utilised only when cryopreservation of semen is successful. To ascertain possible causes of failure of conception with frozen thawed camel semen, it would be worthwhile to evaluate *in vitro* duration of post thaw survival of frozen- thawed camel spermatozoa. Objectives of this study have been to evaluate post thaw motility in cryopreserved camel semen, grade semen samples as fit / unfit for insemination purposes, compare post-thaw motility in duplicates of same semen sample and to evaluate longevity of post thaw motility at 37 and 4 °C at 0,1,2,3,4 and 24 hrs after thawing.

Materials and Methods

Eleven adult and trained Jaisalmeri (Dromedary) camels of our institute varying in age from 7-11 years were taken for this study and a total of 70 semen samples with good pre-freeze motility were subjected to freezing, each sample in duplicate. Semen samples were harvested during winter, which is rutting season for camel in this country. Samples were collected at an interval of 2-3 days from individual camels.

A 30 cm long bovine AV was used. The inner chamber was filled with approximately 750 ml water at 45-50°C, depending upon weather conditions and in order to maintain an internal AV temperature of 41-42°C. Air was infiltrated to maintain adequate pressure. Director's funnel, collection glass tube and insulation bag were applied as used in bovine. Application of lubricant was avoided as the camel has a significant pre-ejaculate liquid prior to intromission of the penis into an artificial vagina.

A female camel restrained in sternal recumbency was used as a dummy. The male was allowed to mount her. An operator approached the mounted male from the left side as males usually fall on the right side after copulation. The AV was held firmly in the left hand of the operator and the erect penis was directed into the AV with the right hand on the sheath throughout the course of copulation to prevent extrusion of the penis from the AV. During copulation, at least two assistants were required to control the male camel to prevent accidental falling of the male on to the operator.

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Color, consistency and volume of semen were recorded, and photographed to show its typical physical characteristics such as frothy nature, gel like consistency, non-miscibility with the extender in freshly ejaculated state, its shuffling on the top of the tube upon adding extender into the semen containing tube and settling in the bottom after few hours leaving a clear transparent layer on the top of the tube. Extender was added followed by cooling of semen with semen tube immersed in water bath transferred to a refrigerating unit. Evaluation for pre-freeze motility was conducted on cooled semen 4-6 h after collection in inverted phase contrast microscope (Nikon, 400 X magnification) with warm thermostatic stage and video monitor to conduct satisfactory evaluation of sperm motility.

The chemical buffer used for semen dilution was prepared as follows:

Tris[1]	30.28 g
Fructose[1]	12.5 g
Citric acid[2]	16.7 g
Caffeine[3]	0.039 g
Distilled water add to	1000 ml

The buffer was autoclaved at 1.1kg/cm^2 pressure for 30 min, cooled and refrigerated until used. Semen extender for dilution of semen was prepared fresh every day as follows:

Unglycerolated buffer :

Buffer	80 ml
Egg yolk	20 ml
Benzyl penicillin[4]	1000 IU/ml
Streptomycin sulfate	1000 mg/ml

Glycerolated buffer :

Buffer	68 ml
Egg yolk	20 ml
Glycerol[2]	12 ml
Benzyl penicillin[4]	1000 IU/ml
Streptomycin sulfate[5]	1000 mg/ml

The semen extenders were maintained at 37°C in an incubator prior to collection of semen. Semen was extended initially at the rate of 1 part : 1 part with unglycerolated diluent. Final dilution was 1 part : 3 part with glycerolated dilutor within 30 min. The diluted semen samples (2 ml) were packaged after extension into labelled cryovials of 2 ml capacity. These cryovials were placed into a glass beaker containing water (25°C) for cooling into a refrigerator unit. It was cooled within 2 h and kept at this temperature for further 3 h.

Freezing of semen was carried out in automated liquid nitrogen based programmable freezer KRYO 10-1.3 (Planer Products Ltd., UK) using the following protocol as used by Almquist (1969) for dairy cattle.

Cooling rates (Start temperature 4°C)

From 4 to -15°C -1°C per min

From -15 to -60°C -4°C per min

From -60 to -100°C -20°C per min

Finally, the cryovials were plunged and stored in liquid nitrogen until required.

Thawing of semen involved immersion of cryovials in a water bath of 40°C for 2 min. Post-thaw motility was studied using same equipment at 0, 1, 2, 4 and 24 hr in the duplicate semen samples incubated at 37 and 4°C. Samples were categorised into 3 viz. those showing post thaw motility >30%, those showing > than 20 but <30%, and those showing below 20%. Average pre-freeze and post thaw motility were compared and reduction of motility due to freezing and thawing was enumerated.

Statistical Analysis:

One way analysis of variance was used to study the effect of animals on pre-freeze motility of 70 samples from 11 male camels. Post-thaw motility of duplicate vials of same semen sample frozen in same batch and preserved in same conditions were compared by paired t-test. Post-thaw motility of semen vials incubated in 2 different conditions were also compared by at different time intervals after thawing.

Results

Pre-freeze sperm motility (PFM)-

The average pre-freeze sperm motility varied from 47 ± 4.37 to 70 ± 4.08 per cent and it did not differ statistically in between male camels (Table 1).

Maximum post thaw motility (PTM)-

The maximum post thaw motility observed in different samples ranged from 0-55 %.

As shown in table 1, 37% cryopreserved semen samples exhibited > 30 % post thaw motility, another 14% exhibited 20-30% post-thawmotility while remaining 48% exhibited lower or nil post-

1.S.D. Fine Chem. Ltd., India, 2. E. Merck (India) Ltd., India, 3.Hi Media Laboratories Ltd., India 4.Alembic Ltd., India 5.Sarabhai Chemicals, India

Table 1. Pre Freeze and post thaw motility of 11 camel semen.

Camel No.	No. of Sample	Pre-freeze motility Mean ± S.E.	No. of Sample with PTM>30%	No. of Sample with PTM 20% -<30%	No. of Sample with PTM<20%
1	9	60.55 ± 3.76	6	2	1
2	5	47 ± 4.37	0	0	5
3	7	64.28 ± 3.85	3	1	3
4	9	62.77 ± 3.23	2	3	4
5	4	70 ± 4.08	2	0	2
6	3	68.33±10.14	1	1	1
7	5	57 ± 5.84	1	0	4
8	6	65.83 ± 2.72	3	1	2
9	6	64.33 ± 3.54	1	2	3
10	8	56.87 ± 4.73	3	0	5
11	8	59.28 ± 5.45	4	0	4
Total	70		26 (37%)	10 (14%)	34 (48%)

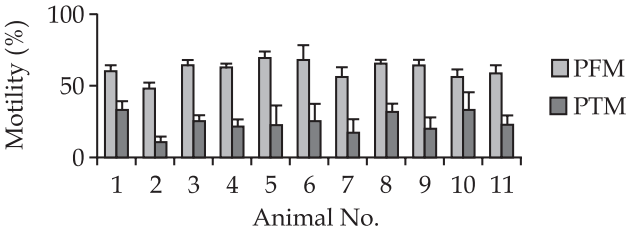

Fig 1. Pre-Freeze and post-thaw motility of cryopreserved camel semen from eleven camels.

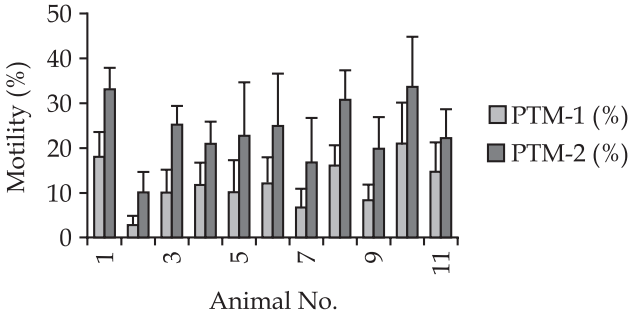

Fig 2. Post-thaw motility of camel semen differs in duplicate vials of semen sample cryopreservation in same batch.

thaw motility revival. Overall mean ± sem for post-thaw motility and grading wise mean ± sem for individual animals have been shown in table 4.

Comparison of pre freeze motility with maximum post-thaw motility:

A comparison of PFM and PTM for 11 camels have been presented in Fig 1, which shows that sperm motility was decreased from 23.5 to 47.5 % as a result of single freeze thaw procedure of camel spermatozoa.

Post-thaw motility in duplicate vials of same semen sample frozen in same batch:

Post-thaw motility of same semen sample cryopreserved in duplicate vials in same batch differ significantly as revealed by 49 duplicate sample studied, results of which are presented in Table 2 and depicted in Fig 2. Out of 49 duplicate samples studied, 20 samples exhibited wide variation and difference was significant in 4/11 males.

Longevity of post thaw motility:

Thirty five thawed semen samples, which exhibited greater than 25 % post thaw motility were incubated either at 37°C (n=17) or at 4°C (n=18) and sperm motility was examined at 0, 1, 2, 3, 4 and 24 hr after thawing. The results are presented in Table 3 and depicted in Fig 3, which shows that sperm motility was preserved for longer time at 4°C as compared to 37°C. As shown in the Table 3, at 0 hr, the post thaw motility in the 2 groups (34.7 ± 1.63 vs. 35.5 ± 1.89) was not different but at 1, 2, 3, 4 and 24 hr of incubation it was significantly greater (P> 0.01) in samples incubated at refrigeratory temperature than at 37°C. At 37°C, the reduction in motility was about 50% than at 0 hr. At 2,3,4 and 24 hr after incubation, almost 93, 99 and 100 % spermatozoa lost motility. At 4°C incubation, the percent decline was 17, 30, 35.8, 44.1 and 65.5%, at 1, 2, 3, 4 and 24 hr of incubation.

Table 2. Variability in post thaw motility of 49 cryopreserved duplicate semen samples of camels.

S. No.	N	PFM (%)	PTM-1 (%)	PTM-2 (%)	T Value	Significance
1	7	60.55±3.76	17.85±5.65	32.85±4.86	3.074	.05
2	2	47±4.37	2.5 ± 2.5	10.0±5.0	3.000	NS
3	5	64.28±3.85	10.00±5.26	25.4±4.14	3.405	.05
4	7	62.77±3.23	11.71±4.97	20.71±5.28	3.163	.05
5	2	70±4.08	10.00±7.07	22.5±12.5	1.667	NS
6	3	68.33±10.14	12.33±5.36	25.00±11.54	1.952	NS
7	3	57±5.84	6.66±4.40	16.66±10.13	1.000	NS
8	5	65.83±2.72	16.2±4.11	31.00 ± 6.20	4.09	.05
9	5	64.33±3.54	8.4±3.23	19.4±7.73	2.32	NS
10	3	56.87±4.73	21.00±9.45	33.35±12.01	1.939	NS
11	7	59.28±5.45	14.85±6.29	22.14±6.38	1.722	NS
Overall	49		12.83±1.72	24.46±2.12	8.09	.01

Table 3. Post thaw motility of 35 cryopreserved camel semen samples at various intervals after thawing at 37 and 4°C incubation.

S.No.	37° C	4° C	37° C	4° C	37° C	4° C	37° C	4° C	37° C	4° C	37° C	4° C
	0 h	0 h	1 h	1 h	2 h	2 h	3 h	3 h	4 h	4 h	24 h	24 h
1	40	40	10	35	0	35	0	35	0	30	0	18
2	30	40	15	40	8	40	0	30	0	25	0	20
3	40	35	18	30	0	30	0	25	0	20	0	3
4	30	35	2	30	0	15	0	15	0	10	0	3
5	30	30	25	25	2	20	0	20	0	20	0	10
6	30	40	18	35	3	20	0	20	0	20	0	20
7	35	25	10	25	0	25	0	15	0	10	0	10
8	30	35	18	30	0	30	0	25	0	20	0	3
9	40	45	37	40	0	40	0	40	0	40	0	25
10	45	25	30	15	2	8	0	8	0	8	0	5
11	25	35	2	20	0	15	0	15	0	10	0	8
12	45	25	38	20	20	8	2	18	0	18	0	18
13	25	35	10	15	1	10	0	8	0	6	0	5
14	35	25	25	20	2	15	0	13	0	12	0	12
15	35	50	5	50	0	40	0	40	0	40	0	10
16	45	40	30	35	0	35	0	28	0	20	0	2
17	30	50	15	40	0	40	0	40	0	35	0	35
18	-	30	-	25	-	22	-	15	-	13	-	13
Overall	34.7 ± 1.63	35.5 ± 1.89	18.11 ± 2.73	29.44* ± 2.28	2.23 ± 1.21	24.88* ± 2.75	0.11 ± 0.11	22.77* ± 2.52	0	19.83* ± 2.49	0	12.22* ± 2.11

* Significantly different

Table 4. Animal wise and grade wise post-thaw motility (Mean ±S.E.).

Camel No.	Overall mean±sem post thaw motility (range)	Mean±sem for samples >30% motility (range)	Mean±sem for samples >20% but<30% motility (range)	Mean±sem for samples >20% motility (range)
1	25.6±3.6(0- 45)	38.1±1.6 (30-45)	21.2±1.2 (20-25)	5±2.0 (0-10)
2	6.1±1.8 (0-15)	-	-	6.1±1.8 (0-15)
3	15.5±3.7 (1-35)	31.2±1.2 (30-35)	20	6±1.5(1-12)
4	15.7±3.2 (0-40)	33.7±2.3 (30-40)	21.6±1.6(20-25)	5.7±1.3(0-10)
5	20±4.9 (5-35)	33.3±1.6 (30-35)	20	10±2.0(5-15)
6	18.6±6.4 (2-45)	45 (25-45)	20±2.9(20-25)	3.5±1.5(2-5)
7	9.3±4.6 (0-35)	35	-	5.7±2.5(0-5)
8	21.4±4.4 (0-45)	40±2.9(35-45)	22.5±1.4(20-25)	6.5±3.6(0-15)
9	13.5±4.3 (2-45)	45	21.6±1.6(20-25)	5.5±1.3(2-10)
10	22.1±5.5 (1-55)	45±4.5 (35-55)	25	6.5±2.1(1-15)
11	20.6±4.7 (1-50)	40±3.8 (30-50)	-	7.6±1.9(1-18)

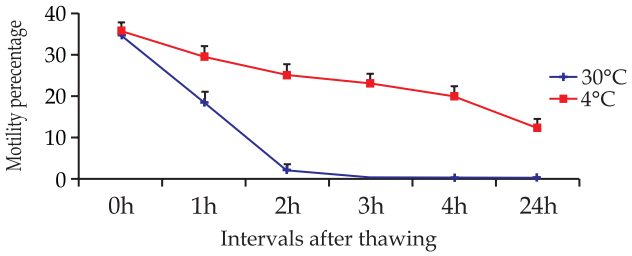

Fig 3. Motility profiles of frozen thawed camel semen at different intervals after thawing incubated at 37 and 4 degree Celcius.

Discussion

Post-frozen motility of 70 semen samples from 11 camels showed an average motility values varying from 47 ± 4.37 to 70 ± 4.08 %. The sperm motility did not differ significantly between camels. As regards to sperm motility in camel semen, it is worthwhile to mention that in freshly ejaculated semen, majority of the spermatozoa are entrapped and not free to move. On storage, spermatozoa exits from their entrapping structure, which has not been yet characterised. The rate of exit of spermatozoa is very slow and 100 % free spermatozoa are never found. At any moment of examination, a proportion of the spermatozoa are free and another proportion entrapped. Therefore, motility estimates are from the former part of the spermatozoa.

Post-thawed motility of frozen thawed camel semen varied from 0-45% in present study. Vaughan (2003) reported 20-40% PTM in Alpaca semen. Santiani *et al* (2005) reported PTM of 20.0± 6.7%, 15.3± 4.1%, 4.0± 1.1% and1.0± 1.4% in Alpaca semen frozen in different extenders. Chen *et al* (1990) reported PTM figures of 43 and 64% in bactrian semen frozen in SYG-2 and SYG-3 extenders, respectively. Sieme *et al* (1990) reported PTM of 40-70%, 30-60%, 40-70%, 30-50%, and 30-60% using different methods of freezing designated by them as stallion, ram, modify boar, dog and bull protocol, respectively. It appears that results of present study are closer to those reported by Vaughan() and Santiani *et al* (2005) for Alpaca semen, while, Sieme *et al* (1990) and Chen *et al* (1990) claimed PTM higher for bactrian camel semen than observed in present study for dromedary.

Post-thawed motility of frozen thawed camel semen is lower than pre freeze motility. A comparison of PFM and PTM showed that sperm motility was decreased from 23.5 to 47.5 % in individual samples, and a total loss of 62% of the motile spermatozoa was estimated as a result of single freeze thaw procedure of camel spermatozoa. Chen *et al* (1990) reported 18-37% decrease in sperm motility in bactrian semen. Decrease in motility of spermatozoa on cryopresevation of semen is commonly reported (Donnelly *et al*, 2001). On an average, for all human and cattle spermatozoa 50% of sperm cells are damaged by freezing and thawing. In general, the recovery of spermatozoa following freezing and thawing is comparatively low as compared to other mammalian cell types for example embryos (asymptote). The reasons of low recovery ascribed to unique structure of sperm cells, ice formation and stresses faced by spermatozoa during freezing have been well described in the literature.

Proportion of semen samples fit for preservation:

Stalhammar *et al* (1994) were of the view that a motility threshold level of 50% in case of dairy bulls, which is often stipulated for approval of the semen after freezing, does not seem to be the optimal choice and advised to reduce the level below which ejaculates should be rejected. Zhao (2000) advised PTM of above 30% in bactrian camels for approval of semen fit for insemination. Chen *et al* (1990) also adopted PTM of 30% as a criterion to approve the bactrian semen for use in AI. If, this criterion is adopted only 37% of semen samples of present study qualify as suitable for insemination. More than 50% of semen samples are then to be rejected. Most of the workers in bactrian, dromedary, and South American Camelids have not reported any significant information on this aspect. But, this rejection rate is much higher than those for dairy bulls, which ranged from 5-15%.

Post-thaw motility of same semen sample cryopreserved in duplicate vials in same batch differ significantly as revealed by 49 duplicate sample studied, 20 samples exhibited wide variation and difference was significant in 4/11 males. Significant male to male differences have been reported almost in all studies, but significant differences in freezability of same semen sample frozen in same batch in duplicate vials into automated liquid nitrogen freezer has been observed and reported for the first time. It is speculated that the empirical methods of cryopreservation developed in 1950's have not been improved significantly since then and remain crude. In particular, it is not common practice to nucleate ice in any controlled manner during sperm cooling. Additionally the control of the cooling rate is often primitive. Samples are commonly suspended in the vapour above liquid nitrogen resulting in significant differences in thermal histories between different samples. A large variation is observed from straw to straw in respect of the rate of cooling and ice nucleation temperature. When plastic ampoules are used in place of straws, the temperature profiles would be

expected to deviate more from the control values than straws. Particulate condition of the vial and vial condition influence the nucleation temperature. With camel semen being gel and usually not undergoing absolute 100% liquefaction, the particulate conditions of different vials of same semen sample may differ significantly. This might result nucleation in different vials at different temperatures. Following nucleation, the temperature used to rise to the melting point and remains close to this temperature for a significant time. The phenomenon is commonly known as latent heat plateau. During this stand still temperature of vial, the atmospheric temperature continues to lower down due to automated programming. This results in different cooling rates in different vials at this point if the temperature of nucleation was different between them and hence difference in sperm motility and viability.

At 37°C, the reduction in motility was about 50% at 1 hr post-thawing than at 0 hr. At 2, 3, 4 and 24 hr after incubation, almost 93, 99 and 100% spermatozoa lost motility. The duration of survival of thawed spermatozoa observed in present study is lower than that reported by Chen *et al* (1990) for bactrian camels, who reported survival period of 5.02 to 8.40 hrs at 37°C for different camels. At 4°C incubation, the percent decline was 17, 30, 35.8, 44.1 and 65.5%, at 1, 2, 3, 4 and 24 hr of incubation. The survival time at 4°C observed in present study is also lower than those of 14.47 to 40 hrs reported by Chen *et al* (1990).

The sperm motility in frozen thawed camel semen is maintained for relatively shorter period when incubated at 37°C as compared to at 4°C. This observation in agreement to that of Zhao *et al* (2004). Brandt and Hoskins (1980) also observed that bovine sperm motility decreased from grade 5 to 0 within 60 minutes of incubation at 37°C. This decrease in sperm motility was correlated with cAMP concentration in sperm cell. The cAMP induces protein kinase mediated phosphorylation of motility protein. Motility revival was found possible with theophylline, the regenerator of cAMP.

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