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Deep-freezing of cattle and buffalo semen with or without equilibration and its fertility trials - A comparative study

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The technique of deep-freezing of bovine semen is continuously being improved to enhance post-thaw recovery and fertility rates among inseminated females. However, very few studies have been supported with the actual fertility trials which are real means of evaluating the semen/sires and the effectiveness of various processing techniques followed. The aim of this study was to know and compare the freezability, post-thaw longevity both at 37° and 5°C, GOT leakage and fertility rates of cattle and buffalo semen frozen with or without equilibration at 5°C.

This investigation was carried out at the Germ Plasm Centre of the Institute utilizing 36 good quality ejaculates (6/bull) obtained at weekly intervals from 3 Friesian and 3 Murrah bulls, managed identically throughout the study. Semen ejaculates with above 70% initial motility were extended at 32°C in standard TFYG diluent keeping 55-60 million sperm/ml. Half the straws (0.5 ml) were frozen in liquid nitrogen vapour soon after 2 hr of gradual cooling to 5°C, i.e. without equilibration, and the remaining straws were equilibrated at 5°C for 2 hr (Dhami and Sahni 1993). Frozen straws were thawed in water-bath at 40°C for 1 min after 24 hr of storage. The sperm forward motility was assessed after dilution, cooling, equilibration and thawing under a phase-contrast microscope fitted with a biotherm (37°C). The glutamic oxaloacetic transaminase (GOT) activity was also determined simultaneously in the supernatant of 1 ml of semen centrifuged (2500 rpm, 20 min) at all these steps (Reitman and Frankel 1957). Two frozen-thawed straws were further incubated at 37°C in water-bath for 1 and 3 hr, and another 2 straws were refrigerated at 5°C (along with thawing water) for 6 and 48 hr of thermal stress and motility was re-assessed as above. Fertility rates of semen frozen without and with 2 hr of equilibration were also determined based on 147 and 352 first inseminations in cows and buffaloes of Gujarat State. Pregnancy was confirmed rectally 90 days later. The data on freezability, post-thaw longevity and GOT leakage were analyzed separately for cattle and buffalo semen using 2 × 2 × 3 factorial RBD and the conception rates were compared by using Chi-square test as per the standard statistical methods.

The findings on the various traits studied in cattle and buffalo semen frozen with and without equilibration and its ANOVA are shown in Tables 1 and 2 respectively.

Freezability and GOT leakage

The pooled mean motility of cattle and buffalo semen was 83.33±1.81 and 85.55±1.22% on dilution. These values declined significantly ($P<0.01$) to 73.06±0.42 and 76.66±0.56% at prefreezing and 51.39±1.14 and 48.61±1.76% at post-freezing respectively. The sperm cell GOT leakage on dilution was 15.85±0.27 and 16.31±0.34

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Table 1. Mean (±SE) freezability, post-thaw incubation (37°C)/ageing (5°C) survival, GOT leakage (μmole/L) and fertility rates of cattle and buffalo semen frozen in tris without or with 2 hr of equilibration at 5°C

Freezing protocols	*Parameters* evaluated	*Cow-bulls*				*Buffalo-bulls*			
		HF-594	HF-625	HF-678	Averge	M-1248	M-1281	M-1296	Average
Without equilibration at 5°C	P F M	75.00±1.29	74.17±2.01	72.50±1.12	73.88±0.86	76.67±1.67	78.33±2.11	77.50±1.71	77.51±1.01
	P T M	48.33±1.67	46.66±4.41	45.00±3.42	46.66±2.21	43.33±5.42	40.83±4.72	50.00±4.08	44.72±2.76
	P T I-1 hr	34.16±2.01	34.16±5.23	28.33±4.77	32.22±2.39	32.50±3.81	28.33±4.77	34.16±2.01	31.66±2.10
	PTI-3 hr	20.00±2.58	19.16±6.25	10.00±1.83	16.38±2.45	12.50±2.14	14.16±3.52	16.66±2.11	14.44±1.51
	PTA-6 hr	41.66±2.47	37.50±4.61	26.66±2.47	35.27±2.37	30.83±3.01	34.16±3.96	33.33±1.67	32.78±1.66
	PTA-48 hr	29.16±4.36	23.33±4.59	11.66±2.47	21.39±2.77	14.16±3.52	20.00±4.47	24.16±3.52	19.44±2.32
	GOT-P F	19.63±0.49	17.00±0.35	17.20±0.39	17.94±0.36	15.85±0.35	20.48±0.49	18.60±0.38	18.34±0.50
	GOT-P T	31.85±0.48	26.33±0.41	26.28±0.53	28.16±0.68	25.03±0.67	31.92±0.45	28.33±0.52	28.42±0.74
	C. Rs. %	59.26	47.37	56.52	53.62	51.28	52.00	63.93	56.66
	No. AI/P	27/16	19/9	23/12	69/37	39/20	50/26	61/39	150/85
With 2 hr of equilibration at 5°C	P F M	72.50±2.14	73.33±1.67	70.83±1.54	72.22±0.01	74.17±1.54	76.67±1.66	76.67±2.11	75.83±1.01
	P T M	55.00±1.85	55.83±3.36	57.50±2.81	56.11±1.74	50.83±4.73	50.00±2.47	56.66±2.47	52.50±2.19
	P T I-1 hr	41.66±2.47	43.33±5.43	31.66±5.43	38.88±2.82	40.00±2.58	36.66±5.27	43.33±2.78	40.00±2.14
	P T I-3hr	25.00±3.16	22.50±5.88	15.83±2.71	21.11±2.44	15.00±2.24	20.83±4.36	24.16±4.36	20.28±2.41
	PTA-6 hr	48.33±3.07	46.66±3.57	39.16±2.01	44.72±1.87	38.33±2.47	41.66±3.80	44.16±2.39	41.38±1.71
	PTA-48 hr	35.00±3.61	31.66±4.41	20.83±2.38	29.16±2.36	19.16±3.71	26.66±3.81	28.33±2.47	24.72±1.92
	GOT-P F	21.32±0.72	18.52±0.49	18.36±0.47	19.36±0.47	17.32±0.34	22.03±0.53	20.15±0.23	19.83±0.51
	GOT-P T	33.73±0.93	27.93±0.66	28.26±0.63	29.98±0.76	27.18±0.66	33.60±0.46	30.20±0.40	30.33±0.69
	C.Rs. %	69.70	52.38	62.50	62.82	58.21	63.29	76.78	65.35
	No. AI/P	33/23	21/11	24/15	78/49	67/39	79/50	56/43	202/132
Pooled	C.Rs. %	65.00	50.00	57.45	58.50	55.66	58.91	70.08	61.65
	No. AI/P	60/39	40/20	47/27	147/86	106/59	129/76	117/82	352/217

PFM/PTM, Prefreeze and post-thaw motility %; PTI, post-thaw incubation (37°C) survival %; PTA, post-thaw ageing (5°C) motility %; GOT-PF/PT, glutamic oxaloacetic transaminase-prefreeze and post-thaw; AI/P, No. of first inseminations and resultant pregnancies; Chi-square test was significant ($P<0.05$) for conception rates (CRs) between bulls and between equilibrations in both the species.

Table 2. Analysis of variance showing the effects of bulls, equilibration times, processing/storage periods and their interactions on freezability, post-thaw longevity (37°C/5°C) and GOT leakage from cattle and buffalo sperm

Source of variation	df	Mean sum of squares and statistical significance							
		Freezability		Post-thaw survival (37°C)		Post-thaw longevity (5°C)		GOT leakage	
		Cattle	Buffalo	Cattle	Buffalo	Cattle	Buffalo	Cattle	Buffalo
Replicates/ejac.	5	113.89**	170.56**	567.85**	267.22**	323.89**	225.00**	18.91**	12.84**
Bulls (B)	2	10.76ns	119.10*	584.72**	168.07**	1252.43**	303.13**	138.35**	190.83**
Equilibrations (E)	1	272.22**	168.05**	583.68**	868.06**	1334.72**	868.06**	47.37**	51.68**
Periods (P)	1	8449.99**	14168.05**	5083.68**	6234.72**	3901.39**	4050.00**	1951.04**	1905.50**
Interaction:									
B × E	2	17.02ns	2.43ns	4.06ns	18.06ns	31.60ns	2.43ns	0.12ns	0.03ns
B × P	2	7.29ns	104.52ns	9.72ns	84.72ns	21.18ns	44.79ns	16.38**	6.86**
E × P	1	555.56**	401.39**	17.02ns	34.72ns	12.50ns	49.99ns	0.72ns	0.76ns
B × E × P	2	10.76ns	4.51ns	30.39ns	5.56ns	3.12ns	13.55ns	0.21ns	0.71ns
Remainders	55	41.92	56.31	68.42	56.47	47.53	48.49	0.47	0.31
C.V. %	-	21.11%	28.22%	50.87%	49.27%	39.99%	39.08%	24.52%	24.16%

*, P<0.05; **, P<0.01; ns, nonsignificant; df, degrees of freedom.

μmole/litre in the extracellular medium, which increased to 18.65±0.34 and 29.06±0.56 μmole/litre in cattle and 19.08±0.42 and 29.38±0.61 μmole/litre in buffalo semen at pre- and post-freezing respectively. Both sperm motility and GOT leakage were significantly (P<0.01) influenced by the bulls, ejaculates, equilibration periods and the processing steps (Table 2). The process of glycerolization and cooling to 5°C followed by 2 hr of equilibration at 5°C over 0 hr, not only increased GOT leakage extracellularly but also improved (P<0.01) the post-thaw recovery, incubation/ageing survival and even fertility rates of frozen semen in both the species (Table 1). These findings very clearly indicated that much of the sperm cell enzymes leaked out into the extracellular medium with loss of viability as a result of cooling, equilibration and freezing-thawing of semen due to osmotic shock, structural damage and increased cell membrane permeability. These observations coincided well with the earlier reports on cattle (Graham *et al.* 1974, Pandit and Garg 1983, Belorkar *et al.* 1993) and buffalo semen (Chinnaiya *et al.* 1979, Jagmohan and Sarma 1988, Dhami and Kodagali 1990). Further, the significant interaction of bull × period (processing steps) for GOT leakage suggested that the sperms from individual bulls behaved differently in their capacity to resist dilution, cooling and freezing shock, and thereby enzyme leakage. The equilibration × period interaction for freezability (Table 2) showed that sperm post-thaw motility could be enhanced significantly by longer equilibration at 5°C, though it reduced the prefreeze motility. These interaction findings also agreed to some of the above reports.

Post-thaw incubation/ageing survival of sperm

In the present study, the average sperm post-thaw motility (51%) of cattle sperm declined significantly (P<0.01) to 35.50±1.88 and 18.75±2.11% after 1 and 3 hr of incubation at 37°C, and to 40.00±1.67 and 25.28±2.07% after 6 and 48 hr of ageing/refrigeration storage at 5°C. The corresponding values for buffalo semen were 35.83±1.35 and 17.22±1.93% on incubation, and 37.08±1.28 and 22.08±1.67% on refrigeration storage respectively. Similar findings on post-thaw incubation survival of cattle and/or buffalo sperm at 37°C (Tuli *et al.* 1985, Chinnaiya and Balkrishnan 1988, Jagmohan and Sarma 1988) and at 5°C (Uwland 1984, Sahni and Mohan 1988) have been reported by others. Further, the post-thaw longevity of sperm at both the storage temperatures was significantly (P<0.01) higher in semen frozen following 2 hr of equilibration over 0 hr in both the species and it was also affected significantly (P<0.01) by the bulls, ejaculates and storage periods. However, none of the interaction was significant for these traits in either of the species (Tables 1, 2). Wiggin and Almquist (1975) and Motwani *et al.* (1986) reported best post-thaw results with 2 or 3 hr of equilibration time over shorter or longer one. Ennen *et al.* (1976) suggested that some equilibration at 5°C is desirable for successful cryopreservation of semen regardless of cooling time. Tuli *et al.* (1981) found significantly better post-thow motility after 4 hr of equilibration than after 2 or 6 hr, whereas Rao *et al.* (1993) suggested 6 hr to be the optimum for buffalo semen. These studies were, however, not supported by fertility trials.

Fertility trials of frozen semen

In our study, the mean conception rates of cattle and buffalo semen frozen without equilibration at 5°C were significantly lower (53.62 and 56.66%) than those frozen after 2 hr of equilibration (62.82 and 65.35%) with a pooled mean of 58.50 and 61.65% respectively. The individual bulls also varied significantly (P<0.05) in their fertility rates among both the species (Table 1). Graham *et al.* (1957) and Dhami and Sahni (1993) reported significant increase in conception rates of bovine frozen semen (from 63.4 to 67.3% and 47.2 to 57.8%) following extension of equilibration period from 4 to 12 hr and 0 to 2 hr respectively. The present overall findings on fertility rates also compared well with the reports of Yassen *et al.* (1985) Chinnaiya and Balakrishna (1988) and Dhami

and Sahni (1993). Further the trend of our findings on various traits suggested that the fertility rate, the ultimate goal of frozen semen, could be predicted on the basis of freezability, post-thaw longevity and/or GOT leakage, and the later traits in turn depended on the initial quality/freezability of semen. These observations compared favourably with those of Saacke and White (1972) and Uwland (1984), who reported significant positive correlations between post-thaw incubation (37°C, 1-2 hr) and ageing (5°C, 24 hr) survival of sperm and its fertility rates. Similarly Pandit and Garg (1983) and Dhami and Kodagali (1990) found negative association between sperm cell GOT leakage and its fertility in bovines.

From the findings, we conclude that the freezability, post-thaw longevity both at 37° and 5°C as well as fertility rates of cattle and buffalo frozen semen were equally good with parallelism in GOT leakage pre-and post-freezing, and that some duration of equilibration/holding (2-3 hr) at 5°C before freezing was essential regardless of cooling time for both cattle and buffalo semen in order to obtain higher post-thaw results in respect of all above parameters studied. GOT estimates were good markers for sperm cell quality and its fertilizability in both the species.

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