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Improvement of low grade ejaculates of Holstein Friesian crossbred bulls by different filtration techniques for cryopreservation

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ABSTRACT

To improve the quality of poor ejaculates, sephadex (FS) and sephadex with ion-exchangers (FS+IE) filtration were used to examine split samples of 18 ejaculates of 6 Karan Fries bulls at various stages of cryopreservation. Data were analyzed using analysis of variance. All semen quality traits improved in both the filtration and further superiority of FS+IE column was observed due to its better efficacy of trapping immotile, dead and abnormal spermatozoa as well as there was decrease in sperm concentration, tail and total abnormalities. The initial, prefreeze and post-thaw motility, viability, sperm cells with intact plasma membranes and sperms with normal acrosomes of FS+IE filtrates were significantly better than the nonfiltered and FS filtered semen samples during different stages of cryopreservation. Therefore, it can be concluded that better quality semen can be harvested from poor ejaculates through FS+IE and FS filtration to improve fertilization potential of the spermatozoa.

Key words: Crossbred bulls, Holstein Friesian, Semen analyses, Semen quality

India is producing 66 million frozen straws covering only 25% of the breedable bovine population and to achieve the target of covering 35% AI, there is a need of 100 million straws by 2015–16. Therefore, one of the alternative measures is harvest of good quality semen without discarding too many poor quality semen ejaculates as crossbred bulls donate nearly 20–30% poor quality semen owing to inherent problem, seasonal and prophylactic stresses rendering them unfit for use in AI. In poor quality semen, large numbers of dead and abnormal sperms are present which release ROS and certain other toxic substances. ROS acts through lipid peroxidation of carbon chain of unsaturated fatty acid to form highly cytotoxic lipid hydroperoxides, which decompose to form end product malondialdehyde, which is highly toxic and is responsible for DNA and protein damage finally leading to cell death as well as damage the fertilization potential of other healthy sperms. ROS production can be minimized through removal of dead and immotile spermatozoa using sedimentation bovine serum gradient, percol density gradient, swim up procedure, glass wool filtration, glass bead filtration, sephadex filtration and sephadex ion- exchanger fitration and centrifugation techniques (Mustafa *et al.* 1998, Ahmad *et al.* 2003, Januskauskas *et al.* 2005) to improve semen quality and fertility. Therefore, we planned to study the effect of filtration on semen quality parameters at various stages of cryopreservation.

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MATERIALS AND METHODS

The present investigation was conducted on Karan Fries (Tharparkar × HF crosses between 50 to 75% exotic inheritance) maintained at the Institute.

Semen collection and initial evaluation: Semen was collected in bovine artificial vagina pre-warmed at (42–45°C) with smooth neoprene liner (IMV-005331). On the day of collection, 2 successive ejaculates were taken with 20 to 30 min gap and each ejaculate was preceded by a period of sexual preparation consisting of at least 2 false on once a week schedule. Each ejaculate was evaluated for volume and initial motility and the ejaculates (18) having initial motility between 55–65% were selected for this study. Sperm concentration was determined using a haemocytometer. The semen was diluted with Tries-citric acid egg yolk glycerol extender. Sephadex (sephadex G–100) and sephadex ion-exchanger filters were prepared as per Ahmad *et al.* (2003) with some modifications. Higher efficacy of sephadex G-100 for improving semen quality in previous studies is the basis for selection of sephadex G-100. Ejaculates (18) were divided into 3 aliquots, 1 each for sephadex filtration, sephadex with ion exchanger and control without filtration after extension (1:4) at 30°C. The extended semen samples were filtered using the columns. After filtration, the concentration of spermatozoa in filtered and nonfiltered semen sample was adjusted to 20 million motile sperm/ml after evaluation of concentration. The split

semen samples were cryopreserved as per standard freezing protocol followed at ABRC. Two frozen straws were thawed at 37°C for 30 sec for evaluation of post-thaw motility. Semen was pooled in micro-centrifuge tube and kept in digital control heat blocks at 37°C till further examination.

Semen analyses: In different stages of cryo-preservation (AD, AG, AE and PF) at –196°C semen motility, non-eosinophilic count, HOST and acrosome integrity were evaluated for control, FS and FS+IE filtrates. Initial progressive motility rating was scored using 200× magnifications with phase contrast microscope equipped with a heated stage. Percent progressive motility (0–100%) was measured at 5 representative areas of the slide. The average of the 5 scores for each category was recorded. If the difference between 2 consecutive counts exceeds 10%, two new counts were performed. Non-eosinophilic (live) spermatozoa (%) were assessed under bright field 100× oil immersion objectives using eosin-nigrosine staining. The same slide made for eosin-nigrosine staining was used for screening morphological abnormalities. About 200 spermatozoa were counted under bright field 100× oil immersion objectives in different fields and percentage of abnormal spermatozoa was calculated by dividing the number of head, mid piece, tail and total abnormalities by the total spermatozoa counted and multiplying the figure by 100. Sperm membrane integrity was assessed using the hypo-osmotic swelling test. Acrosome integrity was carried out by giemsa staining.

Statistical analyses: The effect of filtration on different sperm variables at various stages of cryo-preservation was analyzed by analysis of variance technique. Prior to the analysis, proportionality data (motility, percent non-eosinophilic count, HOST, acrosome integrity and abnormality data) were transformed using the arcsine transformation [asin (sqrt (percent/100))] with adjustment to allow for zero values. Comparison between different treatment groups was done by Fisher's Least Significant Difference (LSD) test. The differences at $P < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The results of the cryopreserved semen at various stages of cryopreservation after Sephadex and Sephadex with ion-exchangers filtration are presented in Tables 1– 2.

Sperm concentration (× 10^6/ml): There was significant ($P<0.05$) decrease in mean spermatozoa concentration after sephadex (FS) and sephadex ion-exchange (FS+IE) filtration as compared to non-filtered samples and it is the reflection of the effective trapping of dead, immotile and abnormal spermatozoa may be due to either agglomeration of sephadex particle with immotile or dead spermatozoa or binding of sephadex particles with any protein present on capacitated spermatozoa. However, no binding between sephadex particles and sperm cells was reported by Anzar and Graham (1993), in our experiment we found trapped spermatozoa in filtration column. Better efficacy of FS+IE filtration column is evident from the results, but the trapping mechanism is not clear. It is speculated that positively charged dead sperms interact with the negatively charged CM-cellulose and are trapped (Anzar and Graham 1993). The results of low sperm concentration after FS and FS+IE filtration is in similar line as reported for cattle (Anzar and Graham 1993, Bhakat *et al.* 2014) and buffalo spermatozoa (Ahmad *et al.* 2003).

Semen quality: In KF bulls, mean sperm motility, non eosinophilic count, HOST and acrosome integrity increased significantly ($P<0.05$) at all the stages of semen cryopreservation in FS and FS+IE filtration as compared to non-filtered samples. The values of the above semen quality parameters were significantly higher ($P<0.05$) in FS+IE filtrates than FS filtrates at different stages of semen processing and preservation. The results of improvement of motility and viability post-filtration are in consonance with the reports of separation using various grades of sephadex in cattle (Kumar *et al.* 2003) and buffalo (Maurya and Tuli 2003) semen. The motility, spermatozoa with stronger plasma membranes and acrosome integrity after FS+IE filtration was as good as previously reported in Holstein bulls (Anzar and Graham 1996, Anzar *et al.* 1997), crossbred bulls (Bhakat *et al.* 2014) and buffalo bulls (Ahmad *et al.* 2003). HOST and acrosome integrity is highly correlated with fertilization ability of the sperm. The post filtration semen quality of FS+IE filtrates than the FS filtrates and non-filtered semen at different stages of cryopreservation further depict the greater efficacy of ion-exchange filtration and higher fertilization potential may be due to higher post thaw motility, more spermatozoa with normal acrosomes, stronger plasma membranes, better capacity to cope up with cryopreservation and less sperm abnormalities. In KF bull sperm abnormalities were observed at different stages of cryopreservation after FS and FS+IE filtration. There was no significant difference in head and mid-piece abnormality between any of the treatment at any stage of cryopreservation under study. The tail abnormality showed a significant decline ($P<0.05$) after FS and FS+IE filtration during all the stages of cryopreservation as compared to control. These tail sperm abnormalities were much lower ($P<0.01$) in FS+IE filtrates than that in the FS filtrates at all stages of semen processing. As the tail abnormality constituted the major portion of total

Table 1. Effect of filtration at room temperature on sperm concentration (x10^6/ml) of Karan Fries bull semen

Parameters	Treatment					
	C		FS		FS + IE	
	Mean	SE	Mean	SE	Mean	SE
concentration (million/ml)	1097.69[a]	24.58	839.31[b]	30.5	755.47[c]	31.36

C, Control; FS, sephadex filter; FS + IE, sephadex with ion-exchangers. Means (±SEM.; N,18) with different superscripts within same row differ significantly ([abc]$P<0.05$).

Table 2. Effect of filtration on semen quality of Karan Fries bull semen during different stages of cryopreservation

Parameters (%)	Stages of cryopreservation	Treatment					
		C		FS		FS + IE	
		Mean	SE	Mean	SE	Mean	SE
Motility	After filtration	60.61^{A}	0.40	71.18^{B}	0.63	75.29^{C}	0.61
	After glycerolization	54.56^{A}	0.60	66.05^{B}	0.81	70.68^{C}	0.75
	After equilibration	52.32^{A}	0.68	62.96^{B}	0.84	68.56^{C}	0.86
	Post-freezing	25.69^{a}	0.93	37.39^{b}	0.52	40.76^{c}	0.65
Non-eosinophilic count	After filtration	68.18^{a}	0.82	74.78^{b}	1.00	78.97^{c}	1.08
	After glycerolization	63.60^{a}	1.05	69.43^{b}	1.01	74.57^{c}	0.81
	After equilibration	61.08^{a}	1.08	67.79^{b}	1.06	73.62^{c}	0.93
	Post-freezing	33.09^{a}	1.17	39.43^{b}	1.05	46.11^{c}	1.04
HOST	After filtration	57.80^{a}	0.74	63.23^{b}	1.02	69.49^{c}	1.09
	After glycerolization	53.26^{a}	0.98	58.91^{b}	1.06	64.90^{c}	1.16
	After equilibration	52.63^{a}	0.92	58.17^{b}	1.03	64.09^{c}	1.13
	Post-freezing	24.88^{a}	0.98	29.64^{b}	1.27	36.93^{c}	0.82
Acrosome integrity	After filtration	71.03^{a}	0.87	76.34^{b}	1.12	81.42^{c}	1.24
	After glycerolization	65.62^{a}	1.09	73.77^{b}	1.15	79.58^{c}	1.22
	After equilibration	57.86^{a}	1.16	72.69^{b}	1.11	78.01^{c}	1.15
	Post-freezing	30.68^{A}	1.27	37.61^{B}	1.18	48.88^{C}	0.84
Head	After filtration	2.94	0.51	2.34	0.67	1.78	0.79
	After glycerolization	2.96	0.51	2.42	0.70	1.98	0.68
	After equilibration	3.39	0.50	2.76	0.68	2.17	0.64
	Post-freezing	5.44	0.51	3.92	0.70	3.11	0.60
Mid piece	After filtration	2.53	0.49	2.15	0.63	1.53	0.77
	After glycerolization	3.22	0.49	2.18	0.62	1.47	0.68
	After equilibration	3.54	0.46	2.79	0.64	1.82	0.68
	Post-freezing	3.86	0.45	3.08	0.60	2.51	0.65
Tail	After filtration	12.03^{A}	0.77	7.70^{B}	1.16	3.82^{C}	1.02
	After glycerolization	11.86^{A}	0.72	6.91^{B}	1.32	3.72^{C}	1.10
	After equilibration	13.32^{A}	0.84	8.13^{B}	1.29	4.16^{C}	1.11
	Post-freezing	25.72^{a}	0.92	21.57^{b}	1.02	13.13^{c}	0.75
Total	After filtration	17.59^{a}	1.06	12.31^{b}	1.50	7.26^{c}	1.48
	After glycerolization	18.16^{a}	1.02	11.72^{b}	1.59	7.36^{c}	1.38
	After equilibration	20.37^{a}	1.12	13.85^{b}	1.61	8.34^{c}	1.39
	Post-freezing	35.22^{a}	1.23	28.84^{b}	1.42	18.94^{c}	1.14

C, Control; FS, sephadex filter; FS + IE, sephadex with ion-exchangers. Means (±SEM; n=18) with different superscripts within same row differ significantly ($^{abc}P<0.05$, $^{ABC}P<0.01$) between treatments.

abnormality, so the same trend was found in total abnormality as that of in tail abnormality. In the current study, sephadex ion-exchange filtration proved quite effective in trapping morphologically abnormal spermatozoa. Effective removal of abnormal spermatozoa from cattle (Vyas *et al.* 1992), crossbred cattle (Bhakat *et al.* 2014), boar (Bussalleu *et al.* 2009) and buffalo semen (Goyal *et al*. 1996) after passing it through sephadex columns was also reported earlier. Significant reduction in tail and total abnormality after FS and FS+IE filtration depicted the fact that immotile and abnormal spermatozoa is trapped efficiently in the filtration column as sperm motility is largely dependent on the normal functioning of the tail region of spermatozoa, whereas filtration showed nonsignificant reductions of head and mid-piece abnormalities may be owing to less interference of sperm with sperm motility.

Reduction in tail abnormalities after sephadex filtration was reported in cattle (Anzar and Graham 1996) and buffalo (Ahmad *et al.* 2003). Adverse effect of dead spermatozoa on potential fertility of companion cells is quite evident (Anzar and Graham 1993, Goyal *et al.* 1996) as semen samples with high percentage of primary and secondary sperm abnormalities resulted in low fertility. The process of freezing has great impact on motility, sperm abnormalities, normal acrosomes and intact plasma membranes as compared to glycerolization and equilibration stages of cryopreservation and this is in agreement with previous observations (Goyal *et al.* 1996). There is increase in large numbers of dead and abnormal sperms after cryopreservation and dead spermatozoa normally releases ROS and certain other toxic substances. ROS acts through lipid peroxidation of carbon chain of unsaturated fatty acid to form highly cytotoxic lipid hydroperoxides, which decompose to form end product malondialdehyde, which is highly toxic and is responsible for DNA and protein

damage finally leading to cell death as well as damage the fertilization potential of other healthy sperms. During cryopreservation and thawing process there was considerable damage to the motility apparatus, plasma membrane and acrosomal cap may be due to leakage of enzymes responsible for sperm motility, weaker plasma membrane of spermatozoa, dehydration, ice-crystal formation during freezing as well swelling and corrugation of the anterior part of the acrosome. Considerable irreversible loss occurs after cryopreservation, which cannot be avoided.

Our findings were comparable to the findings of others, if any differences were there these may be due to use of various types of ion-exchanger with different grades of sephadex (Sephadex G-10, DEAE-52, positively charged cellulose and CM-52, negatively charged cellulose- Ahmad *et al.* 2003, Sephadex G-15–120, DEAE cellulose and CM cellulose, Anzar and Graham 1993, Anzar and Graham, 1996 and sterilized cotton-1.0 to 1.5 thick, DEAE cellulose and CM cellulose, Mustafa *et al.* 1998), however, we have tried commercially available sephadex-ion exchanger combination [Sephadex-diethyl amino ethane-52 (DEAE-52, positively charged) cellulose and Sephadex-carboxy methyl-52 (CM-52, negatively charged) cellulose)] to study the effect of filtration on semen quality parameters. In our experiment distribution of sephadex was more uniform throughout the column, but in other studies they layered sephadex separately in the bottom.

We can draw the conclusion that sephadex ion-exchange filtration columns are very effective in removal of immotile, dead and abnormal spermatozoa and improve the prefreeze and post thaw semen quality. The system may be adopted by the semen banks to utilize the low grade ejaculates for production of frozen semen keeping in mind the minimum standard protocol of Govt. of India regarding post thaw motility and fertility trails need to be compared for suitable results. The reproductive efficiency of bulls can be improved without discarding too many poor semen ejaculates. The technique can be used after post vaccination latent period till normalcy restored and in seasonal deterioration of semen quality.

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REFERENCES

Ahmad Z, Anzar M, Shahab M, Ahmad N and Andrabi S M H. 2003. Sephadex and sephadex ion-exchange filtration improves the quality and freezability of low grade buffalo semen ejaculates. *Theriogenology* **59**: 1189–202.

Anzar M, Graham E F and Iqbal N. 1997. Post-thaw plasma membrane integrity of bull spermatozoa separated with a sephadex ion exchange column. *Theriogenology* **47**: 845–56.

Anzar M and Graham E F. 1993. Filtration of bovine semen. 2. factors affecting the recovery rate of spermatozoa. *Animal Reproduction Science* **31**: 197–204.

Anzar M and Graham E F. 1996. Role of sperm motility and acrosome integrity in the filtration of bovine semen. *Theriogenology* **45**: 513–20.

Bhakat M, Mohanty T K, Gupta A K, Mohanty A K, Abdullah M. 2014. Effect of filtration of low grade ejaculates on semen quality parameters at refrigerated temperature (4–7°C). *Advances in Animal and Veterinary Sciences* **2** (11): 625–31.

Bussalleu E, Pinart E, Rivera M M, Briz M, Sancho S, Yeste M, Casas I, Fàbrega A, Rigau T, Rodriguez-Gil J E, Bonet S. 2009. Effects of matrix filtration of low-quality boar semen doses on sperm quality. *Reproduction in Domestic Animals* **44** (3): 499–503.

Goyal R L, Tuli R K, Georgie G C and Chand D. 1996. Comparison of quality and freezability of water buffalo semen after washing or sephadex filtration. *Theriogenology* **46**: 679–86.

Januskauskas A, Lukoseviciute K, Nagy S, Johannisson A and Rodriguez-Martinez H. 2005. Assessment of the efficacy of Sephadex G-15 filtration of bovine spermatozoa for cryopreservation. *Theriogenology* **63**: 160–78.

Maurya V P and Tuli R K. 2003. Post thaw thermal resistance test on motility and acrosomal integrity of filtered and non- filtered frozen semen of Murrah buffalo bulls. *Asian Australasian Journal of Animal Science* **16** (10): 1424–28.

Mustafa G, Anzar M and Arslan M. 1998. Separation of motile spermatozoa from frozen-thawed buffalo semen: swim-up vs filtration procedures. *Theriogenology* **50**: 205–11.

Vyas S, Mohan G, Dhami A J and Sahani K L. 1992. Effect of filtration through sephadex and glass wool on the quality and freezability of semen of crossbred bulls. *Indian Journal of Animal Sciences* **62***:* 341–43.