Journal of Animal Research: v.12 n.1, p. 65-68. February 2022

DOI: 10.30954/2277-940X.01.2022.9

Flow Cytometric Evaluation of Acrosomal integrity of Buffalo Spermatozoa at Different Stage of Cryopreservation using Different Extenders

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Received: 09 Jan., 2022 **Revised:** 28 Jan., 2022 **Accepted:** 30 Jan., 2022

ABSTRACT

With an objective to evaluate the value of different extenders semen was collected from Murrah buffalo bulls (n=6) by artificial vagina and subjected to dilution using different extenders *viz* Tris egg yolk citrate (TEYC), LDL, tocopherol (TEYC with 1 mg/mL tocopherol) and sericin (TEYC with 0.25% sericin) based extenders. Semen was cryopreserved and sperm with intact acrosome were evaluated by flow cytometry immediately after dilution, at equilibration stage and after cryopreservation. There were non-significant differences in the proportion of live sperm with intact acrosome (frozen-thawed semen) for all the diluters studied except for buffalo bull number 5333 which evidenced significantly lower (P>0.05) proportion of live sperm with intact sperm in the tocopherol based extender. The overall percent live sperm with intact sperm was significantly higher (P<0.001) for LDL based extender as compared to TEYC, tocopherol, and sericin extenders at the equilibration stage and in frozen-thawed semen. It was concluded that the LDL based extender is best for cryopreservation of buffalo semen.

HIGHLIGHTS

- Sperms with intact acrosomes are not significantly different at three stages of cryopreservation.
- Tris supplemented with LDL yields the highest percent live sperms with intact acrosome.

Keywords: Acrosome, buffalo, LDL, flow cytometry, sericin, vitamin E

Spermatozoa have three types of membranes, which are plasma membrane, mitochondrial membrane, and acrosomal membrane. The membranes contain polyunsaturated fatty acids and hence are very susceptible to oxidative stress, especially during freezing procedures (Chelucci *et al.,* 2015). In all mammals, the capacitation and the subsequent acrosome reaction of spermatozoa represent essential steps for successful fertilization and formation of a zygote. The determination of the ability of spermatozoa to activate the acrosome reaction is supposed to be a useful parameter in evaluating infertility (Prihantoko *et al.,* 2020). Neild *et al.* (2005) suggested that acrosome reaction is a process of releasing a penetrating enzyme that allows spermatozoa to penetrate zona pellucida and to fertilize oocytes. However, if the acrosome reaction takes place before spermatozoa reach fertilization site, the spermatozoa will lose their ability to fertilize oocytes. Spermatozoa must be acrosome-intact to have the ability to fertilize oocytes. The acrosome is considered a large secretory granule that contains several

How to cite this article: Satish, Purohit, G.N., Sharma, R.K., Kumar, P., Jerome, A., Kumar, D., Bala, R., Verma, N., Vyas, J. and Mehta, J.S. (2022). Flow Cytometric Evaluation of Acrosomal integrity of Buffalo Spermatozoa at Different Stage of Cryopreservation using Different Extenders. *J. Anim. Res.,* **12**(01): 65-68.

Source of Support: None; **Conflict of Interest:** None

enzymes including glycohydrolases, proteases, esterases, acid phosphatases, and aryl sulfatases. The release of these hydrolytic enzymes degrade the zona pellucida allowing the spermatozoa to penetrate it and join with the oocyte in a process called AR, which is dependent on calcium (Ca^{2+}) (Tello-Mora *et al.,* 2018). Acrosomal integrity is very crucial in fertilization processes, the present study aimed at evaluating the quality and acrosomal integrity of buffalo spermatozoa at different stages of cryopreservation for semen diluted with different extenders.

MATERIALS AND METHODS

Semen collection and dilution

Semen was collected from buffalo bulls (n=6) during early morning using artificial vagina (Purohit *et al*., 1998). The collected semen was subjected to routine physical examination determination of sperm concentration with Accucell photometer and motility assessment under a warm-stage phase-contrast microscope. Ejaculates having more than 70% individual motility and 500×10^6 sperm/ml were used for further processing. Semen samples of individual buffalo bulls (4 replicates of each bull) were diluted with four different buffalo specific extenders namely Tris egg yolk extender (TEYC) (Purohit *et al*., 1998), LDL based extender (Kumar *et al*., 2016) containing Tris and LDL, the sericin based extender (TEYC supplemented with 0.25% sericin from Sigma-Aldrich, USA) and the tocopherol based extender (TEYC supplemented with 1mg/ml (+)-α-Tocopherol acetate- Sigma, USA). Semen was then cryopreserved using routine procedures adopted at the institute. After 24 hours of cryo-storage, semen was thawed and evaluated again.

Estimation of acrosomal integrity

Pisum sativum agglutinin (PSA) binds to a-mannose and agalactose moieties of the acrosomal matrix. Since PSA cannot penetrate an intact acrosomal membrane, only acrosome-reacted or damaged spermatozoa will stain. It is having the Excitation: l_{max} = 495 nm Emission: l_{max} = 525 nm. Supravital stain Propidium iodide (PI) is used along PSA to study the intactness of the spermatozoa using flow cytometry. The propidium iodide dye cannot pass through the intact plasma membrane. But it passes through the degenerated spermatozoa and stains its nuclei (Garner *et al*., 1997). PI stain excited using 488 nm laser rapid staining cell. These stain enter into the cell via a broken plasmalemma, emitting red fluorescence (PI: 636 nm) when they bind to nucleic acids (Gillan *et al*., 2005). For each sample, approximately 2×10^6 cells were suspended in 1 ml warm phosphate-buffered saline medium (370 C). Cells were incubated at 37°C for 10 min after adding 5 µL of stock solution of PNA (1.5 Mm) to 300 µL of sample aliquot (1.5 µM final concentration). Then 0.5 µL of PI was added and incubated for 5 minutes. Resuspension was carried out by gently flicking the tubes. PSA is routinely tagged to either FITC or Alexa Fluor probes and fluoresces upon contact with acrosome reacted sperm (Casey *et al*., 1993). All the data were collected and analyzed using CytExpert software (v.2.3) (CytoFLEX, Beckman Coulter-Life Sciences). (Excitation: l_{max} = 495 nm Emission: l_{max} = 525 nm). The proportion of spermatozoa showing intact acrosome was recorded and the data was analyzed by statistical tests described previously (Snedecor and Cochran, 1992).

RESULTS AND DISCUSSION

There were non-significant differences in the proportion of live sperm with intact acrosome (frozen-thawed semen) for all the diluters studied except for buffalo bull number 5333 which evidenced significantly lower ($P>0.05$) proportion of live sperms with intact sperms in the tocopherol based extender (Table 1) suggesting that variation across extenders is minimum. However, the overall proportion of live sperm with intact acrosome was significantly higher ($P<0.001$) for LDL based extender as compared to TEYC, sericin and tocopherol based extender at the equilibration stage and in frozen thawed semen (Table 2).

Beneficial effects of buffalo bull semen extender supplementation with sericin have been previously documented (Kumar *et al*., 2015). Similarly beneficial effects of supplementation of buffalo bull semen extender with vitamin E have been reported on acrosome intact sperms of HF bulls (Batool *et al*., 2012). The findings of the present study validate previous studies on buffalo bull semen that utilized the LDL-Tris extender (Kumar *et al*., 2016; Dalal *et al*., 2020; Patil *et al*., 2020). One study suggested that phospholipids released in the extender, following the disruption of LDL during the

Table 1: Percent live sperm with intact acrosome (Mean ± SEM)in the frozen thawed semen of individual buffalo bull using different semen extenders (6 bulls ×4 replicates =24)

Bull Identification no.	Percent live sperm with intact acrosome (Mean ± SEM)				Significance
	TEYC	LDL based Extender	Sericin (0.25 %)	Tocopherol (1mg/ml)	
1315	45.5±3.95	56.75±4.33	49.25±2.29	45.5±5.17	**NS**
4995	35±6.75	45.75±4.15	38.50±3.38	28.50±5.81	**NS**
5246	37.25±9.12	51.00±5.37	44.50±6.61	36.75±4.27	**NS**
5320	47.00±5.79	50.75±2.93	46.75±3.09	37.50±6.69	**NS**
5333	43.00±2.68^{ab}	53.00±2.38^{a}	46.75±2.50^{ab}	38.75±4.42^{b}	*
5414	43.50±5.11	52.25±3.82	44.5±6.08	50.25±4.23	**NS**
Overall	41.88±2.32^{b}	51.58±1.58^{a}	45.04±1.72^{b}	39.54±2.36 ^{b}	**

NS: Non-significant; * Significant at 5% level; **Significant at 1% level.

Table 2: Percent live sperm with intact acrosome (Mean ± SEM) at different stages of cryopreservation process in different semen extenders (6 bulls × 4 replicates = 24)

Stage of cryopreservation	Percent live sperm with intact acrosome (Mean ± SEM)				Significance
	TEYC	LDL based Extender	Sericin (0.25 %)	Tocopherol (1mg/ml)	
Fresh diluted semen	69.58±3.44	74.08±3.82	72.33±3.55	71.13±3.09	NS
Equilibrated semen	62.71±1.27 ^{b}	71.46±1.12^{a}	69.29±1.13^{a}	63.21±1.41^{b}	**
Frozen-thawed semen	41.88±2.32^{b}	51.58±1.58^{a}	45.04±1.72^{b}	39.54±2.36 ^{b}	**

NS: Non-significant; **Significant at 1% level.

freeze-thawing process, could form a gel-like protective film on the spermatozoa, which protects the lipid–protein complex of cell membranes and thereby safeguards the spermatozoa (Akhter *et al*., 2011). A proposed mechanism of protection suggests that LDL seizes the deleterious proteins present in seminal plasma thus improving the freezability of spermatozoa (Manjunath *et al*., 2002 and Bergeron and Manjunath, 2006). Thus, the LDL based extender was found to be the best for cryopreservation of Murrah buffalo bull semen.

CONCLUSION

It was concluded that the LDL diluter is best for cryopreservation of buffalo semen.

ACKNOWLEDGEMENTS

The permission and help extended by the Director, Central institute for research on buffaloes and the Dean, College of Veterinary and Animal Science, Bikaner during the entire study period is thankfully acknowledged. The authors highly acknowledge the financial support extended under AICRP on "Nutritional and physiological interventions for enhancing reproductive performance in animals (NUPHYREP)" during the research program at ICAR-CIRB, Hissar.

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