Original Research

Effect of Varying Osmolarity of Tris Extender on Seminal Attributes of Buffalo Bulls during Refrigeration

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Abstract

The present study investigated the effect of varying osmolarity of tris egg yolk citrate glycerol extender on seminal attributes of buffalo semen at various hours of refrigeration at 5 ºC. Twenty four ejaculates having mass motility ≥ 3+ from 4 bulls (6 ejaculate from each bull) were collected. Each ejaculate was divided into four group's viz., Groups I, II, III and IV, diluted with extenders having osmolarities of 240, 260, 280 and 300 milliosmol/kg (mOs/kg), respectively, to 80×10^6 sperm/ml. After dilution, semen samples were filled in French mini straws and kept at 5°C and evaluated at 0, 24, 48 and 72 hours for various seminal attributes such as individual motility, viability, acrosomal integrity, and hypo-osmotic swelling test. No significant difference in individual motility at 0 hour was observed among all the groups. However at 24, 48 and 72 hours, the individual motility was significantly (P<0.05) higher in groups I and II as compared to groups III and IV. Other seminal attributes such as viability, acrosomal integrity and hypo-osmotic swelling test were significantly (P<0.05) higher in group II (260 mOsm/kg) as compared to other groups. It is concluded that seminal characteristics such as individual motility, viability, hypo-osmotic swelling (HOS) test and acrosomal integrity were significantly affected when the osmolarity of the extender was changed from 260 to 240, 280, and 300 mOsm/kg.

Key words: Murrah Bulls, Osmolarity, Seminal Attributes, Tris Egg Yolk Extender

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Introduction

Artificial insemination (AI) and frozen semen technology played a vital role in improvement of milk production of the buffalo (*Bubalis bubalis*). From four to five decades, cryopreserved semen has been used

for artificial insemination in the buffaloes, however, the conception rate is lower as compared to cattle (Barile, 2012; Anzar *et al.*, 2003). It is presumed that sperm of buffalo are more prone to cryopreservation damages as compared to cattle and in general around 50% of buffalo sperm get damaged during freeze-thaw process leading to reduced motility, viability, acrosomal integrity, membrane integrity (Lone *et al.*, 2016a), chromatin integrity (Khan and Ijaz, 2008; Lone *et al.*, 2017), seminal antioxidants (Lone *et al.*, 2016b) and increased concentration of sperm oxidants (Balamurugan *et al.*, 2018; Lone *et al.*, 2018) leading to reduced fertilizing potential of sperm. Dilution of semen with suitable buffer is of great importance for the maintenance of the fertilizing potential of the sperm (Rasul *et al.*, 2000). Various extenders have been tried for semen preservation and tris (hydroxymethyl aminomethane) has successfully been used as an organic buffer for deep freezing of buffalo semen (Ijaz *et al.*, 2009; Reddy *et al.*, 2010; Lone *et al.*, 2016c). One of the crucial factors responsible for limited formation of ice crystals inside the cells is limited cell dehydration, and higher cell dehydration leads to increased sperm abnormalities (Agca *et al.*, 2002). Thus the osmolarity modification of the extender may be a turning point as it determines the efflux of the water during freeze-thaw procedures (Iguer-Ouada and Verstegen, 2001; Aisen *et al.*, 2002). Previously it was revealed that osmolarity of the semen may vary according to the season of the year and the variation in the osmolarity may affect semen quality in buffalo bulls during the various seasons of the year (Bhakat *et al.*, 2015). Scanty information is available regarding the effect of osmolarity of extender on seminal characteristics of buffalo bulls during various hours of refrigeration. Hence the present experiment was aimed to study the effect of varying osmolarity of the tris extender on seminal attributes of buffalo bulls during refrigeration.

Materials and Methods

Experimental Design

Semen was collected from four Murrah buffalo bulls, 4–6 year old, maintained at Artificial Breeding Research Centre (ABRC), ICAR-National Dairy Research Institute. The bulls were reared under the similar feeding and management conditions during the entire duration of the study.

Semen Collection

Semen was collected during the morning hours using an artificial vagina as per the standard method. 24 ejaculates from 4 bulls (6 from each bull) were selected on the basis of mass activity (+3 and above) and individual progressive motility (70% and above). The mass activity of the semen sample was determined by assessing the motility of the spermatozoa following semen collection. Motility was graded on the scale of 0 to +5 (Tomar *et al*., 1966).

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Semen Processing and Preservation

Immediately after ejaculate collection, it was divided into Group I, II, III and IV, diluted with Egg yolk-Tris-Glycerol (EYTG) extenders (Tris 24.22 g / L, citric acid monohydrate 13.6 g / L, fructose 10 g / L, benzyl penicillin 10^6 IU / L, streptomycin sulphate 1g / L, 20 % egg yolk, 6.4 % glycerol and millipore water added upto 1 L) to 80 million sperm / mL having osmolarities of 240, 260, 280 and 300, mOsm/kg, respectively. Osmolarity was determined by WECOR vapour pressure Osmometer (WESCOR model 5500, INC, USA), which was calibrated with 100-, 290- and 1000-mOsm standards and used to measure the osmolarity. After, dilution, semen samples were filled in French mini straws (0.25 mL) and kept at 5°C and evaluated at 0, 24, 48, and 72, hours for various seminal attributes such as individual motility, viability, acrosomal integrity, and hypo-osmotic swelling response. The details for the procedure of these parameters are given below-

Assessment of Sperm Motility and Live Sperm Count

For assessing sperm motility, a uniform drop of semen was placed on a clean grease free slide maintained at 37 °C. Then a clean cover slip was put on the semen drop and observation was done at 400× magnification of phase contrast microscope (Nikon Eclipse E600, Japan). The number of sperm moving in forward direction were estimated and expressed in percentage. In three different fields semen samples were analysed and a mean estimate of the three readings was taken as a final value of the sperm motility. Eosin-nigrosin stain was used to determine live sperm count as per the method described by Campbell *et al*. (1953). For preparation of stain 5 g of Eosin-Y (water soluble) and 10 g of Nigrosin were dissolved separately in 100 mL of 2.9% sodium citrate solution. The mixture was boiled for 15 min. The amount of volume which got evaporated during boiling was replaced with the buffer. Finally stain was prepared by mixing 10% Nigrosin solution and 5% Eosin-Y solution in the ratio of 4:1 in a container. The mixture was thoroughly shaken and filtered through Whatman filter paper. One drop of semen sample was mixed with three drops of stain and the semen-stain mixture was allowed to rest for about 1 min. After 1 min, a thin smear was prepared on a clean, grease free slide, air dried and then observed at 1000× magnification of phase contrast microscope. The sperm which appeared colourless or white were considered as live and those appeared partially or completely pink coloured were considered as dead. A total of 200 spermatozoa were counted in each slide and percentage of live sperm was determined.

Acrosomal Integrity and Hypo-Osmotic Swelling (HOS) Response

The acrosomal integrity was evaluated by Giemsa staining as per the method described by Watson (1975). After staining, to determine percent intact acrosome (PIA), a total of 200 sperm were counted at 1000× magnification of phase contrast. Functional integrity of sperm plasma membrane was assessed by the hypo-

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osmotic swelling test (HOST) as per the procedure described by Jeyendran *et al*. (1984) with some modifications. Briefly, to 100 µL of semen sample was added 1 mL of the HOST medium (4.9 g of sodium citrate and 9.9 g of fructose were dissolved in 1000 mL of distilled water) in an effendorf tube, followed by its incubation at 37 °C for 1 h. After incubation, a drop of semen sample was taken on a clean grease free slide and a cover slip was put and examined at 400× magnification. Sperm showing visible coiling of tail were considered as HOS responsive spermatozoa. To determine percent HOS responsive spermatozoa, a total of 200 spermatozoa were counted.

Semen Analysis

Individual motility was assessed subjectively under phase contrast microscope equipped with a warm stage (37 ºC) at 400× magnification. The live sperm percentage and abnormality was estimated by differential staining technique using Eosin-Nigrosin stain (Campbell *et al.,* 1953). Acrosomal intactness was determined by Giemsa stain (Watson, 1975). Hypo-osmotic swelling test (HOST) was carried out according to the method described by Jeyendran *et al*. (1984).

Statistical Analysis

Data were analyzed using one way ANOVA by Statistical Analysis System (SAS, 2011) Software Programme, version 9.3 and results were expressed as Mean ± SE.

Results and Discussion

The overall mean ±SE of various seminal attributes at 0, 24, 48 and 72 hours of refrigeration has been depicted in Table 1. No significant difference in individual motility at 0 hour was observed among all groups. However at 24, 48 and 72 hours, the individual motility was significantly ($P<0.05$) higher in groups I & II as compared to groups III & IV. Other seminal attributes such as viability, acrosomal integrity, and hypo-osmotic swelling response were significantly ($P<0.05$) higher in group II (260 mOsm/kg) as compared to other groups.

It has been seen that osmolarity of the extender influences the seminal characteristics; our study revealed that change in osmolarity of the dilutor affects the sperm characteristics of the buffalo semen during refrigeration which corroborates the findings of Mughal *et al.* (2017). Our study demonstrated that reduction in osmolarity from 300 to 260 mOsm/kg resulted in improvement in various seminal characteristics such as motility, viability, HOS response and acrosomal integrity, however when the osmolarity was reduced to 240 mOsm/kg, there was a significant reduction in motility, viability, HOS response and acrosomal integrity. Previous studies have revealed that there was significant reduction in motility, viability, HOS response, acrosomal integrity and increased DNA damage, when the osmolarity of the extender was reduced from 275 to 255 mOsm/kg (Mughal *et al.,* 2017).

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Table 1: Seminal attributes of buffalo semen diluted with extenders of various osmolarities at various hours of refrigeration (Mean ± SE)

Seminal Attributes (%)	Period	Group I (240 mOsm/kg)	Group II (260 mOsm/kg)	Group III (280 mOsm/kg)	Group IV (300 mOsm/kg)
Individual motility	0 h	69.56^{a}±0.26	69.70^{a}±0.26	68.43^{a}±0.26	69.48^{a}±0.26
	24 h	35.94^{a}±0.22	36.38^{b}±0.28	33.95^{c}±0.22	33.10^{c}±0.29
	48 h	24.84^{a}±0.26	25.06^{a}±0.26	22.29^{b}±0.32	22.05^{b}±0.28
	72 h	7.65^{a}±0.25	7.83^{a}±0.25	6.74^{b}±0.24	6.51^{c}±0.30
Viability	0 h	70.68^{a}±0.28	71.18^{b}±0.28	70.53^{ac}±0.27	70.23^{c}±0.28
	24 h	37.26^{a}±0.19	39.80^{b}±0.17	32.66^{c}±0.19	31.32^{d}±0.22
	48 h	24.84^{a}±0.27	27.27^{b}±0.19	21.54^{c}±0.23	21.11^{d}±0.21
	72 h	10.77^{a}±0.19	12.23^{b}±0.21	8.40^{c}±0.22	8.15^{d}±0.27
HOS Response	0 h	63.29^{ac}±0.24	63.70^{b}±0.24	63.06^{c}±0.24	62.99^{c}±0.24
	24 h	33.13^{a}±0.24	35.85^{b}±0.24	29.35^{c}±0.41	27.71^{d}±0.23
	48 h	17.30^{a}±0.30	18.54^{b}±0.31	14.63^{c}±0.31	13.98^{d}±0.29
	72 h	7.36^{a}±0.27	8.02^{b}±0.24	6.56^{c}±0.26	6.21^{d}±0.30
	0 h	73.25^{a}±0.22	73.60^{b}±0.22	72.71^{c}±0.22	72.45^{c}±0.22
	24 h	51.90^{a}±0.24	53.22^{b}±0.22	48.32^{c}±0.22	47.06^{d}±0.22
	48 h	29.83^{a}±0.26	30.74^{b}±0.32	26.89^{c}±0.25	26.13^{d}±0.23
	72 h	11.61^{a}±0.24	12.59^{b}±0.20	9.98^{c}±0.19	9.62^{d}±0.22

Means bearing different superscripts within the same row differed significantly (P<0.05).

The plasma membrane surrounding the sperm holds various organelles and intracellular components and maintains chemical gradient of ions and soluble components. Plasma membrane also has got some specific proteins that are involved in the transport of ions or components (Schurmann *et al*., 2002). So any change in the integrity of the plasma membrane may lead to sperm death or reducing fertility level of the sperm. Buffalo spermatozoa revealed better semen quality after dilution and cryopreservation, when the semen was diuted with the extender of osmolarity of 260 mOsm/kg. In frozen-thawed bull semen, highest percentage of motile spermatozoa was found when semen was diluted with extenders having osmolarities ranging from 270 and 340 mOsm/kg (Liu *et al.*, 1998b). Due to solute loading and outpouring of water phenomena, it is better that extenders should be towards hypotonic side in terms of osmolarity as compared to hypertonic side. Maintaining osmolarity of medium as 300 mOsm/kg in bovine semen yielded better results in terms of membrane integrity and sperm motility (De Pauw *et al*., 2003). Percentage of motile spermatozoa declined sharply, when the osmolarity of the modified Tyrode's solution was increased to >500 mOsm (Liu and Foote, 1998). Dilutors needed for semen preservation are designed in such a way that they should be nearly isotonic to semen, in order to reduce the effects on metabolism and survival of spermatozoa (Bredderman and Foote, 1969). Osmolarity environment is a vital factor that needs to be considered during cryopreservation of semen. Anisoosmotic solutions not only lead to induction of stress in cells, but also determine outcome of process of cryopreservation along with the

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freezing/thawing processes, there are important osmotic changes due to differential freezing medium, leading to flux of water through the cell membrane (Curry and Watson, 1994).

Conclusion

It is concluded that buffalo sperm are sensitive to the osmotic pressure of the extender used for dilution of semen. Seminal characteristics such as individual motility, viability, hypo-osmotic swelling (HOS) response and acrosomal integrity were significantly affected when the osmolarity of the extender was changed from 260 to 240, 280, and 300, mOsm/kg. A marked change in the osmotic pressure of the semen dilutor may alter freezability and fertility of buffalo sperm. It is recommended that osmolarity of the dilutor used for preservation of buffalo semen at refrigeration temperature should be closer to the osmolarity of the seminal plasma.

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Conflict of Interest

The authors declare that they don't have any conflict of interest.

References

1. Agca Y, Gilmore J, Byers M, Woods EJ, Liu J and Critser JK. 2002. Osmotic characteristics of mouse spermatozoa in the presence of extenders and sugars. Biology of Reproduction. 67: 1493-1501.
2. Aisen EG, Medina VH and Venturino A. 2002. Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. Theriogenology. 57: 1801-1808.
3. Anzar M, Farooq U, Mirza MA, Shahab M and Ahmad N. 2003. Factors affecting the efficiency of artificial insemination in cattle and buffalo in Punjab, Pakistan. Pakistan Veterinary Journal. 23: 106-113.
4. Balamurugan B, Ghosh SK□□, Lone SA□□, Prasad JK□, Das GK, Katiyar R, Mustapha□ AR, Kumar A and Verma MR. 2018. □Partial deoxygenation of extender improves sperm quality, reduces lipid peroxidation and reactive oxygen species during cryopreservation of buffalo (Bubalus bubalis) semen. Animal Reproduction Science. 189: 60-68.
5. Barile VL. 2012. Technologies related with the artificial insemination in buffalo. Journal of Buffalo Science. 1: 139-146.
6. Bhakat M, Mohanty TK, Gupta AK, Prasad S , Chakravarty AK and Khan HM. 2015. Effect of season on semen quality parameters in murrah buffalo bulls. Buffalo Bulletin. 34: 100-112.
7. Bredderman PJ and Foote RH. 1969. Volume of stressed bull spermatozoa and protoplasmic droplets and the relationship of cell size to motility and fertility. Journal of Animal Science. 28: 496-501.
8. Campbell, RG, Hancock JL and Rothschild L. 1953. Counting live and dead bull spermatozoa. Journal of Experimental Biology. 30: 44-45.
9. Curry MR and Watson PF. 1994. Osmotic effects on ram and human sperm membranes in relation to thawing injury. Cryobiology. 31: 39-46.

DOI 10.5455/ijlr.20180327052829

10. De Pauw I, Van Soom A, Mintiens K, Verberckmoes S and De Kruif A. 2003. In vitro survival of bovine spermatozoa stored at room temperature under epididymal conditions. Theriogenology. 59: 1093-1107.
11. Iguer-ouada M and Verstegen JP. 2001. Long term preservation of chilled canine semen: effect of commercial and laboratory prepared extenders. Theriogenology. 55: 671-684.
12. Ijaz A, Hussain A, Aleem M, Yousaf MS and Rehman H. 2009. Butylated hydroxytoluene inclusion in semen extender improves the post-thawed semen quality of Nili-Ravi buffalo (Bubalus bubalis). Theriogenology. 71: 1326-1329.
13. Jeyendran RS, Vander, Ven HH., Parez-pelaez M, Crabo BG and Zaneweld LJD. 1984. Development of an assay to assess the functional integrity of the human membrane and its relationship to other semen characteristics. Journal of Reproduction and Fertility. 70: 219-228.
14. Khan MIR, Ijaz A. 2008. Effects of osmotic pressure on motility, plasma membrane integrity and viability in fresh and frozen-thawed buffalo spermatozoa. Animal. 2: 548-553.
15. Liu Z and Foote RH. 1998a. Bull sperm motility and membrane integrity in media varying in osmolality. Journal of Dairy Science. 81(7): 1868-1873.
16. Liu Z, Foote RH and Brockett CC. 1998b. Survival of bull sperm frozen at different rates in media varying in osmolality. Cryobiology. 37: 219-230.
17. Lone SA, Prasad JK, Ghosh SK, Das GK, Kumar N, Balamurugan B, Katiyar R and Verma MR. 2016a. Effect of cholesterol loaded cyclodextrin (CLC) on lipid peroxidation and reactive oxygen species levels during cryopreservation of buffalo (Bubalus bubalis) spermatozoa. Asian Pacific Journal of Reproduction. 5: 476-480.
18. Lone SA, Prasad JK, Ghosh SK, Kumar N, Bhat SA and Das GK. 2016b. Effect of cholesterol loaded cyclodextrin on activity of antioxidants during cryopreservation of buffalo (*Bubalus bubalis)* semen. Indian Journal of Animal Sciences. 86(11): 1255–1258.
19. Lone SA, Prasad JK, Ghosh SK, Das GK, Balamurugan B, Sheikh AA, Katiyar R and Verma M.R. 2016c. Activity of enzymatic antioxidants and total antioxidant capacity in seminal plasma of Murrah bulls during cryopreservation. Journal Animal Research. 6(3): 405-410.
20. Lone SA, Shah N, Yadav HP, Wagay MA, Singh A and Sinha R. 2017. Sperm DNA damage causes, assessment and relationship with fertility: A Review. Theriogenology Insight. 7(1): 13-20.
21. Lone SA, Prasad JK, Ghosh SK, Das GK, Balamurugan B and Verma MR. 2018. Study on correlation of sperm quality parameters with antioxidant and oxidant status of buffalo bull semen during various stages of cryopreservation. Andrologia. 2018: e12970.
22. Mughal DH, Ijaz A, Yousaf MS, Wadood F, Farooq U, Mahmood SA and Riaz A. 2017. Effect of osmotic pressure on spermatozoa characteristics of cryopreserved buffalo bull (Bubalus bubalis) semen. Journal of Applied Animal Research: 1-4.
23. Rajoriya JS, Prasad JK, Ghosh SK, Perumal P, Kumar A, Kaushal S, Singh M. 2013. Effects of seasons on enzymatic changes and cholesterol efflux in relation to freezability in Tharparkar bull semen. *Asian Pac. J. Reprod*. 2, 280-288.
24. Rasul Z, Anzar M, Jalali S, Ahmad N. 2000. Effect of buffering systems on post-thaw motion characteristics, plasma membrane integrity and acrosome morphology of buffalo spermatozoa. Animal Reproduction Science. 59: 31-41.
25. Reddy NSS, Mohanarao GJ and Atreja SK. 2010. Effects of adding taurine and trehalose to a tris-based egg yolk extender on buffalo (Bubalus bubalis) sperm quality following cryopreservation. Animal Reproduction Science. 119: 183-190.
26. SAS Institute Inc. 2011. SAS® 9.3 System Options: Reference, Second Edition. Cary, NC: SAS Institute Inc.
27. Schurmann A, Axer H, Scheepers A, Doege H and Joost HG. 2002. The glucose transport facilitator GLUT8 is predominantly associated with the acrosomal region of mature spermatozoa. Cell Tissue Research. 307: 237-242.

DOI 10.5455/ijlr.20180327052829

28. Tomar N., Mishra BS and Johari, CB 1966. Seasonal variations in reaction time and semenproduction and prediction of some semen attributes on initial motility of spermatozoa in Hariana and Murrah bull. Indian Journal of Dairy Sciences. 19: 87-93.
29. Watson PF. 1975. Use of Giemsa stain to detect changes in the acrosome of frozen ram spermatozoa. Veterinary Record. 97: 12-15.

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