Original Article

Buffalo Bulletin (April-June 2024) Vol.43 No.2
https://doi.org/10.56825/bufbu.2024.4323936

SEMINAL CHARACTERISTICS AND EFFICACY OF EGG YOLK FREE SEMEN EXTENDER ON CRYOPRESERVATION OF MURRAH BUFFALO BULL SEMEN

Vikash Kumar Jaiswal, Rajendra Prasad Tiwari, Girish Kumar Mishra*, Pingla Bhatt, Sanjeev Sahasrabudhe and Ajit Kumar Nair

Received: 11 September 2020
Accepted: 01 April 2024

ABSTRACT

The experiment was undertaken to study the efficacy of egg yolk free semen extender on cryopreservation of buffalo semen. A total of 24 semen ejaculates from Murrah bulls (5 to 6 years) maintained at Central Semen Station, Anjora, Durg, Chhattisgarh were divided into two aliquots and were diluted using Tris-fructose-egg yolk-glycerol (TFYG) and egg yolk free commercial extender (EYFCE, AndroMed). The overall average Murrah bull fresh semen volume (mL), sperm concentration (million/mL), initial progressive motility (%), live sperm (%), abnormal spermatozoa (%), intact acrosome (%) and HOS reactive sperm (%) were 4.90±0.49, 1286.92±100.23, 75.00±0.67, 81.45±0.61, 10.70±0.51, 80.16±0.43 and 68.83±0.40, respectively. There was significant difference between bulls in semen volume ($P<0.01$), individual motility ($P<0.01$) and total sperm abnormalities ($P<0.05$). The semen extended with TFYG and EYFC extenders were cryopreserved in french mini-straw (0.25 mL) using programmable bio-freezer. The mean values of post thaw motility (%), live sperm (%), abnormal sperm (%), intact acrosome (%), HOST (%) of Murrah bull semen extended with TFYG and EYFCE were 45.83±0.77 vs 49.16±1.33, 57.41±0.54 vs 58.62±0.73, 12.54±0.46 vs 12.45±0.63, 69.62±0.82 vs 71.45±0.64 and 56.45±0.52 vs 57.37±0.55, respectively. Post thaw motility, live spermatozoa and intact acrosome were significantly higher ($P<0.01$) in frozen thawed semen extended with as EYFCE compared to TFYG in Murrah bull semen. It is concluded that EYFCE results in improvement in post thaw semen characteristics *viz*. post thaw motility, live sperm percent and intact acrosome.

Keywords: *Bubalus bubalis*, buffaloes, egg yolk free dilutor, freezability, fresh semen, Murrah buffalo bulls

INTRODUCTION

Cryopreservation is a non-physiological method for adaptation of biological cells to the osmotic and thermal shocks that occur during the dilution, cooling, freezing, and thawing (Watson *et al*., 1992). Damage occurring during the freezing and thawing procedures mainly affects the cellular plasma membranes, mitochondria, nucleus

Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science and Animal Husbandry, Anjora Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Chhattisgarh, India, *E-mail: drkodu@gmail.com

(Blesbois, 2007) and sperm viability. The egg yolk (EY) is the most common constituent in semen extenders and yolk (EY) for bovine semen (Foote, 1982). Meanwhile, extenders based on egg yolk can have negative effects on sperm respiration, motility, fertilizing capacity (Bousseau *et al*., 1998) and makes the evaluation of sperm difficult (Singh *et al*., 2012). Soyabean can be used as an alternative to conventional extender that contain skimmed milk and or egg yolk (Papa *et al*., 2011) with improved post-thawed sperm characteristics (Singh *et al*., 2012). Moreover, sperm motility and viability were better in soya lecithin-based extender compared to milk, tris-citric egg yolk and egg yolk-citrate extender for buffalo sperms stored at 5ºC (Akhter *et al*., 2011). A soyabean lecithin-based extender has been developed and utilized for bovine semen (Van Wagtendonk *et al*., 2000). Many commercially available extenders containing a substitute for egg yolk have been used for the preservation of bovine, ovine and caprine semen (Aires *et al*., 2003; Stradaioli *et al*., 2007; Celeghini *et al*., 2008). The literature on efficacy of egg yolk free semen extender is scanty. Therefore, the present study was designed to assess the semen characteristics and comparative suitability of egg yolk free semen extender over egg yolk semen extender on freezability of Murrah bull semen.

MATERIALS AND METHODS

The study was conducted on Murrah bulls of 5 to 6 years age, maintained in identical feeding and management regimes according to minimum standard protocol (MSP) of Government of India at Central Semen Station, Anjora, Durg, Chhattisgarh. A total 24 ejaculates from Murrah bulls were collected by using artificial vagina maintained at 42 to 45ºC during February to April. The semen was collected from all experimental bulls twice a week during morning hours between 7.00 to 8.30 A.M. Fresh semen was used for pre freeze seminal evaluation of ejaculate volume (Kedia *et al*., 2014), sperm concentration (Mishra *et al*., 2012), initial progressive motility (Ahmad, 1994), percent live spermatozoa count (Campbell *et al*., 1953), percent sperm abnormalities (Kedia *et al*., 2014), acrosomal integrity (Watson, 1975) and hypo osmotic swelling test (Jeyendran *et al*., 1984). Each ejaculate was divided into two aliquots; one was diluted in Tris-Fructose-Yolk-Glycerol (TFYG) extender (Davis *et al*., 1963) and other was diluted in Egg yolk free commercial extender (EYFCE, AndroMed, Minitub GmbH). Briefly, the commercial Egg yolk free extender was prepared fresh for use by dilution (1:4) with Millipore water according to manufacturer's instructions for bull ejaculates. Egg yolk free extender contains phospholipids, TRIS, citric acid, sugar, antioxidants, buffers, glycerol, antibiotics, and purest water. The semen samples with acceptable semen characteristics of volume (>1 mL), sperm concentration (>500 million per mL) and initial progressive motility (>70%) were diluted with a calculated quantity of both extenders to ensure 20 million sperms in French mini straw (0.25 mL). Filled and sealed straws following standard procedure of equilibration at 4ºC for 4 h and freezing through programmable bio-freezer for 8 to 10 minutes were cryopreserved in liquid nitrogen (-196°C). Post thaw evaluation for percent post thaw motility (Sardar, 2007), live spermatozoa, sperm abnormalities, intact acrosome and HOS reactive spermatozoa in cryopreserved semen were assessed as procedure described for fresh semen characteristics after 24 h of freezing.

Mean values of seminal characteristics were determined as per the standard statistical

method. Significance within species between extenders was tested by paired t-test. Significance within species between bulls were tested by independent mean 't' test as per the procedure by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Fresh seminal characterestics of Murrah bull

Ejaculated semen volume

The average semen volume in Murrah bulls was 4.90±0.49 mL. The volume of semen differed significantly (P≤0.01) between bulls (Table 1). The seminal volume of Murrah is in close agreement with the breeds of Murrah (Dhami *et al*., 1998); Surti (Doshi, 1991) and Jaffrabadi and Mehsana buffalo (Patel *et al*., 2012) bulls. Lower values were in Murrah (Bhakat *et al*., 2015) and Surti buffalo (Jani, 1982) bulls. However, higher values were in Murrah (Gokhale *et al*., 2003) and Jafarabadi buffalo (Kerur *et al*., 1979) bulls. Significant difference (P<0.01) in seminal volume between bulls might be due to variation in the androgen dependent accessory glands particularly those affected by testosterone concentration. Ejaculate volume is a breed characteristic that depends upon the body weight, scrotal size and weight, reproductive health, age of bulls, method and frequency of collection, nutrition, season, and management (Nazir, 1988).

Sperm concentration

The sperm concentration was 1286.92±100.23 million per mL in Murrah bull semen (Table 1). The sperm concentration of the Murrah is found to be in close agreement with the findings of Kerur *et al*. (1979); Dhami *et al.* (1998) in Murrah buffalo bulls. However, higher values were reported by Gokhale *et al*. (2003); Bhakat *et al*. (2015). Moreover, the lower sperm concentration values were reported in Murrah (Dhami *et al*., 1998), Surti (Pareek and Paul, 1985), Mehsana and Jafarabadi (Patel *et al*., 2012) bulls. These variations in sperm concentration might be as a result of techniques used while semen collection, season (Tiwari *et al*., 2011), age and feeding of the bulls (Ijaz *et al*., 2009).

Initial progressive sperm motility

The individual initial progressive motility was 75.00±0.67% in and Murrah bull semen. The average individual motility differed significantly between bulls (P<0.01) in Murrah (Table 1) bulls. The individual initial progressive sperm motility of Murrah bulls is in close agreement with the findings in Murrah (Kumar *et al*., 1993; Tomar and Singh, 1996), Jafarabadi (Kerur *et al*., 1979), Surti (Jani, 1982) and Mehsana (Patel *et al*., 2012) breeds of the buffalo bulls. However, initial progressive sperm motility was found higher (Singh *et al*., 2014; Bhakat *et al*., 2015) and lower (Dhami *et al*., 1998; Gokhale *et al*., 2003) in Murrah bulls. The difference in individual sperm motility may be due to individual genetic variations, semen handling and environmental influences (Bailey *et al*., 2003). Moreover, the results can also vary from person to person, as sperm motility is usually assessed under microscope through naked eye (Farooq *et al*., 2013).

Live spermatozoa

The average live sperm percent was 81.45±0.61 in Murrah bull semen (Table 1). The present findings of percent live sperm in Murrah bulls are in close agreement with findings in Surti (Nema *et al*., 1983), Mehsana (Patel *et al*., 2012) and Egyptian (Fayez *et al*., 1987) buffalo bulls.

However, the percent live spermatozoa were found higher (Kumar, 2008; Bhakat *et al*., 2015) and lower (Dhami and Sahni, 1994) in Murrah bulls. Meanwhile, the percent live sperm were also observed higher in Surti (Pareek and Paul, 1985) and lower in Mehsana (Patel *et al*., 2012) and Surti (Dhami and Kodagali, 1988) buffalo bulls. The present finding is well within the acceptable limit and these variations in live sperm count may be attributed to frequency of collection (Tomar, 1984), age of breeding bulls and season (Dhami, 1986).

Total sperm abnormalities

The mean total sperm abnormalities were 10.70±0.51% in Murrah bull semen. The sperm head, mid piece and tail abnormalities were 5.08±0.29, 2.54±0.24 and 3.04±0.20% in Murrah bull semen (Table 1), respectively. The present findings of total percent sperm abnormalities in Murrah semen are found parallel to that of abnormalities in Murrah (Bhakat *et al*., 2015), Mehsana (Patel *et al*., 2012) and Surti (Nema *et al*., 1983) breeds of buffalo. However, these values were lower that of the findings in buffalo (Kumar *et al*., 1993, Singh *et al*., 2014) in bull semen. Our present findings are in permissible limit as acceptable quality of semen should not contain more than 20% abnormal sperm, whereas semen containing >30% abnormal sperm would be considered as poor (Hafez, 1993). Higher abnormal spermatozoa affect the freezability of semen (Pangaonkar and Sharma, 1989), fertility in A.I. bulls (Soderquist *et al*., 1991) and impaired testicular functions as attributed by elevated environmental temperature during summer season (Rao and Rao, 1978).

Intact acrosome

The average percent intact acrosome was 80.16±0.43 in Murrah bull semen (Table 1). There was no significant difference observed between Murrah bulls. The percent intact acrosome of Murrah bulls is in close agreement with the findings in Murrah (Kumar, 2008; Ram *et al*., 2017). Spermatozoa which have lost their acrosome integrity spontaneously after ejaculation or induced by physical damages are unable to bind to oocytes, and consequently unable to fertilize oocyte.

Hypo-osmotic swelling test (HOST)

The average HOST percent was 68.83±0.40 in and Murrah bull semen (Table 1), respectively. There is no significant difference between bulls. The present findings of HOS reactivity are correlated with the findings of HOS reactive spermatozoa in Murrah (Bhakat *et al*., 2015), *Nili-Ravi* (Lodhi *et al*., 2008), Tarai (Tiwari *et al*., 2009) and Jafarabadi (Rana and Dhami, 2002) buffalo. Spermatozoa with damaged or inactive plasma membranes are unable to respond the HOS reactivity (Jeyendran *et al*., 1984).

Efficacy of egg yolk free semen extender for cryopreservation of Murrah bull semen

Post Thaw Motility (PTM)

The mean values of per cent PTM in semen extended with TFYG and EYFCE were 45.83±0.77 and 49.16±1.33 in Murrah bull semen, respectively (Table 2). Post thaw motility was significantly ($P<0.01$) higher in semen extended with EYFCE than TFYG in Murrah bull semen. The present findings of PTM in Murrah bull semen are line with the semen extended with TFYG (Chaudhari *et al*., 2015) and EYFCE - AndroMed (Beran *et al*., 2012), Bioxcell (Asr *et al*., 2011) and Biociphos-Plus (Veerabramhaiah *et al*., 2011). Moreover, EYFCE (soy-lecithin based extenders) significantly improved post thaw motility than egg yolk-based extenders (TFYG- Aires *et al*., 2003; Amirat *et*

al., 2005; Stradaioli *et al*., 2007; Rastegarnia *et al*., 2013). Soy-lecithin based extenders could improve post-thawed sperm characteristics (Singh *et al*., 2012) as their protective role in sperm cryopreservation due to low viscosity and less debris (Zhang *et al*., 2009).

Live spermatozoa

The mean percent live sperm of post thawed semen extended with TFYG and EYFCE were 57.41±0.54 and 58.62±0.73 in Murrah bull semen, respectively (Table 2). There was significantly (P<0.01) higher percent live sperm was observed in semen extended with EYFCE than TFYG in Murrah bull semen. The present findings of percent live spermatozoa in post thawed semen diluted with EYFCE showed better sperm survival in frozen thawed semen than Tris-citrate egg yolk (TCEYE- Rastegarnia *et al*., 2013), Tris-Soy modified (TSM, Arifiantini and Yusuf, 2010), egg yolk-based extender (Triladyl/TFYG - Singh *et al*., 2013) and Optixcell (Chaudhari *et al*., 2015) extender in bovine semen. Soy-lecithin is thought to be a better emulsifier may protect the spermatozoa damage during cryopreservation and thawing (Zhang *et al*., 2009) and improve post thawed sperm characteristics in semen extended with soybean (Singh *et al*., 2012).

Sperm abnormalities

The mean values of percent post thawed abnormal sperm in semen extended with TFYG and EYFCE were 12.54±0.46 and 12.45±0.63 in Murrah bull semen, respectively (Table 2). The head, mid piece, and tail abnormalities in TFYG and EYFCE were recorded as 5.54±0.25 vs 5.66±0.22, 3.04±0.14 vs 3.12±0.17 and 3.95±0.17 vs 3.70±0.18 in Murrah bull semen, respectively. No significant difference in abnormal post thawed spermatozoa was observed in semen extended with EYFCE than TFYG in Murrah bull semen. The present findings of percent post thawed abnormal sperm are corresponding to the values of semen diluted with TFYG and EYFCE (Veerabramhaiah *et al*., 2011; Chaudhari *et al*., 2015) in bovine semen. Similarly, soybean can be used as an alternative source to conventional extender that contain skimmed milk and or egg yolk and improved the post thawed sperm characteristics (Papa *et al*., 2011).

Acrosomal integrity

The mean values of per cent post thaw intact acrosome in semen extended with TFYG and EYFCE were 69.62±0.82 and 71.45±0.64 in Murrah bull semen, respectively. Significantly (P<0.01) higher percent intact acrosome was found in semen diluted with EYFCE than TFYG in Murrah bull semen. The present findings of percent post thawed intact acrosome is in accordance with the findings of the semen diluted with TFYG and EYFCE (Veerabramhaiah *et al*., 2011; Chaudhari *et al*., 2015). Similarity, significantly higher acrosomal integrity was also reported in soy-lecithin based extender than egg yolk-based extenders (Rastegarnia *et al*., 2013; Chaudhari *et al*., 2015). However, egg yolk-based extender may destabilize the sperm acrosomal membrane as well as acrosomal integrity and difficult evaluation of spermatozoa (Amirat *et al*., 2005).

Hypo osmotic swelling test

The mean values of percent post thaw HOS reactive sperm in semen extended with TFYG and EYFCE were 56.45±0.52 vs 57.37±0.55 in Murrah bull semen, respectively. However, no significant difference percent post thaw HOS reactive sperm was observed in Murrah bull semen. The present findings of percent post thaw HOS reactive

Table 1. Characteristics of fresh semen in Murrah buffalo bulls.

SN.	PARAMETERS	BULL 44816	BULL 116627	BULL 114161	BULL 43712	OVERALL
1	Volume (mL)	4.06 ±0.80^{A}	3.05±0.32^{A}	5.20 ±0.30^{AB}	7.30±0.27^{B}	4.90±0.49
2	Concentration (Million/mL)	1372.31±172.37	1255.28±232.85	1395.78±208.64	124.31±219.24	1286.92±100.23
3	Initial progressive sperm motility (%)	77.50±1.11^{B}	75.00±1.29^{B}	75.83±0.83^{B}	71.66±1.05^{A}	75.00±0.67
4	Live sperm (%)	83.00±1.18	81.33±0.95	82.16±1.70	79.33±0.49	81.45±0.61
5	Total sperm abnormalities (%)	9.83±0.49^{a}	10.83±0.49^{ab}	9.16±0.65^{a}	13.00±0.96^{b}	10.70±0.51
a	Head abnormalities (%)	5.00±0.51	5.50±0.67	4.50±0.56	5.33±0.66	5.08±0.29
b	Mid piece abnormalities (%)	2.16±0.47^{a}	2.33±0.21^{a}	1.83±0.30^{a}	3.83±0.47^{b}	2.54±0.24
c	Tail abnormalities (%)	2.66±0.21	2.83±0.30	2.83±0.1	3.83±0.65	3.04±0.20
6	Intact acrosome (%)	81.66±0.71	80.16±0.87	79.33±1.14	79.50±0.42	80.16±0.43
7	HOST (%)	68.83±0.83	69.33±0.84	69.16±0.60	68.00±1.00	68.83±0.40

Mean bearing different (^{ab}) and (^{AB}) superscript between column within row differ significantly ($P<0.05$) and ($P<0.01$), respectively.

Table 2. Effect of tris-fructose-yolk-glycerol extender and egg yolk Free commercial extender on freezability of Murrah bull semen.

SN	Semen parameters (%)	Semen extenders		Level of significance
		TFYGE	EYFCE	
1	Post thaw motility	45.83±0.77[b]	49.16±1.33[a]	P <0.01
2	Live spermatozoa	57.41±0.54[b]	58.62±0.73[a]	P <0.01
3	Total sperm abnormalities	12.54±0.46	12.45±0.63	P>0.05
a	Head abnormalities	5.54±0.25	5.66±0.22	P>0.05
b	Mid piece abnormalities	3.04±0.14	3.12±0.17	P>0.05
c	Tail abnormalities	3.95±0.17	3.70±0.18	P>0.05
4	Intact acrosome	69.62 ± 0.82[b]	71.45 ± 0.64[a]	P <0.01
5	HOST	56.45±0.52	57.37±0.55	P>0.05

TFYGE = Tris-Fructose-Yolk-Glycerol Extender.

EYFCE = Egg Yolk Free Commercial Extender (AndroMed, Minitub, GmBH).

Mean bearing different ([abc]) superscript within row differ significantly (P<0.01).

spermatozoa are in accordance with the findings of the semen diluted with TFYG and EYFCE (Akhter *et al*., 2010; Singh *et al*., 2013) in bovine semen. Significantly higher post thaw HOS reactive sperm were also observed in semen extended with soy-lecithin based than TFYG extender (Rastegarnia *et al*., 2013; Chaudhari *et al*., 2015). The soy-lecithin is a natural mixture of phosphatidyl choline and others fatty acids, provide structural stability to cells (Oke *et al*., 2010). The better HOS reactive spermatozoa in EYFCE extender clearly indicated better protection of spermatozoa against freezing damage. Moreover, fertilizing capacity of spermatozoa is negatively affected by the risk of microbial contamination associated with egg yolk (Aires *et al*., 2003).

The present study indicates that cryopreservation of Murrah bull semen in EYFCE extenders was more beneficial than TFYG in terms of higher progressive motility, live sperm, intact acrosome, HOS reactive sperm and reduce abnormal sperms. Therefore, the soya based ready to use egg yolk free commercial semen extender can be used for cryopreservation of Murrah bull semen.

ACKNOWLEDGEMENTS

Authors are highly thankful to Dean, College of Veterinary Science and Animal Husbandry, Anjora, Durg and Director Veterinary Services (C.G.) for permitting to carry out the research project at Central Semen Station, Anjora, Durg and providing necessary facilities.

REFERENCES

Ahmad, N. 1994. *Clinical and experimental studies of reproductive functions in the ram and male goat with special reference to the*

use of diagnostic ultrasound. Ph.D. Thesis, Department of Large Animal Medicine and Surgery, Royal Veterinary College, University of London, London, UK.

Aires, V.A., K.D. Hinsch, F.M. Schloesser, K. Bognera and S.M. Schloesser. 2003. *In vitro* and *in vivo* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. *Theriogenology*, **60**(2): 269-279. DOI: 10.1016/S0093-691X(02)01369-9

Akhter, S., M.S. Ansari, B.A. Rakha, N. Ullah, S.M.H. Andrabi and M. Khalid. 2011. *In vitro* evaluation of liquid-stored buffalo semen at 5c diluted in soya lecithin based extender (Bioxcell), tris-citric egg yolk, skim milk and egg yolk-citrate extenders. *Reprod. Domest. Anim*., **46**(1): 45-49. DOI: 10.1111/j.1439-0531.2009.01561.x

Amirat, L., M. Anton, D. Tainturier, G. Chatagnon, I. Battut and J. Courtens. 2005. Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. *Reproduction*, **129**(4): 535-543. DOI: 10.1530/rep.1.00011

Arifiantini, R.I. and T.L. Yusuf. 2010. Developing of Tris soy milk diluent for Frisian Holstein bull frozen semen. *J. Biosciences*, **17**(2): 91-94. DOI: 10.4308/hjb.17.2.91

Asr, S.J., R. Beheshti and H. Kohram. 2011. The evaluations of Tris-citrate-egg yolk or Bioxcell extenders on the post-thawed buffalo sperm parameters. *Annals of Biological Research*, **2**(4): 360-365.

Bailey, J., A. Morrie and N. Cormier. 2003. Semen cryopreservation: Success and persistence in farm species. *Can. J. Anim. Sci*., **83**(3): 393-401. DOI: 10.4141/A03-024

Beran, J., L. Stadnik, J. Bezdicek, F. Louda, J. Citek and J. Duchacek. 2012. Effect of sire and extender on sperm motility and share of live or dead sperm in bulls' fresh ejaculate and in AI doses after thawing. *Arch. Tierzucht*, **55**(3): 207-218. DOI: 10.5194/aab-55-207-2012

Bhakat, M., T.K. Mohanty, A.K. Gupta, S. Prasad, A.K. Chakravarty and H.M. Khan. 2015. Effect of season on semen quality parameters in Murrah buffalo bulls. *Buffalo Bull*., **34**(1): 100-112. Available on: https://kukrdb.lib.ku.ac.th/journal/BuffaloBulletin/search_detail/result/288696

Blesbois, E. 2007. Current status in avian semen cryopreservation. *World's Poult. Sci. J*., **63**: 213-222. DOI: 10.1017/S0043933907001419

Bousseau, S., J.P. Brillard, L. Marquant, B. Guienne, B. Guerin, A. Camus and M. Lechat. 1998. Comparison of bacteriological qualities of various egg yolk sources and the *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology*, **50**(5): 699-706. DOI: 10.1016/s0093-691x(98)00175-7

Campbell, R.G., J.L. Hancock and L. Rothschild. 1953. Counting live and dead bull spermatozoa. *J. Expt. Biol*., **30**(1): 44-49. DOI: 10.1242/jeb.30.1.44

Celeghini, E.C.C., R.P.D. Arruda, A.F.C.D. Andrade, J. Nascimento, C.F. Raphael and P.H.M. Roderigues. 2008. Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin. *Anim. Reprod. Sci*., **104**(2-4): 119-131. DOI: 10.1016/j.anireprosci.2007.02.001

Chaudhari, D.V., A.J. Dhami, K.K. Hadiya and J.A. Patel. 2015. Relative efficacy of egg

yolk and soya milk-based extenders for cryopreservation (-196°C) of buffalo semen. *Vet. World*, **8**(2): 239-244. DOI: 10.14202/vetworld.2015.239-244

Davis, I.S., R.W. Bratton and R.W. Foote. 1963. Livability of bovine spermatozoa at 5, -25 and 85ºC in Tris-buffered and citrate-buffered yolk-glycerol extender. *J. Dairy Sci*., **46**: 333-336.

Dhami, A.J. 1986. *Studies on seminal characteristics, biochemical and enzymatic constituents, freezability, enzyme leakage and fertility in Surti buffalo bulls*. M.V.Sc. Thesis, Gujarat Agricultural University, Anand, Gujarat, India.

Dhami, A.J. and S.B. Kodagali. 1988. Seminal characteristics and their interrelationships in Surti buffalo. *Indian Vet. J*., **65**(1): 61-64.

Dhami, A.J. and K.L. Sahni. 1994. Comparative appraisal of physico-morphological and enzymatic attributes of semen and their interrelationships in ox and buffalo bull. *J. Appl. Anim. Res*., **5**(1): 13-20. DOI: 10.1080/09712119.1994.9705992

Dhami, A.J., G. Mohan and K.L. Sahni. 1998. Seasonal influence on the quality and freezability of semen of Friesian and Murrah buffalo bulls. *Indian J. Anim. Reprod*., **19**(1): 55-58.

Doshi, M.B. 1991. *Effect of Clomiphene Citrate (Fertivit-300) on sexual behaviour and semen quality in Surti buffalo bulls*. M.V.Sc. Thesis, Gujarat Agricultural University, Anand, India.

Farooq, U., A. Ijaz, N. Ahmad, H. Rehman and H. Zaneb. 2013. Investigations on semen quality and freezability of Cholistani breeding bulls - A preliminary study from Cholistan desert of Pakistan. *J. Anim. Plant Sci*., **23**(2): 359-363.

Fayez, I., M. Marai, A.H. Daader and A.E. Navsar. 1987. Some physical and biochemical attributes of buffalo semen. *Egyptian Journal of Animal Science*, **25**(1): 99-104.

Foote, R. 1982. Cryopreservation of spermatozoa and artificial insemination: Past, present, and future. *J. Androl*., **3**(2): 85-100. DOI: 10.1002/j.1939-4640.1982.tb00651.x

Gokhale, S.B., M. Mushtaque, N. Phadke, L. Dinodkar and G.S. Ambhore. 2003. Studies on the effect of hydrogen ion concentration of extender on semen characters of Murrah buffalo bulls. *Indian Journal of Animal Reproduction*, **24**(2): 158-160.

Hafez, E.S.E. 1993. *Reproduction in Farm Animals*, 6th ed. Lea and Febiger, Philadelphia, USA. p. 405-439.

Ijaz, A., A. Hussain, M. Aleem, M.S. Yousaf and H. Rehman. 2009. Butylated hydroxytoluene inclusion in semen extender improves the post-thawed semen quality of *Nili-Ravi* buffalo *(Bubalus bubalis). Theriogenology*, **71**: 1326-1329. DOI: 10.1016/j.theriogenology.2008.12.023

Jani, V.R. 1982. S*tudies on deep freezing of bull and buffalo semen for freezability, enzyme leakage and fertility*, M.V.Sc. Thesis, Gujarat Agricultural University, Anand, India.

Jeyendran, R.S., H.H. Van der Ven, M. Perez-Pelaez, B.G. Grabo and L.Z.D. Zaneveld. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil*., **70**(1): 219-228. DOI: 10.1530/jrf.0.0700219

Kedia, N.K., R.P. Tiwari, G.K. Mishra, M.R. Poyam, A.K. Pandey, A.K. Nair and S.A.

Sahasrabudhe. 2014. Characteristics and freezability of Tharparkar bull semen. *Indian J. Anim. Sci*., **84**(4): 382-388. DOI: 10.56093/ijans.v84i4.39837

Kerur, V.K., A.H. Serasia and B.K. Bhavsar. 1979. Effect of vitamin-A feed supplementation on semen production of Jafarabadi buffalo bulls. *Indian Vet. J*., **56**: 1020-1022.

Kumar, S. 2008. *Seminal Characterestics and freezability of Murrah bulls semen during spring and summer season*, M.V.Sc. Thesis, Submitted to Indira Gandhi Krishi Viswavidyalaya, Raipur, India.

Kumar, S., K.L. Sahni and G. Mohan. 1993. Freezing of buffalo semen in milk, tris and sodium citrate dilutors with different levels of yolk and glycerol in relation to pH of dilutors. *Indian J. Anim. Sci*., **63**(5): 499-504.

Lodhi, L.A., M. Zubair, Z.I. Qureshi, I. Ahmad and H. Jamil. 2008. Correlation between hypo-osmotic swelling test and various conventional semen evaluation parameters in fresh *Nili-Ravi* buffalo and Sahiwal cow bull semen. *Pak. Vet. J*., **28**(4): 186-188.

Mishra, G.K., R. Tiwari, S.U. Rehman, K.S. Rathore, R.B. Singh, S.K. Saxena and M.U. Siddiqui. 2012. Effect of seasons on semen production performance of Jersey bulls. *Indian J. Dairy Sci*., **65**(6): 497-500.

Nazir, M. 1988. Semen evaluation and sperm morphology. *Monograph on Reproductive Pattern of Riverine Buffaloes and Recommendations to Improve Their Reproductive Performance at Small Farmer Level*. Pakistan Agricultural Research Council, Islamabad, Pakistan.

Nema, S.P., S.B. Kodagali, K. Jankiraman and G.A. Prabhu. 1983. Comparative studies on semen ejaculates (static and motile) in Surti buffalo bulls. *Indian J. Anim. Reprod*., **3**(1): 5-8.

Oke, M., J.K. Jacob and G. Paliyath. 2010. Effect of soy lecithin in enhancing fruit juice/sauce quality. *Food Res. Int*., **43**(1): 232-240. DOI: 10.1016/j.foodres.2009.09.021

Pangaonkar, G.R. and R.D. Sharma. 1989. Spermiogram and cytomorphology of spermatozoa in relation to freezability of Holstein Freisian semen. *Indian Journal of Animal Reproduction*, **10**: 60-63.

Papa, F.O., G.B. Felício, C.M. Melo-ON, M.A. Alvarenga, B.D. Vita, C. Trinque, J.N.P. Puoli-Filho and Jr.J.A. Dell'aqua. 2011. Replacing egg yolk with soybean lecithin in the cryopreservation of stallion semen. *Anim. Reprod. Sci*., **129**(1-2): 73-77. DOI: 10.1016/j.anireprosci.2011.10.006

Pareek, P.K. and C.S. Paul. 1985. Sexual behavior and characteristics of Surti buffalo bulls in Arid Zone of Rajasthan. *The Proceedings All India Symposium on Seminology*, Pantnagar, India.

Patel, J.B., A.J. Dhami and P.A. Patel. 2012. Comparative evaluation of physico-morphological attributes of semen of Jafarabadi, Mehsana (*Bubalus bubalis*) and crossbred (HF X Kankrej) (*Bos indicus*) bulls. *Indian Journal of Field Veterinarians*, **7**(3): 1-7.

Ram, K.L., R.P. Tiwari, G.K. Mishra, S.A. Sahasrabudhe and A.K. Nair. 2017. Effect of heat stress on seminal characteristics of Murrah Buffalo bull semen. *Buffalo Bull*., **36**(2): 369-378. Available on: https://kukrdb.lib.ku.ac.th/journal/BuffaloBulletin/search_detail/result/368842

Rana, C.M. and A.J. Dhami. 2002. Physical

attributes, intact acrosomes, HOS test and freezability of semen of Gir and Jafrabadi bulls. *The 18th Annual Convention of ISSAR and National Symposium*, Indian Veterinary Research Institute, Izatnagar, India.

Rao, T.L.N. and A.R. Rao. 1978. Semen characteristics of crossbred bulls. *Indian Vet. J.*, **55**(9): 692-700.

Rastegarnia, A., A. Shahverdi, T.R. Topraggaleh and V. Shafiepour. 2013. *In vitro* comparison of soybean lecithin-based extenders for cryopreservation of buffalo (*Bubalus bubalis*) semen. *Comparative Clinical Pathology*, **23**(4): 893-900. DOI: 10.1007/s00580-013-1708-6

Sardar, M.J.U. 2007. Environment related variations in the semen characteristics of bulls used for Artificial Insemination (AI) programme in Bangladesh. *University Journal of Zoology Rajshahi University*, **26**: 81-88. DOI: 10.3329/ujzru.v26i0.706

Singh, A.K., P.S. Brar and R.S. Cheema. 2014. Relationships among frozen-thawed semen fertility, physical parameters, certain routine sperm characteristics and testosterone in breeding Murrah buffalo (*Bubalus bubalis*) bulls. *Vet. World*, **7**(9): 644-651. DOI: 10.14202/vetworld.2014.644-651

Singh, A.K., V.K. Singh, B.M. Narwade, T.K. Mohanty and S.K. Atreja. 2012. Comparative quality assessment of buffalo (*Bubalus bubalis*) semen chilled (5°C) in egg yolk- and soya milk-based extenders. *Reprod. Domest. Anim*., **47**(4): 596-600. DOI: 10.1111/j.1439-0531.2011.01928.x

Singh, V.K., A.K. Singh, R. Kumar and S.K. Atreja. 2013. Development of soya milk extender for semen cryopreservation of Karan Fries (crossbreed cattle). *CryoLetters*, **34**(1): 52-61.

Snedecor, G.W. and W.G. Cochran. 1994. *Statistical Methods*, 8th ed. The Iowa State University Press, Iowa, USA.

Soderquist, L., L. Jansson, H. Larsson and S. Einaarsson. 1991. Sperm morphology and fertility in A.I. bulls. *Zentralb. Vet. Riehe A*, **38**(7): 534-543. DOI: 10.1111/j.1439-0442.1991.tb01045.x

Stradaioli, G., T. Noro, L. Sylla and M. Monaci. 2007. Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: comparison between two extenders. *Theriogenology*, **67**(7): 1249-2155. DOI: 10.1016/j.theriogenology.2007.01.009

Tiwari, M., R.B. Prasad and H.B. Gupta. 2009. Physico-morphology and *in-vitro* fertility of semen / spermatozoa of Tarai buffalo semen. *Indian J. Anim. Physiol*., **1**: 11-14.

Tiwari, R., G.K. Mishra, M.K. Shukla, R.B. Singh, S.K. Saxena and M.U. Siddiqui. 2011. Seasonal variations in semen production of Murrah buffalo bulls. *Indian Journal of Animal Reproduction*, **32**(2): 5-7.

Tomar, N.S. 1984. *Artificial Insemination and Reproduction of Cattle and Buffalo*, 3rd ed. Saroj Prakashan, Allahabad, India.

Tomar, S.S. and S.P. Singh. 1996. Studies on reaction time and some of the seminal attributes and their inter-relationships in Murrah buffalo bulls. *Indian J. Anim. Res*., **30**(1): 49-54.

Van Wagtendonk-de Leeuw, A.M., R.M. Haring, L.M.T.E. Kaal-Lansbergen and J.H.G. Den Dass. 2000. Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. *Theriogenology*, **54**(1): 57-67. DOI: 10.1016/S0093-691X(00)00324-1

Veerabramhaiah, K., A.S. Rao, V.H. Rao, K.V. Naidu and S.T.V. Rao. 2011. Efficacy of the

Tris and Biociphos plus extenders on the freezability of Punganur bull semen. *Indian Journal of Animal Reproduction*, **32**(2): 1-4.

Watson, P.F. 1975. Use of a Giemsa stain to detect changes in acrosomes of frozen Ram spermatozoa. *Vet. Rec.*, **97**(1): 12-15. DOI: 10.1136/vr.97.1.12

Watson, P.F., E. Kunze, P. Cramer and R.H. Hammerstedt. 1992. A comparison of critical osmolality and hydraulic conductivity and its activation energy in fowl and bull spermatozoa. *J. Androl.*, **13**(2): 131-138. Available on: https://onlinelibrary.wiley.com/doi/pdf/10.1002/j.1939-4640.1992.tb01644.x

Zhang, S.S., J.H. Hu, Q.W. Li, Z.L. Jiang and X.Y. Zhang. 2009. The cryoprotective effects of soybean lecithin on boar spermatozoa quality. *Afr. J. Biotechnol.*, **8**(22): 6476-6480. DOI: 10.5897/AJB09.1070