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Semen Discard Rate at Fresh and Post-Thaw Stages in Indian Riverine Buffalo (*Bubalus bubalis*)

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ABSTRACT

The quality of spermatozoa at post-thaw stage depends on many factors including the level of lipid per-oxidation (LPO) and the antioxidant profile of the ejaculates at fresh stage. Thus, the present investigation was done to evaluate discard rate of semen ejaculates at fresh and post-thaw stages in association with various semen quality parameters in Murrah buffalo. A total of 79 ejaculates from seven Murrah buffalo bulls formed part of the study. Following application of rigorous prescribed selection criteria it was observed that 17.72% (14/79) ejaculates were discarded at fresh stage due to poor quality. The initially selected ejaculates were processed for cryopreservation as per the minimum standard protocols. The study show satisfactory results for 31 (47.69%) samples for inclusion in the further AI programme (discard rate of 52.31% (34/65) at post thaw. The mean post-thaw motility (PTM; %) of the passed ejaculates was found to be 44.2±1.0. In conclusion, study results show efforts to minimize LPO and enhance total antioxidant capacity (TAC) at various processing stages shall play a vital role in reducing discard rate of the semen samples in buffalo bulls.

Key words: Murrah, Post-thaw, Acrosome integrity, Freezability, ROS

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INTRODUCTION

The elaborate procedure of collection followed by cryo-preservation of semen in domestic species is aimed to dis-seminate valuable germplasm from highly fertile males. To this aim, artificial insemination (AI) has been proved to be the most widespread tool for improvement of the genetic potential of livestock (Bhakat *et.,* 2011). Though India has the largest world buffalo population of 109.85 million (20th Livestock Census 2019) contributing about 49.1% of the total milk produced and 86% of the total exported meat (FAOSTAT, 2018), but it is still an underutilized species. It is largely because of the paucity of the quality male germ-plasm, below par performance in artificial breeding pro-gramme, beside prevalence of traditional buffalo rearing practices. Current statistics show that AI coverage of the breedable bovine population is less than 20% (Project Management Unit NDDB). Such a poor coverage of the breedable buffalo population under AI programme needs to be improved for the better economic returns from this

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much promising species. However, achievement of the stated goal places greater demand on the frozen semen doses from the high producing buffalo population.

The available statistics show that total semen production (cattle and buffalo) in India was 88.18 million doses in 2018-19 from the 28 A and B graded semen stations (Annual Report, National Dairy Development Board 2018-19). It was estimated that demand for frozen semen shall rise to 140 million doses by 2021-22 (Project Management Unit NDDB). The paucity of frozen semen of buffalo bulls of high economic worth is aggravated by several factors such as poor semen freezability (Kumaresan *et al.,* 2006, Andrabi *et al.,* 2008), vulnerability towards the freezing stress (Mughal *et al.,* 2017) and by relatively high discard rate at the time of semen processing stage itself. The available literature shows that about 18.22% (1533/8412) (Zafar *et al.,* 1988); 16.38% (792/4834) (Tiwari *et al.,* 2011) and 32.5% (13/40) (Singh *et al.,* 2013) of the fresh semen ejaculates were found non-freezable in buffalo bull due to poor quality and were discarded at the fresh stage. The high discard percentage of the buffalo semen at the fresh as well as at the post thaw stage accounts for the shortage of the quality germ plasm for the buffalo breeding propagation and limits their economic exploitation.

For production of quality semen with high freezability and fertility rates, evaluation and selection of seminal characteristics which pass the threshold values is must (Bhoite *et al.,* 2005). Thus, the objective of the investigation was to find out the discard rate of ejaculates in Murrah buffalo and their relation with various semen quality parameters.

MATERIALS AND METHODS

The study was conducted at the Germ Plasm Centre, Division of Animal Reproduction, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly (UP). The institute is located at an altitude of 564 feet above the mean sea level at latitude of 28^0 North and longitude of 79^0 East. The place has a subtropical climate and experiences both the extremes of hot and cold weather conditions with the relative humidity ranging between 15 to 85% in different months of the year.

Experimental animals and semen evaluation

In the present study a total of 79 ejaculates from seven Murrah buffalo bulls aged between 4-6 years, were collected. All the bulls were maintained under iso-managerial condition throughout the study. Following semen collection, the semen was examined for volume and consistency. The mass motility was assessed by placing a drop of fresh semen on clean, grease free glass slide without cover slip. The grading was done on the scale of 0 to 5+ (Salisbury *et al.,* 1978). The sperm concentration was determined by Bovine photometer (Accucell, IMV, France).The individual progressive motility (IPM) was assessed by placing a small drop of diluted semen (100 µL of semen was mixed with 200 µL of extender) on pre-warmed glass slide (37°C) under high power objective (400X) of microscope (Motic, China) fitted with thermo-stage after covering with a cover slip. The ejaculates with ≥+3 mass motility, ≥70% IPM and ≥500million-mL^{-1}were only utilized for further processing.

The viability (%) and sperm morphological abnormalities (%) were counted in 200 spermatozoa by making a smear on grease free glass slide with Eosin-Nigrosin staining (Swanson and Bearden, 1951). The functional or biochemical plasma membrane integrity (%) of the spermatozoa was assessed by HOS (Hypo-osmotic swelling) assay and various sperm tail swelling patterns were observed (Jeyendran *et al.,* 1984). Acrosome integrity (%) of the spermatozoa was assessed using Giemsa staining method (Watson, 1975). At least 200 spermatozoa were counted for assessment of plasma membrane integrity and acrosome integrity.

Lipid peroxidation of the spermatozoa in terms of concentration of malondialdehyde (MDA) was assessed in the sperm pellet obtained after centrifugation at 400 g for 10 min and separation of seminal plasma by TBA-TCA (Thiobarbituric acid-Trichloroacetic acid) method (Suleiman *et al.,* 1996). Total antioxidant capacity (TAC) was assessed in the seminal plasma using ferric reducing/ antioxidant power (FRAP) assay as described by Benzie and Strain (Benzie *et al.,* 1996).

The semen sample was extended in Tris-Egg Yolk-Glycerol extender. The extended semen was filled into 0.25mL (French mini) straws using semi-automatic filling machine. The straws were then sealed with PVA (Poly vinyl alcohol, IMV) powder. Following filling and sealing, the straws were kept to equilibrate at 4°C for 4h in cold handling cabinet. The straws were frozen via static vapour freezing and then straws were plunged directly into liquid nitrogen (LN_2, -196°C).

The post-thaw motility was determined following thawing of semen at temperature 37°C for 30s. The other parameters determined at post-thaw stage were spermatozoa viability and morphological abnormality using Eosin-Nigrosin stain, acrosome integrity using Giemsa stain, Hypo osmotic swelling (HOS) test for biochemical integrity of plasma membrane of spermatozoa. Similar to fresh stage lipid per-oxidation (MDA) in sperm pellet and TAC in seminal plasma was also analyzed.

Statistical analysis

The obtained data from the study was analyzed for Mean±SEM using Microsoft Excel 10 after assessment of normality of data. GraphPad Prism version 8.0.1 (244) software was used for making pie chart and bar diagrams for depicting number and percent distribution of the data obtained. Unpaired student's T-test was employed to compare various semen quality parameters at fresh and post-thaw stage.

Table 1. Performance of fresh ejaculates with respect to threshold selection values (No.)

		Rejected			
			Reason of rejection		
Total ejaculate collected	Processed	Total	Mass motility (<+3)	IPM (<70%)	Sperm concentration (<500millionmL^{-1})
79	65 (82.28%)	14 (17.72%)	5 (35.71%)	8 (57.14%)	1 (7.15%)

IPM, Individual Progressive Motility

Table 2. Attributes of selected ejaculates at fresh and the post-thaw stage (Mean±SEM)

Semen quality parameters (SQPs)	Fresh stage (n=65)	Post-thaw stage (n=65)
Volume (mL)	2.58±0.12	-
Mass motility	3.75±0.11	-
Sperm motility (%)	75.84±0.61[a]	34.15±1.51[b]
Sperm concentration (million/mL)	1226.54±53.31	-
Viability (%)	82.70±0.64[a]	40.95±1.49[b]
HOST +ve sperm (%)	73.03±0.86[a]	34.64±1.43[b]
Morphological abnormalities (%)	5.73±0.33[a]	9.21±0.44[b]
Acrosome integrity (%)	77.49±0.92[a]	48.04±1.94[b]
TAC (µmol/mL)	1.46±0.06[a]	0.61±0.02[b]
MDA (µmol/mL/20×10^6 spermatozoa)	0.34±0.02[a]	0.79±0.03[b]

HOST, Hypo-osmotic Swelling Test; TAC, Total antioxidant capacity; MDA, Malondialdehyde

Values bearing superscripts a, b differ significantly with in row (p<0.0001)

RESULTS AND DISCUSSION

The study comprises data from 79 ejaculates. Out of this, 14 (17.72%) ejaculates were discarded at fresh stage due to poor quality (Table 1). The remaining 65 samples were utilized for further examination and processing. The average seminal attributes at fresh and post-thaw stage of Murrah buffalo bulls have been presented in Table 2.

Semen quality parameters at fresh stage

The distribution (number) of ejaculates in terms of mass motility, volume and sperm concentration at fresh stage has been presented as Fig. 1a, 1b and 1c, respectively. The number distribution of ejaculates in terms of IPM, viability, HOST and Acrosome integrity has been depicted as Fig. 2 while, Fig. 3a and 3b represents the number and percent distribution of ejaculates in terms of TAC and MDA, respectively at fresh stage.

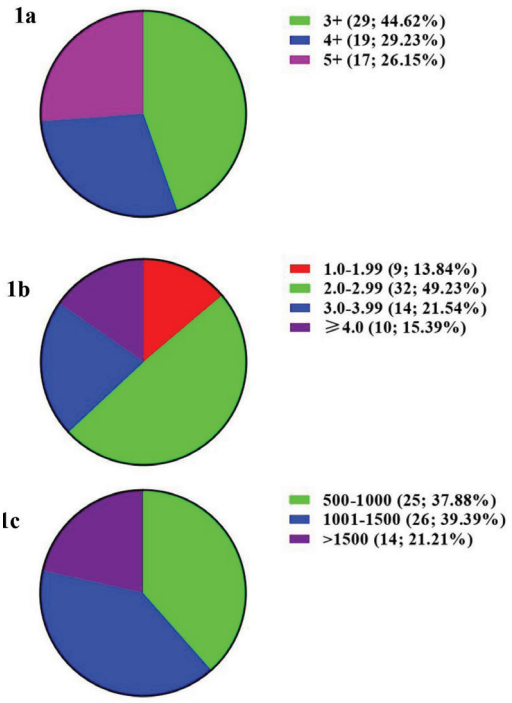

Fig. 1: Number and percent distribution of ejaculates (n=65) in terms of mass motility (1a), semen volume (mL) (1b) and sperm concentration (million/mL) (1c)

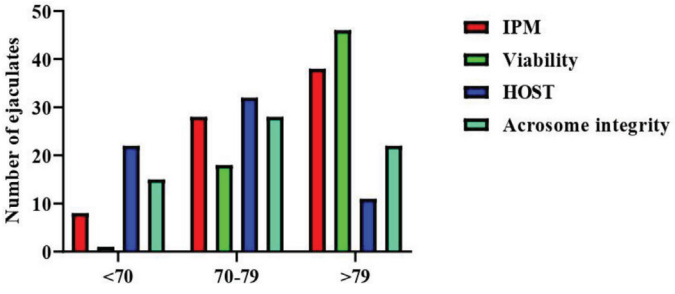

Fig. 2: Number distribution of ejaculates (n=65) in terms of IPM, Viability, HOST and Acrosome integrity (expressed in %)

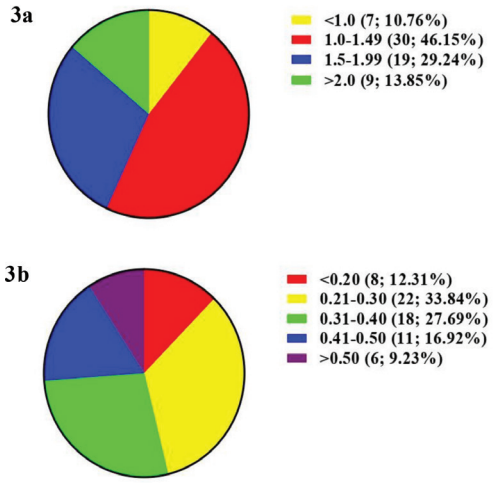

Fig. 3: Number and percent distribution of ejaculates (n=65) in terms of total antioxidant capacity (µmol/mL) (3a) and malondialdehyde concentration (µmol/mL/20 million spermatozoa) (3b) at fresh stage

The volume of 65 ejaculates ranged from 1-6.4mL with mean value of 2.58±0.12 mL. Higher volume was reported by Dhami and Sahni (1994) and Shukla and Mishra (2005). The volume of semen is affected by season, teasing of bull before collection, frequency of collection (Shukla and Mishra 2005, Ravismurugan *et al.,* 2008). The mass motility ranged from 3+ to 5+ with mean of 3.75±0.11 whereas, Similar result was observed by Shukla and Mishra (2005). While Shivahre *et al.* (2015), Vyawanare *et al.* (1989) reported lower mass motility in buffalo semen. The mean value of IPM was 75.84±0.61% with range between 70-90%. Higher IPM as compared to this study was reported by Singh *et al.* (2014) and Pathak *et al.* (2019) while, lower IPM was observed in study of Kumar *et al.* (1993) and Bhakat., (2011). The result for IPM was in concurrence with result of Ghodasara., (2016).The value of sperm concentration ranged between 541.8 and 2159 million$mL^{-1}$with mean of 1226.54±53.31 millionmL^{-1}. The concentration was found similar to Bedi *et al.* (1984) and Dhami and Sahni (1994) but was found higher than earlier reports (Bhakat *et al.,* 2011, Ghodasara *et al.,* 2016). The concentration was lower than report of Bhakat *et al.,* (2015). The spermatozoa viability in the present study was 82.70±0.64%. The result was similar to observation of Lone *et al.* (2016) and Ghodasara *et al.* (2016) but higher than Shivahre *et al.* (2015).

The mean value of spermatozoa viability (%) was 82.70±0.64 which ranged between 68 and 91%. The HOS test evaluates functional integrity of spermatozoa membrane (Jeyendran *et al.,* 1984). The HOS test positive spermatozoa population ranged from 60 to 86% with mean value of 73.03±0.86%. The similar observation was reported in earlier literature (Shivahre *et al.,* 2015). The lower and higher HOS test positive spermatozoa percent was reported by Bhakat *et al.* (2011) and Aguiar *et al.* (1994), respectively. The morphological abnormality percent was 5.73±0.33 with range of 2-12% which similar to result of Dhami and Sahni (1994) and lower than report of Bhakat *et al.* (2015).The intactness of acrosome is essential for capacitation, acrosome reaction and fertiliza (Rautela et al., 2020). The spermatozoa with intact acrosome ranged from 62-91% with mean value of 77.49±0.92%. Higher value was reported by Singh *et al.* (2014) and Shivahre *et al.* (2015). The observation similar to the present study was reported by Bhaket *et al.* (2015).

The mean value of TAC (µmol/mL) was 1.46±0.06 ranging between 0.6 to 2.55 at fresh stage, a value lower than that observed by Lone *et al.* (2018) (1.82±0.13). However, this result does not sync with the results of Kumar *et al.* (2011) (957±57.9 nmol/mL) and Kumar *et al.* (2016) (19.94±0.14 mmol/mL) due to variation in expression units and the procedure followed by different workers. The mean MDA concentration was 0.34±0.02 ranging between 0.02 to 0.75 µmol/mL/20×10^6 spermatozoa at fresh stage is dissimilar to the previous studies *viz*. Nair *et al.* (2006) (1.99±0.26 nmol MDA/10^8); Kadirvel *et al.* (2009) (226.4±24.3 nmol MDA/10^9 sperm); Kadirvel *et al.* (2014) (278.78±18.28 nmol MDA/10^9 sperm); Bansal and Cheema, (2016) (121.26 nmol/µg protein/mL); Lone *et al.* (2016) (254.42±2.85 nmol MDA/10^9 sperm); Lone *et al.* (2018) (255.46±15.83 nmol MDA/10^9 sperm) and Kandiel *et al.* (2017) (27.26±4.62 nmol/mL). The variation in values might be assigned to different samples, expression units and technique followed for estimation of MDA concentration.

Semen quality parameters at post-thaw stage

The number distribution of ejaculates in terms of PTM, viability, HOST and acrosome integrity at post-thaw stage has been depicted as Fig. 4 whereas, Fig. 5 represent the number distribution of ejaculates in terms of sperm morphological abnormalities (%) at fresh as well as post-thaw stage. The number and percent distribution of ejaculates in terms of post-thaw TAC and MDA concentration has been presented as Fig. 6a and 6b, respectively.

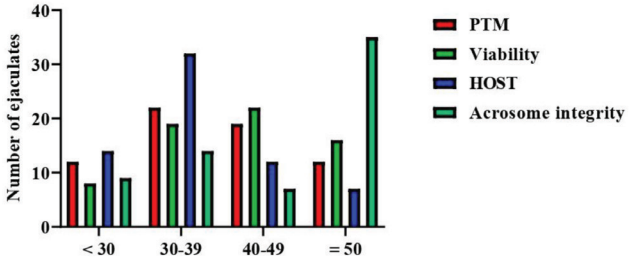

Fig. 4: Number distribution of ejaculates (n=65) in terms of PTM, Viability, HOST and Acrosome integrity (expressed in %)

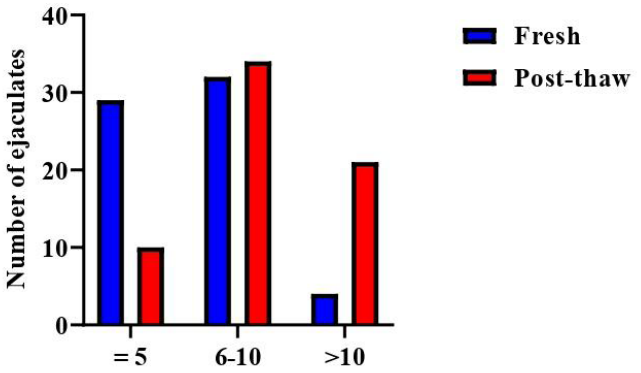

Fig. 5: Number distribution of ejaculates (n=65) in terms of sperm morphological abnormalities at fresh and post-thaw stage (expressed in %)

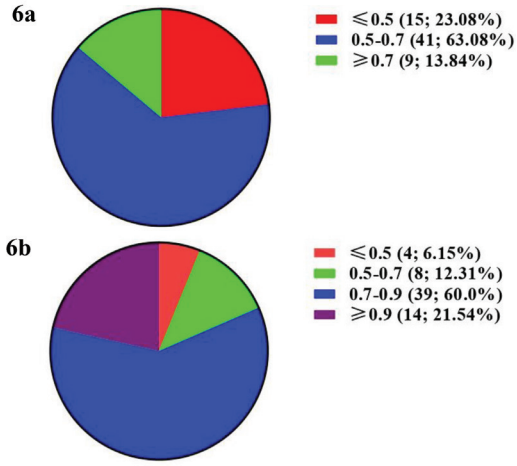

Fig. 6: Number and percent distribution of ejaculates (n=65) in terms of total antioxidant capacity (µmol/mL) (6a) and malondialdehyde concentration (µmol/mL/20 million spermatozoa) (6b) at post-thaw stage

The determination of post-thaw motility is important as it predicts the fertilizing ability of semen. In this study the mean value of post-thaw motility was 34.15±1.51% with range between 10 and 60%. The result was lower than the previous report (Ghodasara *et al.,* 2016) and was similar to Maurya and Tuli (2003) and Singh *et al.* (2013). The viability percent range from 8-65 with mean of 40.95±1.49% which was similar to the result of Sandeep *et al.* (2015). While higher post-thaw motility was observed by Lone *et al.* (2016) and Pathak *et al.* (2019) in Murrah buffalo.

The mean HOST positive spermatozoa population was 34.64±1.43% which ranged from 2-60%.The higher results were observed in previous reports (Pathak *et al.,* 2019, Lone *et al.,* 2016). The morphological abnormality ranged from 3-16% with value of 9.21±0.44%.The result was similar to report of Meena *et al.* (2010) but was lower as compared to result of Singh *et al.* (2013). The mean population acrosome intact spermatozoa were 48.04±1.94 which ranged between 18 and 72%.The result was similar to report of Sandeep *et al.* (2015). While, higher values were reported in earlier literature (Lone *et al.,* 2016, Meena *et al.,* 2010).

The mean value of TAC (µmol/mL) at post-thaw stage was 0.61±0.02 which significantly less (p<0.001) than at fresh stage which agreed with Kumar *et al.* (2011) and Lone *et al.* (2018). The low level of TAC at post-thaw was due to the fact that greater production of free radicals by dead and damaged spermatozoa through enzyme system led to consumptions of neutralizing antioxidants resulting in reduction in total antioxidant capacity of seminal plasma. The MDA (µmol/mL/20×10^6 spermatozoa) concentration at post-thaw stage was 0.79±0.03 ranging between 0.3-0.9 and 0.47-1.38. The mean value of MDA at post-thaw stage was found to be significantly greater (p<0.001) than fresh stage. The increase in lipid per-oxidation at post-thaw stage as compared to fresh is in agreement with earlier reports by Kadirvel *et al.* (2009; 2014); Bansal and Cheema (2016); Lone *et al.* (2016; 2018); Bisla *et al.* (2020b; 2021a; 2021b); Kumar *et al.* (2021; 2022) and Rautela *et al.* (2022) in buffalo semen. This might be due to that the reduction of antioxidant (enzyme & non-enzyme) level in buffalo semen during thawing and freezing (Kadirvel *et al.,* 2014, Kumar *et al.,* 2011) along with susceptibility of per-oxidation of frozen-thawed than fresh bull sperm (Trinchero *et al.,* 1990). Waheed *et al.* (2013) found that MDA was found to be negatively correlated with sperm motility which also supported the results of present study.

Discard rate at fresh and post-thaw stages

The semen discard rate at fresh stage obtained in the present study (17.72%; 14/79) found in agreement with Zafar *et al.* (1988) and Tiwari *et al.* (2011) but quite lesser than Singh *et al.* (2013). The reason for discard or rejection of ejaculates is been depicted in Table 1. The major reason for rejection at fresh stage was non-confirmation of the ejaculates with mass motility and IPM while only one ejaculate did not match the standard of the spermatozoa concentration.

As static vapour freezing was followed, out of 65 ejaculates processed only 31 (47.69%) were found to be having PTM ≥40% and thus were satisfactory to be passed for further AI programme *i.e.* 52.31% (34/65) ejaculates were rejected or discarded at post-thaw stage. The mean post-thaw motility (%) of the passed ejaculates was found to be 44.2±1.0. The reduced value of semen parameters at post-thaw as compared to fresh stage is due to freeze-thaw damage (Bansal and Bilaspuri, 2011) and ROS generation (Kadirvel *et al.,* 2014). The association of ROS produc-

tion, induced oxidative stress due to lipid peroxidation, reduction in antioxidant defense system and thereby ultimately impaired seminal attributes and fertility have been established in different species (Mavi *et al*., 2019a; 2019b; 2020a; 2020b; Bisla *et al*., 2020b; 2021a; 2021b; 2022; Ngou *et al*., 2020; Kumar *et al.,* 2021; 2022; Rautela *et al*., 2022). These damages are inevitable during cryopreservation, however quality improvement of semen can be achieved either by treatment with antioxidants (Ansari *et al.,* 2010), or employing techniques such as partial deoxygenation (Kumar *et al*., 2018), filtration (Maurya and Tuli 2003; Bisla., 2020a) and nano-purification (Bisla *et al*., 2020b; reviewed by Bisla *et al*., 2022).

CONCLUSIONS

The present study show efforts to minimize lipid per-oxidation and enhance TAC at various processing stages shall play a vital role in reducing discard rate of the semen samples in buffalo bulls. The results can be used as valuable information while processing of semen in semen banks.

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CONFLICT OF INTEREST

No conflict of interest was reported by the authors.

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