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Comparative Efficacy of Percoll^{TM} Discontinuous Density Gradient Centrifugation and Glass Wool Filtration Techniques for Spermatozoa Selection in Buffalo (*Bubalus bubalis*)

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ABSTRACT

Dead and damaged spermatozoa cells present universally in the ejaculates of all eutherian mammals exert toxic effect on contemporary healthy cells mostly through generation of excessive free radicals. This is much more evident during extended period of processing, resulting in poor ejaculate quality. The solution lies in depletion of dead/damaged spermatozoa from the neat ejaculates itself. Thus the objective of the study was to evaluate the efficiency of the protocols such as discontinuous Percoll^{TM} density gradient centrifugation (PDGC) and glass wool filtration (GWF) for depletion of dead/damaged spermatozoa from fresh semen in buffalo. Random ejaculates (n=6) of Murrah buffalo bulls were divided into two aliquots after quality assessment: PDGC and GWF protocols (Group I and II, respectively). At the end of the purification protocol, efficiency of the protocols in depleting dead/damaged spermatozoa as reflected by certain quality parameters were evaluated. The mean efficiency (%) of purification protocols based on recovery of spermatozoa was 44.68 and 40.02% for PDGC and GWF, respectively. Moreover significantly (p<0.05) greater values for quality parameters was observed in the Group II (26.4+6.8 *vs* 68.8+4.4 for acrosome integrity (%); 12.68+6.6 *vs* 57.7+7.5 for functional plasma membrane integrity (%); 20.3+5.8 *vs* 80.75+6.7 for viability (%) in Group I and II, respectively). It was concluded that GWF is a better technique than PGDC to filter out dead/damaged spermatozoa from fresh semen with improvement in semen quality and can be a valuable tool in assisted reproductive technology.

Keywords: Glass wool, Percoll^{TM}, Buffalo, Semen, Dead spermatozoa

Mammalian **s**emen is a heterogeneous mixture of live as well as dead spermatozoa. It has been reported that fresh buffalo ejaculates contain 25-30% dead and damaged spermatozoa (Maurya and Tuli, 2003; Mahmoud *et al*., 2013; Shivahre *et al*., 2015). This population of dead and damaged spermatozoa in the processed semen is responsible for the production of reactive oxygen species (ROS) leading to oxidative stress responsible for poor freezability and high discard rate (Roca *et al.,* 2013). The problem is exaggerated further in the buffalo semen due to high lipid per-oxidation rate, less activity of antioxidant system, high content of polyunsaturated fatty acids in the spermatozoa membrane and greater susceptibility to osmotic stress (Khan and Ijaz, 2008).

Thus, to counter the adverse effect investigators have relied on addition of antioxidants or techniques to deplete of the dead and damaged spermatozoa from the fresh

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semen itself. Such spermatozoa selection techniques with varying success in buffalo include filtration techniques *i.e.* Sephadex filtration, glass wool filtration, gradient separation and swim up and swim down technique. The Gradient separation technique is based upon the principle of low density and inability of the dead spermatozoa to pass through the colloid suspension of varying gradient during centrifugation (Oshio, 1988). Percoll^{TM} gradient, which is a colloid solution of 15-20 nm size consisting of silica beads coated with polyvinylpyrolidone, is most commonly used in gradient separation (Pertoft *et al.,* 1978). In glass wool filtration (GWF), the effect is provided by the changes in plasma membrane of dead and damaged spermatozoa followed by binding or agglomeration of dead and damaged spermatozoa with glass fibers (Morrell e*t al.,* 2009). Clusterin which is negative protein, secreted from the seminal plasma and epididymal fluid, is associated with abnormal or damaged spermatozoa, and binds with glass wool to filter out dead and damaged spermatozoa (Ibrahim *et al.*, 2001). Lee *et al.* (2009) observed that glass wool filtration was better technique than Percoll^{TM} discontinuous density gradient centrifugation (PDGC) for the separation of dead bovine spermatozoa. It has been observed that filtration as well as other spermatozoa selection techniques removes leucocytes as well as dead and damaged spermatozoa which are prime source of ROS in semen while selecting the morphologically normal, viable and acrosome intact spermatozoa (Anzar and Graham, 1996; Januskauskas *et al*., 2005). Studies regarding the comparative efficiency of GWF and PDGC of dead and damaged spermatozoa have been done in cattle and other species but the information is meager in buffalo. Therefore, the objective of the present study was to compare the efficiency of PDGC and GWF techniques as revealed by semen quality parameters in buffalo.

MATERIALS AND METHODS

Selection of the ejaculate

Six fresh semen ejaculates were collected randomly from the Murrah buffalo bulls maintained at the ICAR-Indian Veterinary Research Institute, Bareilly. The semen was collected by the artificial vagina (AV) method and after collection examined for the initial semen quality parameters. After the initial examination, the semen sample was divided into two equal parts *viz*., Group I (PDGC) and Group II (GWF).

Discontinuous Percoll^{TM} density gradient centrifugation (PDGC)

The PDGC was performed as per the method described by Srivastava *et al.* (2017) with slight modifications. Ham's F-10 media (Table 1), BSA supplemented media (Table 2), isotonic gradient media (Table 3) and 20 and 40% (v/v) gradient media (Table 4) were prepared as fresh as per the method. Briefly, 1 mL of 40% percoll^{TM} gradient solution was layered in a 15 ml centrifuge tube with layering of 1 mL 20% percoll^{TM} gradient solution over it. 1 mL of the fresh semen ejaculate was layered over it and centrifugation was done at 400 g for 15 min. The spermatozoa pellet obtained was dissolved in 5 mL of BSA supplemented media and again centrifugation was done at 200 g for 5 min twice. The resulting spermatozoa pellet was finally dispersed in the BSA supplemented media and examined for spermatozoa concentration, livability, abnormality, acrosome intactness and functional integrity of plasma membrane.

Table 1: Composition of Ham's F-10 medium for PDGC technique

Ingredient	Quantity
Sodium chloride	7.4 g
Sodium bicarbonate	1.2 g
Potassium chloride	0.285 g
Disodium monohydrogen phosphate	0.154 g
Magnesium sulphate heptahydrate	0.153 g
Potassium dihydrogen phosphate	0.083 g
Calcium chloride dihydrate	0.044 g
D-Glucose	1.1 g
Distilled water (DW)	750 mL
pH adjusted	7.4

Table 2: Composition of BSA Supplement medium for PDGC technique

Ingredient	Quantity
Bovine serum albumin (BSA)	300 mg
Sodium pyruvate	1.5 mg

Sodium lactate (60% v/v)	0.18 mL
Sodium bicarbonate	100 mg
Ham's F-10 medium	50 mL

Table 3: Composition of Isotonic density gradient medium

Ingredient	Quantity
Ham's F-10 medium	10 mL
Density gradient medium (Percoll^{TM})	90 mL
BSA	300 mg
Sodium pyruvate	3 mg
Sodium lactate (60% v/v)	0.37 mL
Sodium bicarbonate	200 mg

Table 4: Composition of 20% and 40% (v/v) density gradient medium

20% density gradient medium		40% density gradient medium	
Ingredient	**Quantity**	**Ingredient**	**Quantity**
BSA supplemented medium	40 mL	BSA supplemented medium	30 mL
Isotonic gradient medium	10 mL	Isotonic gradient medium	20 mL

Glass wool filtration (GWF)

The GWF was performed as per the method described by Engel *et al*. (2001) with some modifications. Brackett and Oliphant (BO) and BO supplemented media were prepared as fresh as per the method (Table 5).

Table 5: Media composition for GWF technique

Brackett and Oliphant (BO) medium		BO supplemented medium	
Ingredient	**Quantity**	**Ingredient**	**Quantity**
Sodium chloride	6.55 g	Caffeine	29.1 mg
Sodium bicarbonate	3.104 g	Heparin	300 µg
Potassium chloride	0.300 g	BSA	300 mg
Sodium dihydrogen phosphate	0.113 g	BO medium	30 mL
Magnesium chloride hexahydrate	0.106 g		
Sodium pyruvate	0.137 g		
Calcium chloride dihydrate	0.330 g		
D-Glucose	2.5 g		
Bovine serum albumin (BSA)	3.0 g		
Distilled water	To 1000 mL		

Briefly, a 5 mL capacity sterilized glass syringe was taken and filled with about 30 mg of glass wool up to 1 cm depth and multiple washing of the column were done with BO supplemented media to remove the excessive glass wool fibers coming outside the column. The assembly was maintained at 37 °C and 1 mL of 1:1 diluted fresh semen (diluted with tris buffer without egg yolk) was layered over the glass wool layer allowing filtration for 5 min. The obtained filtrate was washed with 6 mL BO supplemented media by centrifugation at 300 g for 5 min. The final washed filtrate was suspended in the BO supplemented media and examined for spermatozoa concentration, livability, abnormality, acrosome intactness and functional integrity of plasma membrane.

Semen quality parameters (SQPs)

The mass motility of the semen was assessed by putting a drop of undiluted semen on the grease free glass slide on the pre-warmed thermo-stage and was categorized from 0 to 5+ as per Salisbury *et al*. (1978). The spermatozoa concentration of fresh and after PDGC and GWF was determined by using Bovine photometer (Accucell, IMV technologies). The motility was recorded as percentage of progressively motile spermatozoa after the extension of small amount of fresh semen (100 µL semen with 200 µL of extender) with TYG extender and observed under high power objective (40x) of microscope fitted with thermostatically control stage after covering with a cover slip. The semen sample was extended so that approximately 15 to 20 spermatozoa were visible under the visual field of microscope (Motic, China). The individual progressive motility (IPM) was observed before and after PDGC and GWF procedure.

The spermatozoa viability and morphological abnormalities were assessed by eosin-nigrosin staining method as described by Evans and Maxwell, (1987). A drop of Eosin Nigrosin stain was taken on clean, grease free pre warmed glass slide to which one drop of semen was added, and mixed quickly but gently using a blunt fine glass rod. After 30 s to 1 min a thin smear was made on

the same slide from semen and stain mixer. The smear was air dried immediately and examined under oil immersion objective of Bright field microscope (Motic, China). A total of about 200 spermatozoa were counted in each slide and per cent value calculated. The stained and partially stained spermatozoa were considered as dead/ damaged. Percentage spermatozoa morphological abnormalities were determined using same staining procedure.

Plasma membrane integrity

The spermatozoa plasma membrane integrity was determined by hypo-osmotic spermatozoa swelling test (HOST) as described by Jeyendran *et al.* (1984). The osmolarity of hypo-osmotic solution was kept at 150 mOsm/L. 1 mL of hypo-osmotic solution was taken in a sugar tube, to which, 0.1 mL of diluted semen was added and incubated at 37 ºC in water bath. After incubation a drop of eosin-Y solution was added, small drop of the suspension from the bottom of the tube was placed on clean, grease free glass slide and covered with cover slip. A minimum of 200 spermatozoa were counted at 40X to record different types of tail swelling pattern.

Acrosome integrity

The spermatozoa acrosomal integrity was assessed by Giemsa staining of semen smears as described by Watson (1975). Briefly, a smear of diluted buffalo semen was prepared on a clean, grease free glass slide and air dried. The smear was then fixed in Hancock's fixative for 15 min. The fixed smear was then washed in slow running water for 15 min. After drying the smear was stained in Giemsa working solution for 90-120 min. Slides containing smears were then removed from the stain solution and rinsed quickly in distilled water and air dried. The smear was then examined under oil immersion objective of the microscope to assess acrosome integrity. At least 200 spermatozoa were counted for each slide for estimation of intact acrosome percentage. The acrosome manifesting marked swelling, knobbed, ruffled, or incomplete contour and denudation were recorded as abnormal.

Efficiency of PDGC and GWF

The efficiency of PDGC and GWF techniques was calculated in comparison to the values of various semen quality parameters (SQPs) before and after the PDGC and GWF separately as per the formula given below:

$$\text{Efficiency (\%)} = \frac{\text{SQP after treatment} - \text{SQP before treatment}}{\text{SQP before treatment}} \times 100$$

Statistical Analysis

The normality of data obtained from various SQPs was ascertained prior to statistical analysis. The obtained SQPs data and efficiency (%) was statistically using Graphpad Prism 8.1.2 (332) software via unpaired student's T-test.

RESULTS AND DISCUSSION

The seminal attributes of the randomly selected samples of the Murrah buffalo bulls have been presented in Table 6.

Table 6: Seminal attributes of Murrah buffalo semen (n=6)

Sl. No.	Semen Quality Parameters	Value
1	Mass motility	3.17±0.31
2	Individual progressive motility (%)	61.67±4.77
3	Spermatozoa concentration (million/mL)	1013.1±197.3
4	Viability (%)	87.37±4.17
5	Morphological abnormalities (%)	3.29±0.98
6	Plasma membrane integrity (%)	58.01±6.56
7	Acrosome integrity (%)	61.79±6.40

The mass motility of ejaculates in the present study was almost similar to Alavi-Shoushtari *et al*., 2009 (3.59±0.16) and Singh *et al*., 2013 (3.31±0.17 to 3.52±0.13) but greater than Bhakat *et al*., 2011 (2.88±0.02); Bhakat *et al*., 2015 (2.54±0.70); Shivhare *et al*., 2015 (2.8±0.14) and Henry *et al*., 2017 (2.3±1.3). The variability in the mass motility of semen could arise due to semen quality, season and microscopic examination technique of the individual observer (Bhakat *et al*., 2015). Mass activity of the ejaculates is also affected by spermatozoa concentration and ability of spermatozoa dislocation (Henry *et al*., 2017).

IPM obtained in the present study was comparable to Maurya and Tuli, 2003 (60.75±4.96); Bhakat *et al*., 2015 (60.64±0.02); Shivhare *et al*., 2015 (63.8±2.16) and Hoque *et al*., 2018 (64.41±14.91) but lesser than those reported

by Kadirvel *et al*., 2009 (85.3±2.5), Lone *et al*., 2016 (88.25±0.36) and Lone *et al*., 2018 (85.27±5.25). The results of IPM are subjective and there might be difference in observation between the investigators. The procedure of semen examination, expertise of the evaluator and type of instrument used could lead to variability in findings (Bhakat *et al*., 2015).

Spermatozoa concentration obtained in the present study was similar to Mahmoud *et al*., 2013 (1079.2±21.0); Waheed *et al*., 2013 (1070±0.06); Bhakat *et al*., 2015 (1016.68±21.25) and Kumar *et al*., 2016 (1053.53±48.58) but lesser than the values obtained by Alavi-Shoushtari *et al*., 2009 (1377.14±61.22); Shivhare *et al*., 2015 (1749.7±122.4) and Hoque *et al*., 2018 (1374.31±611.29). Spermatozoa viability obtained in the present study were almost similar to Alavi-Shoushtari *et al*., 2009 (89.68±0.94); Kadirvel *et al*., 2014 (88.43±0.70) and Lone *et al*., 2018 (89.45±4.7) but greater than Maurya and Tuli, 2003 (70.23±2.78); Nair *et al*., 2006 (69.65±0.28); Bhakat *et al*., 2011 (67.2±0.03); Mahmoud *et al*., 2013 (70.9±0.7) and Shivhare *et al*., 2015 (77.3±2.48). The variation in the spermatozoa concentration and viability could be due to bull, age of animal, frequency of collection, false mounting before the collection, housing and feeding management as well as seasonal variation (Bhakat *et al*., 2015; Hoque *et al*., 2018).

The spermatozoa morphological abnormalities (%) obtained in the present study were lesser than Maurya and Tuli, 2003 (19.08±2.04); Alavi-Shoushtari *et al*., 2009 (6.53±4.07); and Shivhare *et al*., 2015 (6.2±0.51); Bhakat *et al*., 2015 (9.47±0.002) and Henry *et al*., 2017 (8.9±0.9). The high percentage of spermatozoa abnormalities in buffalo were found to be associated with high inbreeding of herds (Vale *et al*., 2008) instead of age as in other species (Saeed *et al*., 1990; Zorzetto *et al*., 2016).

The plasma membrane integrity of spermatozoa in the current study was similar to Mandal *et al*., 2003 (58.3±0.02) but lesser than Alavi-Shoushtari *et al*., 2009 (74.12); Kadirvel *et al*., 2014 (74.4±0.8); Shivhare *et al*., 2015 (75.1±1.87); Lone *et al*., 2016 (77.37±0.64) and Lone *et al*., 2018 (77.37±5.38). The acrosome integrity of spermatozoa in the current study was lesser than results of studies of Maurya and Tuli, 2003 (14.85±1.1 damaged acrosome); Alavi-Shoushtari *et al*., 2009 (17.35 % damaged acrosome); Kadirvel *et al*., 2014 (90.7±1.2); Lone *et al*., 2016 (86.95±0.36) and Lone *et al*., 2018 (88.01±3.20). The variability in plasma membrane and acrosome integrity might be due to season (Kale *et al*., 2000), bull, mass activity, progressive motility, spermatozoa count, total spermatozoa with intact acrosome (Prasad *et al*., 1999) and individual fertility level (Jeyendran *et al*., 1984).

The objective of the present investigation was to compare the efficiency of the PDGC and GWF protocols for removal of dead and damaged spermatozoa from buffalo semen. The comparative efficiency (%) of two protocols in terms of total and live spermatozoa recovery has been presented in Fig. 1.

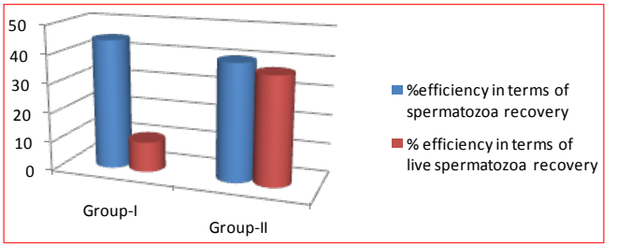

Fig. 1: The efficiency (%) PDGC and GWF protocols in terms of total and live spermatozoa recovery (n=6)

The mean efficiency (%) of the two protocols (Table 7) in terms of total spermatozoa recovery vary non-significantly (p>0.05).

Table 7: The total spermatozoa concentration (million/mL) and percent recovery of spermatozoa (n=6) following PDGC and GWF techniques

Spermatozoa conc. in fresh semen	Group I (PDGC)		Group II (GWF)	
	Spermatozoa Con.	% recovery	Spermatozoa Con.	% recovery
1013.13 ± 197.31[a]	452.70 ± 90.52[b]	44.68	405.47 ± 104.22[b]	40.02

Con., Concentration; Values bearing superscripts a and b differ significantly (p<0.05).

The semen quality parameters obtained after PDGC and GWF have been presented in Table 8. The obtained SQPs are similar to observations of Sherman *et al*. (1981) and Jeyendran *et al*. (1984) suggesting caution because of deleterious effects of glass wool on human spermatozoa. In contrast, Husna *et al*. (2016) observed that post-thaw

quality of the buffalo spermatozoa improved after GWF but had no positive effect on the fertility. Panghal *et al*. (2002) reported effectiveness of GWF in removal of defective spermatozoa from the semen of Murrah buffalo. Filtration through glass wool has been shown to improve semen quality in cattle (Vyas *et al*., 1992; Anzar and Graham, 1996; Mustafa *et al*., 1998).

Table 8: The spermatozoa quality parameters after application of PDGC and GWF techniques (n=6)

Sl. No.	Parameters	Group I	Group II
1	Spermatozoa viability (%)	20.31 ± 5.80^{A}	80.75 ± 6.73^{B}
2	Spermatozoa abnormality (%)	2.78±1.13	6.20±1.14
3	Plasma membrane integrity (%)	12.68 ± 4.63^{x}	57.74 ± 7.54^{y}
4	Acrosome integrity (%)	26.35 ± 6.84^{a}	68.77 ± 4.35^{b}

Values bearing superscripts a and b differ significantly ($p<0.05$); Values bearing superscripts x and y differ significantly ($p<0.01$); Values bearing superscripts A and B differ significantly ($p<0.001$).

In the present investigation, spermatozoa viability ($p<0.001$), plasma membrane integrity ($p<0.01$) and acrosome integrity ($p<0.05$) obtained after GWF were significantly greater than PDGC. In agreement, Van den bergh *et al*. (1997) obtained better spermatozoa recovery with greater intact morphology and less contamination of selected spermatozoa with round cells and dead spermatozoa in the GWF than the PDGC.

Several authors reported selection of spermatozoa by filtration through a Sephadex column (Januskauskas *et al*., 2005) and separation by PDGC (Saeki *et al*., 1991) permitted improvements in the quality of bovine semen. However, in cases of poor semen quality (Johnson *et al*., 1996), high viscosity (Sakkas *et al*., 2003), or cryopreserved ejaculates (Coetzee *et al*., 1994), the technique of filtration through GWF proved to be comparatively advantageous (Engel *et al*., 2001). Percoll™ samples have been termed as "dirty" due to presence of more debris, round cells and dead spermatozoa (Paulson and Polakoski, 1997). Vyas *et al*. (1992) observed that the quality of bovine semen could be improved after glass wool filtration in terms of motility, membrane integrity and a fewer abnormalities. However, free polyvinylpyrrolidone with detrimental action on the plasma membrane as well as on acrosome and mitochondrial membranes (Avery and Greve, 1995; Strehler *et al*., 1998) might be a major concern using polyvinylpyrrolidone based Percoll™ for semen purification.

CONCLUSION

The study results show that GWF is a better technique than PGDC to filter out dead/damaged spermatozoa from fresh semen with improvement in semen quality and therefore can be preferred in techniques involving assisted reproduction in buffaloes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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