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A comparative analysis of sperm selection procedures prior to cryopreservation for Nili-Ravi buffalo bull (*Bubalus bubalis*) semen-: Assessment of its impact on post-thaw sperm functional quality

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ABSTRACT

Sperm selection techniques have been developed to get sperm suspensions enriched in motile and functional cells. Studies show that selection before cryopreservation improves post-thaw quality of cryopreserved sperm but information on buffalo bull sperm is scarce. The study was aimed to 1) perform a comparative analysis of sperm selection procedures; Swim-Up (SU), Sephadex™-G15 Filtration (S-G15) or Glass Wool Filtration (GWF) for total and motile cell recovery, 2) to assess the impact of sperm selection prior to cryopreservation on sperm quality (motility, morphology, cell membrane and normal apical ridge, viability and livability, chromatin integrity) and sperm functionality (Embryo Cleavage after IVF with selected sperm) in post-thawed suspensions of buffalo bull sperm. Semen was collected from 5 Nili Ravi buffalo bulls maintained at the Semen Production Unit Qadirabad, District Sahiwal, Pakistan. Ejaculates were divided into four aliquots for SU, S-G15 and GWF and control. After sperm selection, total and motile sperm recovery was highest in GWF samples (total sperm = 84.08 ± 8.39%; motile sperm = 80.42 ± 3.57%). An improvement ($P < 0.05$) in all post-thaw parameters was observed in S-G15-selected sperm and, in some parameters in GWF-filtered sperm suspensions compared to control. The highest ($P < 0.05$) embryo cleavage rate (%) was achieved with frozen-thawed sperm selected with S-G15 prior to cryopreservation (44.72 ± 4.18) compared to control (21.98 ± 3.00). In conclusion, post thaw sperm quality was improved after sperm selection from fresh buffalo bull semen through S-G15 and GWF procedures compared to SU and control while, the fertility rate (cleavage rate) was improved with sperm processed using the S-G15 procedure.

1. Introduction

Sperm selection mechanisms occur throughout nature, some of which depend on self-selective sperm qualities,

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while others involve a selection imposed by the female reproductive tract (Holt and Look, 2004). Sperm parameters involved in this selection are motility, morphology, chromatin integrity, ability to undergo capacitation and acrosome reaction (Henkel, 2012). Based on such selection, about 10% of the total ejaculated spermatozoa enter the cervix, 1% the uterus and 0.1% the Fallopian tube (Henkel, 2012). However, for artificial insemination programs, pre-freeze sperm selection is solely based on sperm motility and concentration in the ejaculate that still contains dead and abnormal sperm cells, an inevitable source of reactive oxygen species (ROS), which destabilize the sperm membrane through lipid peroxidation. Such oxidative stress is detrimental to sperm viability, plasma membrane, acrosomal (Hallap et al., 2004; Suarez, 2007) and DNA (Chatterjee et al., 2001) integrity, which ultimately impairs sperm fertilizing ability (Said et al., 2005). Therefore, it has become imperative to remove deformed/damaged and dead spermatozoa, the additional source of ROS before processing of semen ejaculate for cryopreservation. It is relevant to mention that removal of these ROS sources resulted in improved post-thaw quality and fertility in bovine (Lee et al., 2009; Arzondo et al., 2012).

Based on natural sperm selection in the female reproductive tract, several biomimetic techniques have been developed that select good quality spermatozoa and reduce the number of dead, moribund and weakly motile sperm from the cell suspension. Among these procedures, the swim up method selects spermatozoa based on sperm motility and morphology, while, filtration is based on the ability of the sperm to move in addition to their interaction with the filter substrates, among which are glass fibers, Sephadex™ beads or membrane pores (Mogas et al., 1998). It is considered that non- viable sperm tend to adhere to the matrix more than motile cells. Sephadex™ either allows immotile and dead sperm to agglomerate because of changes in surface charges (Ahmad et al., 2003), or a protein present on damaged spermatozoa binds to the Sephadex™ particles (Samper et al., 1995). Similarly, glass wool filtration separates dead sperm cells that adhere to the glass and selects motile spermatozoa due to their movement through glass wool fibers (Henkel and Schill, 2003). Some studies have reported that filtration eliminates leukocytes and dead or dying sperm that are the source of ROS and selects morphologically normal (Anzar et al., 1997), viable, and acrosome-intact sperm (Januskauskas et al., 2005).

Selection of functionally intact spermatozoa from a fresh ejaculate has been successfully demonstrated in cattle before processing for cryopreservation using glass wool filtration (Arzondo et al., 2012), swim up (Somfai et al., 2002) and Sephadex™ filtration (Januskauskas et al., 2005). To our knowledge, information about the efficiency of aforementioned methods to select motile sperm from buffalo semen, and simultaneous impact of these procedures on sperm functional status after freeze-thawing is lacking. The present study aimed to evaluate the effect of sperm selection procedures (Swim Up, Sephadex-G15 Filtration and Glass Wool Filtration) on efficacy of total/motile sperm recovery, and to select functionally intact spermatozoa of Nili-Ravi buffalo fresh semen ejaculate. The study also assessed the impact of sperm selection procedures prior to cryopreservation on sperm quality parameters, viz; motility, plasma membrane integrity, normal apical ridge, live/dead ratio, viability (live sperm with intact acrosome), chromatin integrity and *in vitro* fertilizing capacity of frozen-thawed semen.

2. Materials and methods

2.1. Experimental design

Semen was collected from five mature Nili Ravi buffalo bulls maintained at the Semen Production Unit, Qadirabad District Sahiwal, Pakistan. Two consecutive ejaculates per week were collected from five buffalo bulls with an artificial vagina (42° C) for a period of three weeks (three replicate; six ejaculates per bull; total number of ejaculate; thirty). At least one ejaculate from each bull met the criteria of (volume >1 mL, sperm concentration >1.5 billion/mL, motility >60%, abnormalities<20%) for further processing in experiment. The qualifying ejaculates were divided into four aliquots; the first aliquot was untreated (control), the other aliquots were subjected to the swim-up (SU), Sephadex™ filtration (S-G15) and glass wool filtration (GWF) sperm selection methods.

2.2. Methods

2.2.1. Extender preparation

All sperm suspensions were diluted in Tris(pH 7.0)-Citric Acid Buffer Extender prepared as follows: 3.0 g Tris-(hydroxymethyl)-aminomethane and 1.56 g Citric Acid, dissolved in 73 mL distilled water, and supplemented with 0.2% Fructose, 7% glycerol, and 20 mL Egg Yolk (osmotic pressure 320 mOsmol/kg). Antibiotics (1000 μg/mL streptomycin sulphate and 1000 IU/mL benzyl penicillin) were added to the buffer extender.

2.2.2. Sperm selection procedures

2.2.2.1. Swim up method (SU). The swim up selection method was performed in sperm TALP (tyrode albumin lactate pyruvate) medium (pH 7.3-7.4) pre-warmed at 37 °C for one hour prior to use. About 250 μL of fresh semen sample was deposited at the bottom of four 15 mL tubes containing 3 mL calcium-free medium. Tubes were incubated at a 45° angle for 30 min. During this time period, motile sperm moved to the upper layer. The upper layer from each tube was removed and transferred into another 15 mL-Falcon tube and centrifuged at 1600 rpm for 10 min. The sperm pellet was re-suspended in Tris/Citric Acid buffer Extender. Sperm concentration and motility were assessed before and after the selection procedures and recovery rate was estimated.

2.2.2.2. Sephadex gel filtration (S-G15). Tris-Citric Acid buffer extender without egg yolk supplement was used to prepare 20% slurries of Sephadex™ G-15. Sephadex™ particles were allowed to swell overnight at 4° C for column preparation. The filtration column was prepared in a 10 mL-disposable plastic syringe. A hole was drilled at 8 mL level of the syringe barrel to allow removal of air trapped in the barrel. A small amount of glass wool was compressed

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with the plunger to the bottom of the barrel to prevent loss of the Sephadex™. Plastic tubing was attached to the tip of the syringe and clamped. The free end was inserted into the filtrate collection tube. The extended semen was gently layered on to the column with a 6 mL syringe fitted with a 20-guage needle and filtered at a rate of 1.5 mL/ min. The extended semen was filtered through the column at room temperature (28–30° C). Sperm concentration and motility was assessed before and after the selection procedure and recovery rate was estimated.

2.2.2.3. Glass wool filtration (GWF). A glass wool column was prepared by gently inserting 30 mg of glass wool (microfiber code 112; John Manville, USA) into the barrel of a 3 mL syringe which was compressed to a final thickness of 3 mm. Before GWF, samples were diluted with two volumes of sperm washing media (SWM: Modified Ca^{+2} free Sperm-TALP: Mustafa et al., 1998) and mixed by gently pipetting up and down. The column was washed and pre-equilibrated with SWM until the filtrate observed under microscope was free of glass wool fibers or no change was noted between washes. The diluted sperm suspension was centrifuged for 6 min at 300*g* and re-suspended in 2 mL of SWM. The washed sperm suspension was then placed gently on top of the glass wool column and allowed to filter by gravity with the first three drops discarded and remaining filtrate collected. Sperm concentration and motility were assessed before and after the selection procedures and recovery rate was estimated.

2.2.3. Assessment of sperm recovery after sperm selection procedures

For all procedures, total and motile sperm recovery rates were estimated as follows.

$$\text{Total sperm recovery rate (\%)} = \frac{\left(\text{Sperm concentration } \left(\times 10^6/\text{ML}\right) \times \text{volume (mL)}\right) \text{ after selection}}{\left(\text{Sperm concentration } \left(\times 10^6/\text{ML}\right) \times \text{volume (mL)}\right) \text{ before selection}} \times 100$$

$$\text{Total motile sperm recovery rate (\%)} = \frac{\left(\text{Sperm concentration } \left(\times 10^6/mL\right) \times \text{progressive motile sperms (\%)} \times \text{volume (mL)}\right) \text{ after selection}}{\left(\text{Sperm concentration } \left(\times 10^6/mL\right) \times \text{progressive motile sperms (\%)} \times \text{volume (mL)}\right) \text{ before selection}} \times 100$$

2.2.4. Sperm freezing and thawing procedures

Diluted suspensions were cryopreserved and thawed using standard techniques as previously reported by Qadeer et al. (2014). Briefly, sperm suspensions were adjusted to a final concentration of 60×10^6/mL spermatozoa using Tris-Citric Acid extender at 37° C. The sample was cooled to 4° C and equilibrated at 4° C for 4 hrperiod and filled in 0.5 mL French straws with a suction pump at 4° C in a cold cabinet unit. The straws were kept on liquid nitrogen vapors for 10 min, plunged, stored in liquid nitrogen and transported to the Buffalo Research Institute (Pattoki, Kasur, Pakistan), where sperm post-thaw quality was evaluated. After 24 h, straws were thawed in a water bath at 37° C for 30 s and incubated in a water bath for assessment of post-thaw semen quality.

2.2.5. Post-thaw semen analysis

Sperm progressive motility was evaluated with phase contrast microscopy (400× magnification). Plasma membrane integrity was evaluated by means of the Hypo-osmotic swelling (HOS) assay, as previously described (Jeyendran et al., 1984). The HOS solution was prepared by adding 0.73 g sodium citrate and 1.35 g fructose to 100 mL distilled water (osmotic pressure ~190 mOsmol/kg). To assess sperm plasma membrane integrity, 50 μL of the sperm suspension was mixed with 500 μL of HOS solution and incubated for 30–40 min at 37° C. After incubation, an aliquot (10 μL) of the solution was placed on a warm slide and a droplet (10 μL) of eosin (0.5% (w/v) sodium citrate 2.92%) was mixed for 10 s. A coverslip was placed on a mixture and evaluated with phase contrast microscopy (400× magnification). For each sperm suspension under analysis, a total of 200 spermatozoa were observed in at least 5 different fields. Clear heads, tails and swollen tails indicating intact, biochemically active sperm membrane, while pink heads, tails and un-swollen tails indicating disrupted, inactive sperm membranes were noted (Tartaglione and Ritta, 2004). Sperm viability and livability were evaluated by means of a dual staining procedure, as previously described (Kovac and Foote, 1992). Briefly, an equal volume of Trypan-blue and semen were placed on a clean slide at room temperature and mixed. Smears were air-dried and fixed with formaldehyde-neutral red for 5 min. Slides were rinsed with distilled water and Giemsa stain (7.5%) was applied for 4 h. Slides were rinsed, air dried and mounted with mounting media. Two hundred sperm were evaluated in each smear by phase contrast microscopy at 1000× magnification and results were expressed as percentage of viable spermatozoa of total cells evaluated. Unstained spermatozoa and purple acrosome were considered viable with an intact acrosome, while sperm with blue staining were considered dead. Sperm viability was expressed as the percentage of live sperm with intact acrosome and sperm livability as the ratio of live/dead sperm expressed as a percentage. Sperm DNA damage was studied by DNA fragmentation test as previously described (Mello, 1982). Sperm smears were air dried and fixed in 96% ethanol-acetone (1:1) at 4 °C for 30 min. Acid hydrolysis with 4N HCl was carried out at 25 °C for 10–30 min. The smears were then rapidly rinsed in distilled water three times for every two minutes. The preparations were stained with toluidine blue for 10 min. Smears were evaluated under a light microscope at 1000× magnification. At least 200 spermatozoa were assessed in each sample and results

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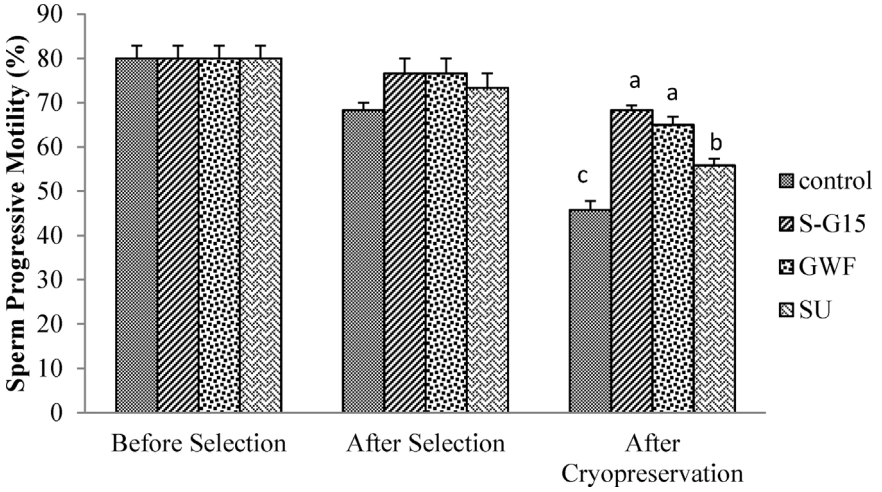

Fig. 1. Effect of different sperm selection techniques on sperm progressive motility (Mean ± SE) at different stages of cryopreservation, before and after filtration and after freezing of buffalo bull spermatozoa. The control bars indicate before and after dilution and freezing. Bars showing different letters differ significantly ($P < 0.05$) Before Selection: Aliquote's Sperm Progressive motility. After Selection: Control; dilution, S-G15; dilution + selection, GWF; dilution + selection, SU; dilution + selection. After Cryopreservation: Post-thaw sperm progressive motility.

were expressed as percentage of spermatozoa with functional DNA of total cells evaluated. The evaluation was done based on the sperm staining pattern, as follows: lightly stained spermatozoa were considered as having functional DNA, while cells depicting a dark stain were considered to have a non-functional DNA. Sperm morphology was evaluated by formaldehyde-citrate solution (2.9 g Tri-Sodium Citrate dihydrate and 1 mL of 37% solution of formaldehyde in 99 mL distilled water). Briefly, 100 μL of sample was mixed with 500 μL of formaldehyde-citrate solution and two hundred sperm were evaluated under a phase contrast microscope at 1000× magnification using oil immersion (Andrabi et al., 2008). The number of sperm abnormalities of the head, midpiece and tail were scored. In addition, the normal sperm apical ridge was evaluated in these samples. Sperm with an intact acrosome and a clear marked head were considered to have a normal apical ridge, while blunt ended cells were considered to have a disrupted apical ridge.

2.2.6. Assessment of sperm performance after sperm selection procedures through in-vitro fertilization (IVF)

The IVF procedure was done as previously reported (Mehmood et al., 2007). Briefly, Buffalo (*Bubalus babalis*) ovaries were obtained from a local abattoir immediately after slaughter and transferred to the laboratory in 2–3 h after slaughter in a thermos containing sterilized pre-warmed phosphate buffer saline (PBS; pH 7.0) After arrival, ovaries were rinsed immediately in fresh PBS. Oocytes were aspirated from follicles (2–8 mm) using an 18-gauge needle and cumulus-oocyte complexes (COCs) were selected based on the presence of multilayered compact cumulus cells and homogeneous ooplasm. Selected COCs were rinsed twice in PBS and once in maturation media. Ten to fifteen COCs were matured in 100-μL droplets of maturation medium (TCM 199 containing 10% FCS, 0.5 μg/mL FSH, 0.5 μg/mL LH and 1 μg/mL β-estradiol) under mineral oil and kept in CO_2 incubator at 38.5 °C for 24 h.

Selected frozen thawed sperm were used for IVF. For this purpose, three straws from SU, S-G15, GWF processed sperm and control were thawed in water bath at 37 °C for 30 s. Spermatozoa with maximum motility were collected by swim up technique (Parrish et al., 1986). Briefly, 250 μL of thawed semen was deposited at the bottom of each tube containing 3 mL of pre warmed sperm wash medium (TALP: modified calcium-free Tyrode's Albumin Lactate Pyruvate with 6 mg/mL BSA fraction-V). Tubes were incubated at an angle of 45° for 30 min. Supernatant from each tube was removed, transferred into another tube and centrifuged at 1600 rpm for 10 min. The pellet obtained after centrifugation was assessed for sperm motility and concentration. Concentration was determined using improved Neubauer counting chamber. Subsequently, sperm pellet was resuspended in pre-warmed fertilization TALP supplemented with 0.1 mM hypotaurine, 0.2 mM penicillamine, 0.01 mM epinephrine and 10 μg/mL heparin to get a final concentration of 2×10^6 live sperm mL^{-1}.

After 24 h of maturation, buffalo oocytes were washed in fertilization media and were placed in fertilization droplet (5 COCs/50 μL droplet) of pre warmed fertilization media under mineral oil with final sperm concentration of 2×10^6 mL^{-1}. The oocyte and spermatozoa were co-incubated at 38.5 °C under 5% CO_2 with maximum humidity for 20 h (Gasparrini et al., 2008).

At 20 h post-fertilization, the sets of 20 presumptive zygotes were washed with TCM 199 containing FCS and cultured in 100-μL droplets of TCM 199 containing 10% FCS at 38.5 °C and 5% CO_2 in air. During culture, the fertilization and embryo developmental rates were assessed in terms of cleavage rates of early embryos at 48 h post-insemination.

2.2.7. Statistical analysis

All semen parameters and additional characteristics evaluated throughout the study were expressed as mean ± standard error of the mean (SEM). Data on the effect of pre-freezing selection methods on post-thaw

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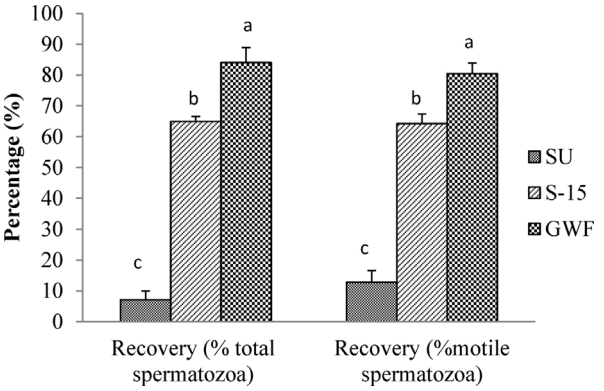

Fig. 2. Effect of different sperm selection techniques on total and motile sperm recovery rates (Mean ± SE) of buffalo bull spermatozoa after filtration. Bars showing different letters differ significantly (P < 0.05).

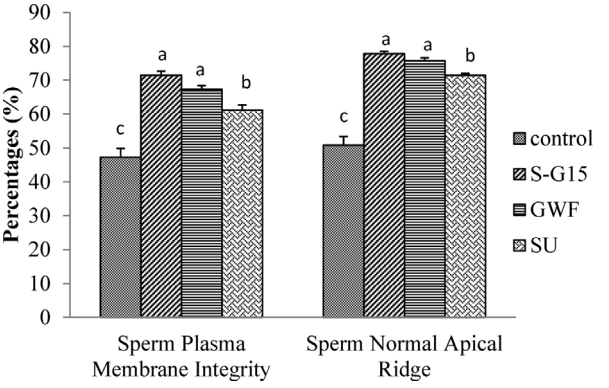

Fig. 3. Effect of different sperm selection techniques on post thaw sperm plasma membrane integrity and normal apical ridge (Mean ± SE) in buffalo bull spermatozoa. Bars showing different letters differ significantly (P < 0.05).

sperm functional parameters were analyzed by means of the ANOVA and least significant difference test was used to compare the treatment means. The data on cleavage rate were analyzed using a Chi-square test. Differences between groups were considered statistically significant at $P < 0.05$. The SPSS-20 Software was used for analysis.

3. Results

3.1. Sperm recovery after selection procedures

Buffalo bull semen samples when subjected to the selection procedures, sperm concentration (million spermatozoa/mL) and motility (%) were comparable in sperm selected through sephadex (824.5 ± 53.7; 76.67 ± 3.33), GWF (1175.86 ± 11.32; 76.67 ± 3.33), SU (927 ± 196.71; 73.33 ± 3.33) and control (866.2 ± 362.8; 68.33 ± 2.0) (Fig. 1). As a result of these studies, a higher ($P < 0.05$) recovery of total and motile spermatozoa was obtained in the order; GWF > S-G15 > SU (Fig. 2).

In addition to this analysis, Sperm progressive motility (mean%) was analyzed before and after selection and after cryopreservation (Fig. 1). Sperm progressive motility did not differ (P > 0.05) among treatments before and after selection; however, after freeze- thawing, sperm motility was significantly improved ($P < 0.05$) in sperm selected by different procedures compared to the control, in the order: S-G15 = GWF > SU > control.

3.2. Post-thaw sperm parameters

The effect of different sperm separation procedures on post-thaw percent progressive motility, plasma membrane integrity and normal apical ridge of buffalo bull spermatozoa are presented in Figs. 1 and 3. Post-thaw sperm progressive motility was higher ($P < 0.05$) in S-G15 (68.33 ± 1.05) and GWF (65.83 ± 1.54) than in SU (55.83 ± 1.53) and control (45.8 ± 2.01). A similar pattern was found for sperm plasma membrane integrity and normal apical ridge (the percentage of cells depicting sperm acrosome integrity). Post-thaw sperm plasma membrane integrity was higher ($P < 0.05$) in S-G15 (71.5 ± 1.17) and GWF (67.33 ± 1.08) than in SU (61.16 ± 1.47) and control (47.3 ± 2.56). Similarly, post-thaw sperm normal apical ridge were higher ($P < 0.05$) in S-G15 (77.83 ± 0.65) and GWF (75.67 ± 0.96) than in SU (71.50 ± 0.56) and control (50.83 ± 2.56).

The percentage of sperm viability was found higher ($P < 0.05$) in spermatozoa selected by means of the S-G15 (66.5 ± 1.40), GWF (61.17 ± 3.73) and SU (60.17 ± 2.08) compared to the controls (46.0 ± 1.46) (Fig. 4). Percent sperm chromatin integrity showed similar trend using S-G15 (98.5 ± 0.22), GWF (98.0 ± 0.25) and SU (98.0 ± 0.36) filtration procedure prior to sperm freezing, however these values were higher (P < 0.05) compared to the control (96.7 ± 0.42) (Fig. 4). Sperm livability (%) was higher ($p < 0.05$) in samples processed using S-G15 (86.66 ± 2.71) and GWF (80.16 ± 3.69) compared to SU (76 ± 2.60) and control (67.5 ± 1.70) (Fig. 4). Post-thaw percentage of total abnormal sperm, head and mid-piece abnormalities were found significantly lower ($p < 0.05$) in sperm processed through S-G15, GWF and SU compared to control whereas tail abnormalities did not differ ($p > 0.05$) in SU, S-G15, GWF and control, (Fig. 5).

A higher ($P < 0.05$) cleavage rate (%) was found in embryos generated after oocyte insemination with spermatozoa from samples selected after S-G15 (44.72 ± 4.18) than GWF (28.97 ± 4.07), SU (21.33 ± 1.94) and control (21.98 ± 3.00). The cleavage rate observed in embryos from spermatozoa selected by GWF and SU samples prior to cryopreservation were comparable to control ($P > 0.05$).

4. Discussion

Sperm passing through the female reproductive tract are subjected to several selection mechanisms (Suarez, 2007) and ultimately, morphologically normal sperm with progressive motility and normal DNA are selected for successful fertilization (Henkel, 2012). However, when ejaculates are selected for cryopreservation using routine procedures, dead/abnormal spermatozoa are also cryopreserved along with normal sperm that are inevitable source of the lipid peroxidation and reactive oxygen species (ROS) production (Shamsi et al., 2008; Zini and Sigman, 2009;

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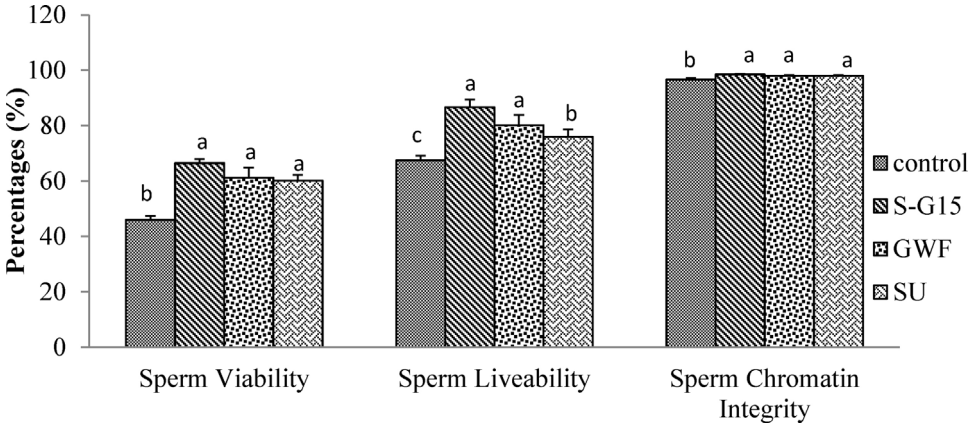

Fig. 4. Effect of different sperm selection techniques on post thaw sperm viability, livability and chromatin integrity (Mean ± SE) in buffalo bull spermatozoa. Bars showing different letters differ significantly (P < 0.05).

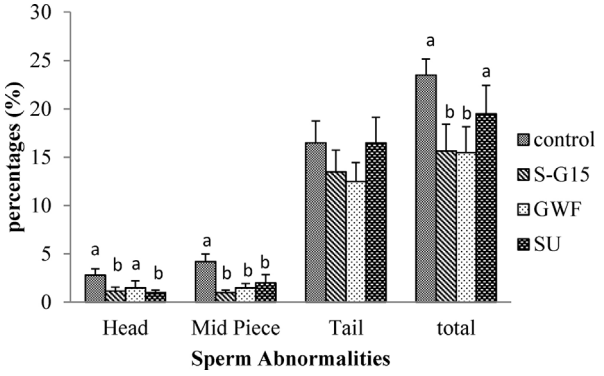

Fig. 5. Effect of different sperm selection techniques on post thaw sperm abnormality (Mean ± SE) in buffalo bull spermatozoa. Bars showing different letters differ significantly (P < 0.05).

Agarwal and Sekhon, 2010). Since, oxygen radicals are readily exported from the sperm cells, they can disrupt the plasma membrane of normal companion cells (Aitken, 1989). Removal of such detrimental sperm population from the ejaculates through sephadex and glass wool filtration has resulted in improved semen quality in cattle (Ajeet et al., 2003; Januskauskas et al., 2005) and Murrah buffalo bulls (Panghal et al., 2002; Maurya and Tuli, 2003). Relevant to the Swim Up technique, higher expression of the mRNA encoding the P450 aromatase (a molecular marker of sperm motility in other species) has been reported in motile sperm compared to non-motile sperm in buffalo (Tiwari et al., 2008).

The concentration of motile sperm after selection procedures correlates well with fertilization rate (Horvath et al., 1989; Rurangwa et al., 2004). In the present study, sperm selection by GWF was found better in terms of recovery of total and motile sperm, followed by Sephadex™ (S-G15) filtration compared to SU. The high recovery of motile sperm after GWF and Sephadex filtration is the reflection of effective trapping of dead, abnormal and immobile spermatozoa due either to physico-chemical reaction of sephadex particle with immotile or dead spermatozoa leading to agglomeration (Graham et al., 1976) or the binding of the protein present on capacitated spermatozoa with the sephadex particles (Samper et al., 1995). On the other hand, glass wool filtration separates motile sperm cells through densely packed glass wool fibers (Mortimer and Mortimer, 1992). Mustafa et al. (1998) harvested maximum number of motile spermatozoa from frozen-thawed buffalo semen using filtration compared to swim-up procedure. Our results on sperm recovery rate are also in line with studies by Marc et al. (1997) who reported higher sperm recovery using glass wool filtration in human.

In the present study, sperm selected through S-G15, and GWF, prior to freezing retained a significantly higher post-thaw sperm quality viz., sperm progressive motility, plasma membrane integrity, normal apical ridge, viability, livability, morphology and chromatin integrity compared to SU and sperm processed through routine procedure (control). It has been reported that sperm selection by sephadex is done on the basis of complex interacting properties of sperm plasma membrane, the medium suspending the sperm and the Sephadex™ particles (Graham et al., 1976). It is possible that positively charged dead spermatozoa get attached to the negatively charged sephadex beads and get trapped, while motility of negatively charged normal spermatozoa facilitates the passage through beads. The GWF is mainly based on the fact that dead and dying sperm are sticky and get attached to glass fiber even in the presence of high concentration of proteins (Mortimer and Mortimer, 1992). The results on sperm motility, acrosome integrity, viability and livability of sperm processed through sephadex filtration procedure in present study were comparable to that of previous studies on bovine (Vyas et al., 1992; Anzar and Graham, 1993, 1995, 1996). Ahmad et al. (2003) reported improved semen quality and freezability of low grade ejaculates whereas Kumar et al. (1999) showed improved freezability and sperm penetration into homologous cervical mucus after sephadex filtration in buffalo. Filteration through Glass wool has been reported to improve sperm quality in cattle (Vyas et al., 1992; Anzar and Graham, 1996; Mustafa et al., 1998), human (Engel et al., 2001) and dog (Mogas et al., 1998).

Sperm morphology is the variable most consistently related to *in vitro* fertilization success rate (Kobayashi et al.,

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1991) and has consistently been used as the best indicator of male fertility. In the present study, head, mid-piece and total abnormalities were found lower in sperm suspension filtered by sephadex compared control, an indication of efficient removal of immotile or dead spermatozoa. Elimination of abnormal spermatozoa by sephadex may have minimized the oxidative stress resulted from exposure to concentrated enzymes found in unextruded cytoplasmic droplet of dead sperm (Gomez et al., 1996) which concomitantly improved the post thaw quality.

Fertilization potential of spermatozoa is highly correlated with sperm progressive motility (Alper et al., 1985; Ron-El et al., 1991; Robinson et al., 1994), concentration (Biljan et al., 1994; Calvo et al., 1994; Robinson et al., 1994) intact acrosome, plasma membrane integrity, chromatin integrity (Evenson et al., 1994; Saacke and White, 1972; Garner and Hafez, 1987; Osinowo et al., 1982; Saacke et al., 1980) and sperm morphology (Kruger et al., 1986; Enginsu et al., 1992; Grow et al., 1994). In our study, spermatozoa selected through sephadex filtration showed improved post-thaw quality and ultimately yielded significantly higher fertilization rates as assessed by cleavage rate. Following sephadex, GWF showed higher fertilization rates compared to SU and control. The lower cleavage rate after oocyte insemination with sperm processed through SU procedure in present study might be due to the stress associated with longer time period required for the SU process that have compromised post-thaw semen quality (Somfai et al., 2002) and ultimately yielded lower cleavage rates as has previously been reported in human (Ron-El et al., 1991).

The processing time required for pre freeze sperm selection is of relevance as cryopreservation process further reduces the viability and longevity of the sperm (Zavos and Centola, 1992). Sephadex and glass wool filtration take less processing time for pre freeze sperm selection which is important for sperm longevity and viability.

The results of this study illustrate that filtration of Nili Ravi buffalo bull semen prior to cryopreservation through sephadex-G15 or glass wool is efficient in improving post-thaw semen quality through removal of immotile, dead and abnormal spermatozoa. Further, on the basis of *in vitro* fertilization rate, sephadex-G15 filtered cryopreserved sperm yielded higher rate of cleaved embryos. In view of the ever increasing demands of semen for breed improvement programme, the sephadex or glass wool filtration techniques, can be used as a routine procedure to harvest high quality semen, provided field fertility results are compared and found favourable. Also, this technique can be used to minimize seasonal deterioration of semen quality.

In conclusion, post thaw sperm quality was improved after sperm selection from fresh buffalo bull semen through S-G15 and GWF procedures compared to SU and control while, the fertility rate (cleavage rate) was improved with sperm processed through S-G15 procedure.

Conflict of interest

None of the authors have any conflict of interest to declare.

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