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Effect of Sperm Separation Methods on Morphology and Functions of Frozen Buffalo Spermatozoa

Abdel-Razek Kh. Abdel-Razek[1], Hassan A. Hussien[1], Waleed Senosy[2], Mohamed S. Yousef [1]

[1]*Department of Theriogenology, Faculty of Veterinary Medicine, Assiut University, Egypt*
[2]*Department of Theriogenology, Faculty of Veterinary Medicine, New Valley, Assiut University, Egypt*

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ABSTRACT

This work was planned to compare three methods for selection of active buffalo spermatozoa, examine the effects of these separation methods on morphology, viability and functions of spermatozoa used for IVF purposes. Ten frozen straws per trial (10 times) were pooled and divided into 4 aliquots: A) First aliquot was considered as control without any separation method. B) Second aliquot was subjected to sperm selection by density gradient method (percoll:PureSperm) using 40-80% double density gradient.. C) The third aliquot was subjected to swim-up in sp-TALP. D) The fourth aliquot was subjected to washing by centrifugation with sp-TALP. The percentage of motility increased for Percoll, swim up and washing than control (86.0, 73.0, and 66.5 vs. 56.5) respectively. Sperm abnormalities percentage was significantly decreased after Percoll, swim up and sperm wash separation methods. Spermatozoa obtained by swim up and Percoll had the highest percentage of intact membrane. Different spermatozoa separation methods significantly increased the lytic activity of the recovered spermatozoa. Live spermatozoa percentage with reacted acrosome significantly increased after both swim up separation and washing. The percentage of dead spermatozoa with reacted acrosome significantly decreased after percoll separation but it did not change when the swim up method was used. Finally it is concluded that, density gradient centrifugation using PureSperm® could be considered as the method of choice for selection of frozen thawed buffalo spermatozoa and presumably with a high potential fertilizing ability, density gradient centrifugation using PureSperm® could be considered as the method of choice for selection of frozen thawed buffalo spermatozoa.

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Introduction

Assisted reproductive technologies such as artificial insemination (AI), multiple ovulation and embryo transfer (MOET) and in vitro fertilization (IVF) have been introduced to improve reproductive efficiency in buffaloes (Madan *et al*., 1994; Nandi *et al*., 2002). AI has been adopted in buffaloes since many years ago (Basirov, 1964); however, it remains unpopular because of poor fertility rate with frozen–thawed semen (Ahmad *et al*., 2003; Senatore *et al*., 2004; Shukla and Misra 2007). It is reported that buffalo spermatozoa are more susceptible to hazards during freezing and thawing than cattle spermatozoa, thus resulting in lower fertilizing potential (Raizada *et al*.. 1990; Andrabi *et al*., 2008). Mammalian spermatozoa are characterized by marked morphological heterogeneity in an ejaculate. Dead and abnormal spermatozoa have toxic (Shannon and Curson, 1972) and lytic (Lindemann *et al*., 1982) effects on viable spermatozoa, and consequently reduce fertility (Saacke and White, 1972). Toxicity of dead sperm is associated with an amino acid oxidase which becomes active only after death of sperm. A metabolic product of this enzyme, peroxide, is responsible for the toxic effect of dead sperm (Shannon and Curson, 1972). In natural mating, cervical mucus differentially selects motile spermatozoa and acts as a barrier to immotile ones (Saacke, 1984).This cervical selection is by passed in AI and IVF procedures. Sperm preparation methods have a significant effect on IVF results (Parrish *et al*., 1995). The first successful separation of motile from immotile spermatozoa was performed by passing diluted semen through a layer of small glass beads (Bangham and Hancock, 1955), glass wool (Maki-Laurila and Graham (1968), pyrex beads (McGrath *et al*., 1977), Percoll gradients (Lessley and Garner, 1983), bovine serum albumin gradients (Zavos,1985), the swim-up method (Parrish and Foote, 1987) and sephadex filtration method (Maki-Laurila and Graham, 1968; Graham *et al*., 1976). Unlike in cattle bull, methods of sperm separation received little attention in buffalo bull. Sperm separation methods have a very important role in successful IVF. Such selection of spermatozoa separates motile sperm from non-motile, removes seminal plasma, cryoprotective agents, other background materials and debris (Zavos, 1992). At the same time these methods initiate capacitation of sperm (Centola *et al*., 1998) Separation of seminal plasma and its favorable effects on sperm survival in fluid and frozen state preservation of semen

*Corresponding author: A Kh Abdel-Razek
E-mail address: razek2@yahoo.com

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have been reported by several workers (Shah, 1993; Ahmad *et al*., 1997; Khan *et al*., 1998) but there is a little information about beneficial effects of seminal plasma removal and effect of sperm preparation methods on functions and morphology of buffalo spermatozoa. The aim of the present was to compare three methods for selection of active buffalo spermatozoa, examine their effects on morphology, viability, acrosomal integrity and determining the most efficient separation method for improving buffalo sperm quality.

Materials and methods

Lots of frozen buffalo semen straws were delivered from semen freezing center and transported in liquid nitrogen tank to the laboratory. Frozen straws containing 0.25 ml ($35X10^6$ spermatozoa/straw) were thawed in water bath at 37ºC for 30 seconds. From each lot, one straw was examined for sperm progressive motility and abnormality. The straws were considered when sperm motility at least 50% and abnormal sperm percentage not exceed 25%. After thawing, semen samples were pooled. Then pooled samples were divided into four aliquots: first aliquot was considered as control without any separation methods, the aliquots were subjected to sperm selection by: density gradient method (Percoll method) using PureSperm® for the second aliquots, The third aliquot was subjected to swim-up procedure according to Allamaneni *et al*. (2005). The forth aliquot was subjected to washing procedure according to Dode *et al.* (2002), The sperm quality parameters were evaluated for control and separated sperm which include motility assessment according to Khan and Ijaz, (2008) and sperm morphology by using phase contrast light microscope where a wet smears of unstained buffered formalin solution-fixed spermatozoa examined according to Mathias and Yusuf (1985) and Sukhato *et al*. (1988). Evaluation of sperm viability and acrosome reaction were carried out using trypan blue/giemsa stained film according to Kovacs and Foote (1992); Presicce *et al*. (2003) and Boccia *et al*. (2007). Sperm membrane integrity was assessed in all samples using a hypo- osmotic swelling test (HOST) according to Jeyendran *et al*. (1984) and Ijaz *et al*. (2009). Assessment of Gelatinolytic or Proteolytic activity was determined using x-ray fixed film. The film was cut into 2x7 cm strips and fixed on clean glass slides by Canada balsam. Immersed in veronal acetate (pH 8.2) containing 0.05% gluteraldehyde for 2 min. Spermatozoa was placed on the prepared fixed gelatin membrane and spread with a cover slip. Slides placed inside a humidity saturated incubator at 40°C for 48 hours. The proteolytic activity was detected as a localized lightening of the blue gelatine membrane around the acrosome of the spermatozoa (modified by El-Sheltawi ,1989).

Data were presented as mean and standard error (mean ± SE). Results from different methods of separation were compared with the control group. Statistical significance was determined by analysis of variance (ANOVA) and t test using Statistical Package for the Social Sciences for Windows, SPSS, (2007). Statistically significant differences were set at $P \leq 0.05$.

Results

The results revealed that spermatozoa recovered by the different separation methods showed a significant increase in the percentage of motility compared to control samples and also there were significant differences among different separation methods ($P<0.05$). The percentages of recovered motility were 56.50±1.50, 86.0 0±1.50, 73.00±2.49 and 66.50±1.50 136% for control, percoll, swim up and washing; respectively. Sperm abnormalities percentages were decreased significantly after recovery from the three separation methods. Abnormal sperm percentage in recovered semen decreased in treated samples in comparison with control but abnormal sperm recovered by washing showed significant increase than the other separation methods (Table 1). Total sperm viability percentage using percoll separation method was significantly higher ($P<.0.05$) than swim up and washing methods. There was no significant difference in the percent of viable spermatozoa recovered after swim up and washing versus the control. On the other hand, the proportion of recovered viable spermatozoa by swim up was significantly higher ($P<0.05$) than that separated by washing.

Table 1. Percentages of sperm motility and abnormalities of frozen thawed buffalo semen after different methods of separation (n=10).

Methods of selection	Sperm motility %	Sperm abnormalities %
Control	56.50 ± 1.50^{d}	18.80 ± 1.94^{a}
Percoll	86.00 ± 1.50^{a}	7.80 ± 0.70^{c}
Swim up	73.00 ± 2.49^{b}	8.80 ± 0.57^{c}
Washing	66.50 ± 1.50^{c}	13.40 ± 1.23^{b}

Data represent mean ± SEM, Means, within the same column, with different superscripts are significantly different at $P<0.05$.

The total alive spermatozoa were numerically decreased after washing compared with control. The results of total alive spermatozoa percentage were 87.00±1.97, 76.00±4.78, 62.40±2.20 and 69.30±1.80 after Percoll, swim up, washing separation and before treatment, respectively (Table 2).

Table 2. Percentages of sperm viability after different methods of separation of frozen thawed buffalo spermatozoa (n=10)

Methods of selection	Total alive %	Total dead %
Control	69.30 ± 1.80^{bc}	30.70 ± 1.80^{ab}
Percoll	87.00 ± 1.97^{a}	13.00 ± 1.97^{c}
Swim up	76.00 ± 4.78^{b}	24.20 ± 4.75^{b}
Washing	62.40 ± 2.20^{c}	36.70 ± 2.15^{a}

Data represent mean ± SEM, Means, within the same column, with different superscripts are significantly different at $P<0.05$.

The percentage of total reacted spermatozoa significantly ($P< 0.05$) increased after swim up separation (from 23.60 ±1.90 to 31.80±3.21%) while there was no significant increase after washing (27.20 ± 2.55%) and percoll 19.50±2.52%. The percentage of total spermatozoa with intact acrosome was 80.50±2.52, 68.20±3.21, 68.90±4.98 and 76.40±1.90 after percoll, swim up, washing separation and control samples, respectively (Table 3). Only the sperms separated by Percoll showed a significant increase in comparison with other methods of separation (Table 3). Percoll method significantly improved ($P<.0.05$) the proportion of live spermatozoa with an intact acrosome (from 61.40±3.13 in control to 72.00±2.53 % after percoll separation). On the other hand, the live intact spermatozoa significantly decreased after washing 47.40±2.48 %, while there was no significant difference between control and swim up treatment. The same table showed that, there were no significant changes of dead intact ratio after swim up or percoll separation and control, while it was significantly increased after washing .The results of dead intact spermatozoa percentage were 8.50±1.38, 13.90±3.10, 25.50±2.29 and 15.00±1.38 after percoll, swim up, washing separation and control respectively (Table 4). The samples separated by swim up showed the highest value of alive reacted spermatozoa (21.70±2.57%) with a significant difference ($P<0.05$) when

compared with that produced by percoll, washing and control(Table 5). Moreover, live spermatozoa with reacted acrosome significantly increased (P<0.05) to 15.00±2.18% after both percoll and washing separation compared to control (7.90±1.21%).The percentage of dead spermatozoa with reacted acrosome significantly decreased (P<0.05) after percoll separation (from 15.70±0.96 to 4.50±0.96%) but no significant difference of dead reacted spermatozoa observed after swim up , washing separation and control in which the value of dead reacted spermatozoa percentage were 10.30±2.68%, 12.20±2.36 and15.70±0.96% respectively.

Table 3. Total reacted and intact spermatozoa after separation of frozen thawed buffalo spermatozoa by different methods (n=10)

Methods of selection	Total reacted %	Total intact %
Control	23.60±1.90 ^{b}	76.40±1.90^{ab}
Percoll	19.50±2.52 ^{b}	80.50±2.52^{a}
Swim up	31.80±3.21^{a}	68.20±3.21^{b}
Washing	27.20±2.55 ^{ab}	68.90±4.98^{b}

-Data represent mean ± SEM, Means, within the same column, with different superscripts are significantly different at P<0.05.

Table 4. Alive and dead intact spermatozoa after separation of frozen thawed buffalo spermatozoa by different separation methods (n=10)

Methods of selection	Live intact %	Dead intact %
Control	61.40±3.13^{b}	15.00±1.38^{b}
Percoll	72.00±2.53^{a}	8.50±1.38^{b}
Swim up	54.30±4.48^{b}	13.90±3.10^{b}
Washing	47.40±2.48^{c}	25.50±2.29^{a}

Data represent mean ± SEM, Means, within the same column, with different superscripts are significantly different at P<0.05.

Table 5. Alive and dead reacted spermatozoa after separation of frozen thawed buffalo spermatozoa by different separation methods (n =10)

Methods of selection	Alive reacted %	Dead reacted %
Control	7.90±1.21^{c}	15.70±0.96 ^{a}
Percoll	15.00±2.18^{b}	4.50±0.96 ^{b}
Swim up	21.70±2.57^{a}	10.30±2.68 ^{a}
Washing	15.00±2.25^{b}	12.20±2.36^{a}

Data represent mean ± SEM, Means, within the same column, with different superscripts are significantly different at P<0.05.

Table 6. Plasma membrane integrity and lytic activity after separation of frozen thawed buffalo spermatozoa by different separation methods (n=10)

Methods of selection	HOST %	Lytic test %
Control	23.30±1.15^{b}	9.00±1.19^{c}
Percoll	30.00±0.60^{a}	34.80±2.42^{a}
Swim up	31.70±2.30^{a}	33.40±1.86^{a}
Washing	25.80±0.87^{b}	22.60±1.83^{b}

Data represent mean ± SEM, Means, within the same column, with different superscripts are significantly different at P<0.05.

The results of separation methods effects on sperm membrane integrity (Fig. 1) by HOS test are recorded in Table 6. The percentage of sperm with undamaged membrane showed significant increase after separation by percoll and swim up methods. The values were 30.00±0.60 and 31.70±2.30 for percoll and swim up method respectively. The values for control and washing technique were 23.30±1.15 and 25.80±0.87 receptively. The same table demonstrated that separation of sperm with percoll (34.80±2.42) and swim up (33.40±1.86) techniques showed significant increase than that separated by washing (22.60±1.83) or control (9.00± 1.19) samples. The percentage of spermatozoa positive to lytic test (showed lytic halo zone around the head presented in Fig.2) was significantly higher (P<.0.05) after both Percoll and swim up than that recovered from washed samples and control samples. While, there was no significant difference between both Percoll and swim up. Different spermatozoa treatments significantly increased the lytic activity of the recovered spermatozoa if compared to control (Fig. 2).

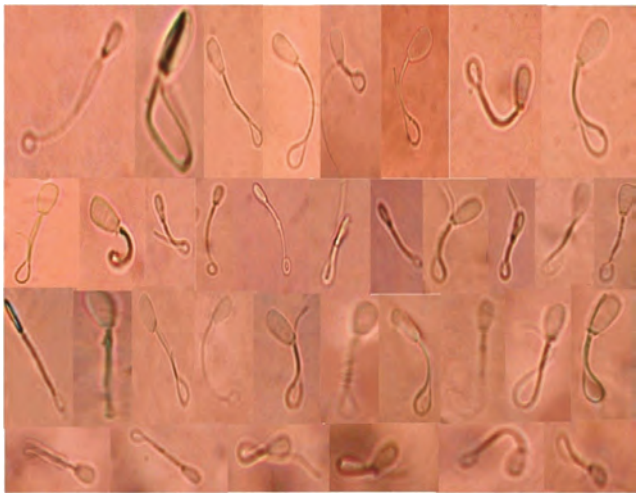

Fig.1. Different types of swelling and curling in buffalo spermatozoa (x400)

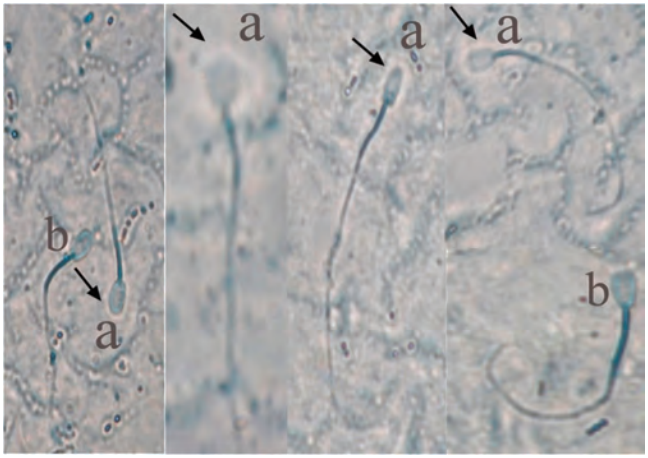

Fig. 2. Acrosin activity of buffalo spermatozoa on a gelatin substrate slide (X ray film). a) Sperm showed acrosin proteolytic actlvity

Discussion

Sperm selection methods are routinely applied for in vitro fertilization (IVF) system for various species. These methods are used in order to remove seminal plasma, cryoprotectant, undesirable spermatozoa, debris and other factors to increase sperm quality characteristics (Parrish *et al.*, 1995; Rodriguez-Martinez *et al.*, 1997). In the current work, there was a signif-

icant difference increase in the motility of spermatozoa after percoll preparation in comparison to initial sperm motility. The result agreed with (Parrish *et al*., 1995; Valcarcel et al., 1996) in frozen thawed semen of ram. On the other hand, Samardzija *et al.* (2006); Tanghe *et al*. (2002) found a lower recovered motility (63%-68%) on frozen thawed bull semen after percoll method. This difference is assumed because the authors had lower initial values of motility than the present work after thawing, also the difference in the density gradient applied where they used percoll® gradient while PureSperm® was used in the present work. There was a significant improvement in the percentage of viable spermatozoa after Percoll method when compared to the control samples. These results coincided with that reported after Percoll separation in fresh human semen (Pousette *et al*., 1986), in frozen- thawed bull semen and in frozen thawed buffalo semen (Abdel- Razek, 2005). Only spermatozoa with normal intact acrosomes can undergo acrosome reaction (AR), which is essential for the spermatozoa to penetrate the zona pellucida and to fuse with the oolemma. Furthermore, the AR must occur at the correct time for the spermatozoa to be able to penetrate the zona pellucida (ZP). A normal sperm sample should contain a high proportion of spermatozoa with intact acrosomes capable of undergoing AR after incubation in capacitating conditions (Takahashi *et al*., 1992). If sperm prematurely capacitated or acrosome reacted, they could have a diminished capacity to penetrate the cumulus mass and fertilize the egg (Yanagimachi, 1994). The present study revealed a high percentage of spermatozoa with intact acrosome in percoll separated samples when compared to control. Similarly, Valcarcel *et al* (1996) reported the ability of discontinuous Percoll separation to extract more spermatozoa with an intact acrosome in frozen–thawed ram semen. However, in bull spermatozoa, this beneficial effect of percoll separation was obtained only on frozen–thawed semen having spermatozoa with low post thaw motility (27–30%) or a low rate of intact membrane (Rodriguez- Martinez *et al*., 1997). The obtained results disagreed with that reported by Cesari *et al*. (2006) and Abdel-Razek, (2005), who found a higher percentage of spermatozoa with partially lost acrosomes obtained after percoll separation. The controversy probably because of the levels of endotoxins produced by the free polyvinyl pyrrolidone (PVP) present in the percoll® gradients used. It is assumed that percoll® induces capacitation-like changes on the sperm plasma membrane. In the present study, a higher percentage of frozen thawed buffalo bull spermatozoa with intact acrosome were successfully separated by density gradient centrifugation using PureSperm® gradient. The results provide evidence that the percoll treatment was capable to reduce the population of acrosome-reacted spermatozoa. In the same time, the study revealed that percoll preparation improved the percentage of alive spermatozoa with intact acrosome when compared with raw samples. Somfai *et al*. (2002) and Samardzija *et al*., (2006) found a significant increase in the proportion of live spermatozoa with intact acrosome for percoll®. Swim-up technique resulted in a significantly higher percentage of motile spermatozoa than control. This result was in a harmony with that recorded by Mehmood *et al*. (2009) in frozen thawed buffalo semen, (Parrish *et al*., 1995) in frozen thawed bull semen and (García-López *et al*.,1996) in fresh ram semen. There was an improvement in sperm motility after swim up of fresh human semen (Michaeli *et al*., 2004). The results of the current work showing an improvement of sperm viability after swim up when compared with control samples. Similar results obtained by Somfai *et al*. (2002) in frozen thawed bull semen and in fresh human semen (Esteves *et al*., 2000). This study showed a higher percentage of spermatozoa with reacted acrosome separated by swim up method. Somfai *et al.* (2002) explained that during one hour incubation some of the migrated spermatozoa undergo acrosome reaction. There was no significant difference in the proportion of alive spermatozoa with intact acrosome before and after swim up preparation which is parallel to those obtained by Samardzija *et al*. (2006). A significant increase in progressive motility of spermatozoa was recovered after washing when compared with control samples. The obtained results agreed with those of Jamil *et al*. (2007); Abdel-Razek (2005); Correa and Zavos (1996). A higher percentage of spermatozoa with reacted acrosome were observed after washing but the difference was not significant, while Abdel-Razek (2005) found a high significant increase in spermatozoa with reacted acrosome after washing compared to control. Although, Goyal *et al*. (1996) reported that spermatozoa with acrosomal reaction were decreased after washing. The present data showed that there was no significant increase in viable spermatozoa proportion before and after sperm washing. This was in conformity with the results of Abdel-Razek (2005) and this support that the washing method used only for sperm separation not for sperm selection. The recovered motility of buffalo bull spermatozoa was significantly higher with Percoll density gradient separation than swim up and washing. Our results agreed with those reported by Van Der Zwalmen *et al*. (1991); Abdel-Razek *et al*. (2001), Abdel-Razek (2005);. Contrary to our results, Mehmood *et al*. (2009) reported that recovered motility (%) from frozen–thawed buffalo semen was higher with swim-up method than percoll method. The results showed that the percentage of dead spermatozoa with reacted acrosome was significantly higher after swim up than after percoll separation. A possible reason for their presence might be the death of spermatozoa that had migrated up to the covering fraction early before the assessment. There is some evidence that processing-related stress occurs during the swim up procedure causing a high mortality rate of migrated spermatozoa (Nevo and Mohan, 1969) and it has been assumed that this stress is related to the lingering time period of the method (Correa and Zavos, 1996). These results were in a harmony with that recorded by Somfai *et al*. (2002); Palomo *et al*. (1999); Brandeis and Manuel, (1993). Opposite to our results Mehmood *et al*. (2009) revealed no significant difference in acrosome intact spermatozoa separated by swim-up and percoll gradient methods. Cesari *et al*. (2006) also, reported that the percentage of sperm with partially lost acrosome by transmission electron microscopy (TEM) was higher in percoll treated sperm than in swim up, according to Yanagimachi (1988), elevated rates of spontaneous AR would have a negative implications for IVF protocols, since reacted sperm are not able to bind to the zona pellucida and fertilize oocytes. In the present investigation, Percoll methods selected a higher percentage of viable spermatozoa with intact acrosomes than Washing and swim up methods. According to Shamsuddin and Rodriguez-Martinez, (1994) there was a negative effect of centrifugation after swim up on sperm viability and motility. In this regard it might be possible that swim up causes the spermatozoa to be more sensitive to centrifugation. Thus it is probable that both factors are responsible for the presence of dead reacted spermatozoa in swim up-treated sperm populations. The same results recorded by Somfai *et al*. (2002); Palomo *et al*. (1999); Brandeis and Manuel (1993). The percentage of bovine spermatozoa forming Halos (proteolytic activity) in gelatin membrane was found to be highly correlated with fertility (Kircher *et al*., 1985). In the current study, the percentage of spermatozoa positive to lytic test was significantly higher after both Percoll and swim up while there was no significant difference between both Percoll and swim up. Tornyov *et al*. (1979) found that freezing of ram semen caused marked reduction of acrosomal proteolytic activity of the spermatozoa. Overall, density gradient centrifugation using PureSperm®

360 could be considered as the method of choice for selection of frozen thawed buffalo spermatozoa. swim up procedure is easier to perform as compared to PureSperm gradient centrifugation. swim up method also can be used for frozen thawed buffalo semen preparation for IVF purposes. Sperm washing is much easier and more economical to perform as compared to other procedures, however it is used only for sperm separation not for sperm selection. Sperm washing not improved the sperm viability or acrosomal integrity.

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