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The Quality of Frozen Buffalo Sperm Following Sexing using Bovine Serum Albumin (BSA) Column and Swim-Up (SU) Methods

Masrizal¹, Tinda Afriani¹, Dwiki Wahyudi³, Savira Saharani², and Ananda^{1*}

¹Department of Livestock Production Technology, Faculty of Animal Science, Universitas Andalas, Padang, 25163, Indonesia

²Undergraduated Program, Faculty of Animal Science, Universitas Andalas, Padang, 25163, Indonesia

³Postgraduated Program, Faculty of Animal Science, Universitas Andalas, Padang, 25163, Indonesia

ABSTRACT

This study assesses the impact of two sperm sexing techniques, the Bovine Serum Albumin (BSA) column method, and the swim-up (SU) method, on frozen buffalo spermatozoa quality. A total of 50 straws of frozen buffalo semen were used in this study. Spermatozoa quality was evaluated before (post thawing) and after the spermatozoa sexing process. Spermatozoa trapped in BSA upper fraction, BSA lower fraction, SU upper fraction, and SU lower fraction were separately evaluated. The parameters measured consisted of spermatozoa motility, viability, intact plasma membrane, intact acrosome cap, and spermatozoa DNA integrity. The results indicated that the quality of post-thawing buffalo spermatozoa remained relatively high, with motility at 41%, viability at 64.48%, intact plasma membrane at 55.42%, intact acrosome cap at 47.12%, and sperm DNA integrity at 74.94%. However, the use of the BSA column method significantly ($p < 0.05$) decreased spermatozoa quality in both the upper and lower fractions, resulting in motility levels of 34% and 32%, viability rates of 49.36% and 44.71%, intact plasma membrane percentages of 44.78% and 37.13%, intact acrosome cap figures of 37.58% and 33.27%, and sperm DNA integrity levels of 74.76% and 72.45%, respectively. In contrast, the application of the SU method proved effective in preserving post-thawing spermatozoa quality, yielding motility rates of 42% and 41%, viability levels of 63.62% and 62.78%, intact plasma membrane percentages of 54.42% and 54.74%, intact acrosome cap figures of 46.94% and 45.74%, and sperm DNA integrity values of 70.57% and 70.01%, respectively. In summary, after freezing, the SU method excel the BSA column method in maintaining the quality of buffalo spermatozoa post-thawing.

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* Corresponding author:

E-mail: ananda@ansci.unand.ac.id

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Introduction

The buffalo (*Bubalus bubalis*) represent substantial ruminant livestock, holding significant potential as a source of animal protein through meat production. Nevertheless, the development of buffalo populations lags behind that of cattle, primarily due to their persistently low birth rate. The adoption of reproductive technology presents a viable solution to this challenge. Several reproductive technologies have been developed to bolster livestock productivity, including Artificial Insemination (AI), Embryo Transfer (ET), and In Vitro Fertilization (IVF) (Lopulalan *et al.*, 2018).

Method of AI is the most successful livestock reproduction technology, widely embraced by breeders due to its cost-effectiveness and efficiency in disseminating superior genetic material. The value of artificial insemination can be further enhanced by controlling the sex of offspring

according to the intended purpose. For instance, in meat production, males are preferred, while females are sought after in breeding programs. The technology used to determine the sex of offspring is known as sperm sexing (Susilawati, 2014), with sex being determined by the presence of X and Y chromosomes in male spermatozoa (Garner and Hafez, 2000).

Sexing technology involves separating X and Y spermatozoa, and the application of spermatozoa sexing biotechnology represents an alternative method developed to predict the sex of offspring, with potential applications in livestock management (Bhalakiya *et al.*, 2018). Determining the sex of offspring before birth holds significant economic advantages as it reduces maintenance costs and supports breeding programs by selecting superior genetic material. The utilization of spermatozoa sexing technology is a strategic choice to enhance the efficiency of livestock

breeding. The separation of X and Y spermatozoa using a Bovine Serum Albumin (BSA) column relies on differences in motility between X and Y spermatozoa when introduced into a BSA solution (Forman *et al.*, 2013) and differences in DNA size between the two types of spermatozoa. The ratio of DNA differences between X and Y spermatozoa in cattle is approximately 3.8% (Welch and Johnson, 1999). The method for sexing spermatozoa using the BSA column has been developed and successfully applied in the field. It has resulted in an 81% sex compatibility rate for dairy calves with a sex chromosome ratio (S/C) of 1.37 (Said *et al.*, 2005) and the production of embryos through IVF, leading to the development of embryos to the morula and blastocyst stages (Kaiin *et al.*, 2008). Notably, Gunawan *et al.* (2015) achieved a sexing success rate of 87% for spermatozoa X and 89.5% for spermatozoa Y, while in Simmental cattle, the rate for spermatozoa Y reached 81.7% (Kaiin *et al.*, 2008). In Bali cattle, a rate of 76.7% was observed for spermatozoa X (Gunawan *et al.*, 2017).

Sperm sexing based on motility and size differences of sperm using albumin column method has been reported by Garner and Hafez (2000), and Yadav *et al.* (2017), respectively. The BSA was used earlier in sperm sexing than egg white. Egg white was first reported as medium instead of BSA in sperm sexing by Saili (1999). BSA, being a natural protein present in semen plasma (Akhter *et al.*, 2014), serves as a viable substitute for egg yolk in semen diluent medium. Using BSA at 10% or 15% concentrations can yield post-thawing quality comparable to that achieved with Tris-yolk. The use of BSA serves multiple purposes, including safeguarding spermatozoa when stored at a temperature of 5°C. Additionally, when included in a capacitation medium, BSA enhances efflux on the plasma membrane of spermatozoa cells (Matsuoka *et al.*, 2006), offers protection to the plasma membrane, and helps maintain the integrity of spermatozoa DNA (Akhter *et al.*, 2014).

The optimal method for preparing spermatozoa involves efficient, rapid, and cost-effective removal of semen plasma without harming the spermatozoa. This process should also eliminate decapacitation factors or reactive oxygen species (ROS) while increasing the number of spermatozoa capable of fertilization. Semen plasma, despite its protective role for spermatozoa in harsh conditions, contains factors that inhibit their fertilization potential and reduce capacitation induction. Additionally, cryopreservation can reduce motility and damage the integrity of the sperm membrane, acrosome membrane, and spermatozoa chromatin (Celeghini *et al.*, 2008; Donnelly *et al.*, 2001). To address these challenges, various methods have been developed to efficiently remove semen plasma or cryoprotectants and select high-quality spermatozoa in thawed frozen semen, thereby enhancing their fertilization capacity (Henkel and Schill, 2003). The swim-up technique is commonly applied in a series of IVF. Due to the smaller size, spermatozoa carrying Y chromosomes are

theoretically expected to swim faster than spermatozoa carrying X chromosomes, allowing them to ascend to the top of the swim tube. In contrast, spermatozoa with an X chromosome tend to be at the bottom of the tube (Azizeddin *et al.*, 2014). Spermatozoa sexing through the conventional swim-up technique has been successfully performed in humans, achieving a success rate of 81% (Check *et al.*, 1989; Khatamee *et al.*, 1991). Developing low-cost alternative spermatozoa sexing procedures is highly desirable in livestock, especially buffalo.

Buffalo semen that has already been processed into frozen semen, can still be utilized to produce individuals of the desired gender. While it is not widely implemented in artificial insemination programs, the production of sexed embryos *in vitro* remains a viable option. Therefore, this study aims to evaluate the semen quality of post-thawing buffaloes by sexing using the bovine serum albumin column method and the swim-up method.

Materials and Methods

Source of Buffalo frozen semen

The frozen semen was provided by Lembang Artificial Insemination Center, Bandung, West Java, Indonesia. Fifty straws of frozen buffalo semen were thawed by submerging them in a 37°C water bath for 30 s. The thawed semen was then transferred to sterile microtubes for evaluation.

Post-thawing spermatozoa analysis

Spermatozoa motility was assessed using a phase-contrast microscope at 400x magnification. Following the method described by Arifiantini (2012), the hypoosmotic swelling test (HOST) was employed to evaluate the integrity of the plasma membrane. The HOST solution consisted of 0.73 g of sodium citrate (Merck) and 1.35 g of fructose (Scharlau) dissolved in 100 mL of distilled water, resulting in an osmolality of 190 mOsmol/kg. After combining 50 µL of the semen sample with 500 µL of pre-warmed HOST solution, the mixture was incubated at 37°C for 30–40 min. Subsequently, a drop of the incubated sample was placed on a slide, covered with a lid, and observed under a 400x microscope. To assess 200 spermatozoa, five fields of view were examined. According to Akhter *et al.* (2014), swelling of spermatozoa tails indicates healthy and functional membranes, whereas unswollen tails suggest damaged, inactive, and non-functional membranes.

To assess spermatozoa acrosome integrity, we used a formaldehyde-citrate solution composed of 2.9 g trisodium citrate dihydrate and 1 mL of a 37% formaldehyde solution diluted in 99 mL of distilled water. A phase-contrast microscope with oil immersion at 400x magnification examined 100 µL of semen mixed with 500 µL of the formaldehyde-citrate solution, including 200 spermatozoa (Ansari *et al.*, 2011). Typically, spermatozoa display an undamaged acrosome and a clear, gleaming head, whereas those with blunt-tipped cells exhibit acrosome damage.

For the viability test, semen was mixed with eosin-nigrosin in a 1:4 ratio, homogenized, smeared, and dried. Phase-contrast microscopy at 1000x magnification assessed 200 spermatozoa in each smear. Results were expressed as the percentage of viable spermatozoa among all cells examined. Viable spermatozoa lacked color, while those with a purple hue were considered non-viable. The percentages represented spermatozoa viability.

A single drop of frozen semen was used to create smears on four glass slides, enabling the observation of intact DNA. These samples were then dried and fixed for 30 min at 4°C in a 1:1 solution of 96% ethanol and acetone. Subsequently, they were hydrolyzed in 0.1 N HCl at 4°C for 5 min and rinsed thrice with distilled water. The smears were stained with 0.05% toluidine blue (TB) for 10 min. Following staining, the samples were cleared with xylene twice, dehydrated twice with t-butanol, and re-washed with distilled water. A cover glass was then applied to each slide, and spermatozoa with high chromatin integrity displayed bright blue heads, while those with low chromatin integrity exhibited dark blue heads. A total of 100 spermatozoa were examined for each sample under light microscope with 400 magnification.

Sperm separation using bovine serum albumin (BSA) method

The frozen semen is processed for sexing spermatozoa using a 5-10% BSA gradient column for 45 min, as described by Kaiin and Gunawan (2017). Following the sexing process, the spermatozoa collected from each gradient of BSA column were then centrifuged in Brackett Oliphant (BO) medium for 10 min at 1800 rpm. Subsequently, the spermatozoa cell pellet was resuspended in 1000 µL of BO medium, and their quality was examined microscopically. This examination included the assessment of motility, viability, the integrity of the plasma membrane, the intactness of the acrosome cap, and the preservation of DNA integrity.

Sperm separation using swim up (SU) method

A physiological NaCl solution was used as the medium in the spermatozoa separation method through swim-up. A volume of 0.25 mL of frozen semen suspension was carefully transferred to a sterile test tube, followed by adding 1.5 mL of physiological NaCl solution through the tube wall. The filled tube was placed upright on a rack and allowed to stand at room temperature for 5 min. Subsequently, the upper and lower layers were separated and transferred to different microtubes. The quality of the spermatozoa was observed microscopically, assessing motility, viability, intact plasma membrane, intact acrosome cap, and intact DNA.

Data analysis

This research employed a completely randomized design (CRD). A total of 50 straws of

frozen buffalo semen from the same batch were divided into five treatment groups: post-thawing only, BSA upper fraction, BSA lower fraction, SU upper fraction, and SU lower fraction, with each treatment consisting of 10 repetitions (10 straws). Analysis of variance was applied to assess the effect of treatment on the measured variables. Differences between treatments were then analyzed using the Tukey test with a confidence level of 95%.

Results and Discussion

The results of observations on post-thawing buffalo spermatozoa motility are presented in Figure 1. The average motility of post-thawing buffalo spermatozoa was 41%. While the motility of frozen thawed sperm following sexing using BSA method significantly reduced the motility of buffalo spermatozoa ($p < 0.05$). Specifically, the sperm motility decreased to 34%, and 32%, respectively for the upper and the lower fraction. Conversely, the swim-up method treatment slightly increased motility in the upper fraction to 42% and did not result in significant changes in motility in the lower fraction, which remained at 41%.

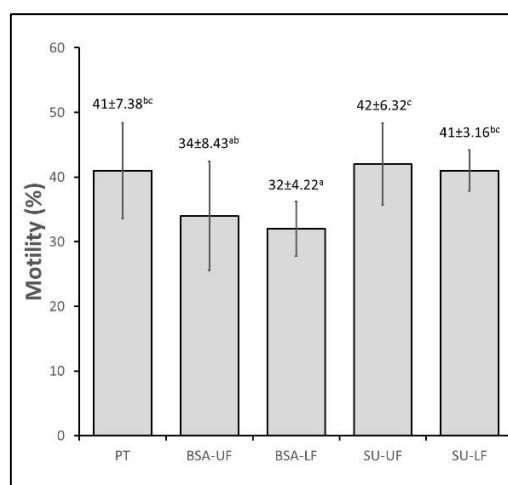


Figure 1. Motility of spermatozoa treated with BSA and SU after the freezing process. ^{a,b} Different superscripts in the same bar indicates differences in motility in each of treatment ($P < 0.05$). PT: post-thawing; BSA-UF: bovine serum albumin – upper fraction; BSA-LF: bovine serum albumin – lower fraction; SU-UF: swim up – upper fraction; SU-LF: swim up – lower fraction.

Observational data on the viability of buffalo spermatozoa are presented in Figure 2. In this study, the viability of post-thawing buffalo spermatozoa was 64.48%, and this result remained consistent after the swim-up process, with viability rates of 63.62% in the upper fraction and 62% in the lower fraction. However, treatment using the BSA column significantly ($p < 0.05$) reduced the viability of buffalo spermatozoa, resulting in a viability of 49.36% in the upper fraction and 44.71% in the lower fraction.

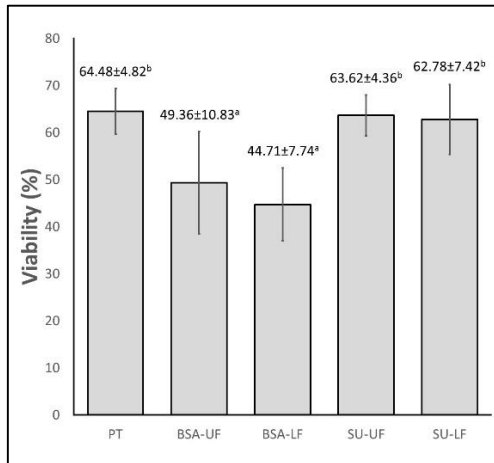


Figure 2. Viability of spermatozoa treated with BSA and SU after the freezing process. ^{a,b} Different superscripts in the same bar indicates differences in motility in each of treatment (P<0.05). PT: post-twaing; BSA-UF: bovine serum albumin – upper fraction; BSA-LF: bovine serum albumin – lower fraction; SU-UF: swim up – upper fraction; SU-LF: swim up – lower fraction.

Observation of the intact plasma membrane of post-thawing buffalo spermatozoa revealed a rate of 55.42% (Figure 3). Similar results were observed in the swim-up treatment, with the upper fraction at 54.42% and the lower fraction at 54.74%. However, contrasting results were observed following treatment using the BSA column (p<0.05), where the upper fraction decreased to 44.78%, and the lower fraction dropped to 37.13%.

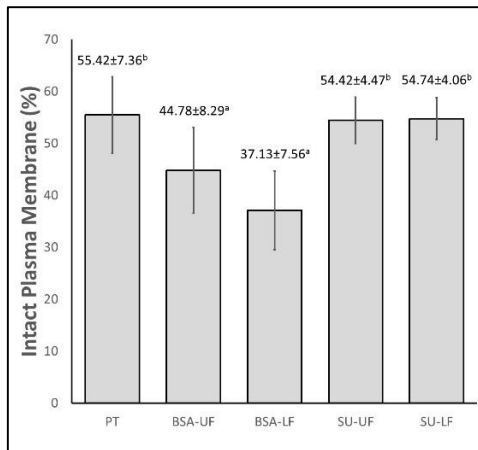


Figure 3. Intact plasma membrane of spermatozoa treated with BSA and SU after the freezing process. ^{a,b} Different superscripts in the same bar indicates differences in motility in each of treatment (P<0.05). PT: post-twaing; BSA-UF: bovine serum albumin – upper fraction; BSA-LF: bovine serum albumin – lower fraction; SU-UF: swim up – upper fraction; SU-LF: swim up – lower fraction.

The percentage of Intact Acrosome Caps in post-thawing buffalo spermatozoa was 47.12% (Figure 4). After treatment with the BSA column, a significant decrease (p<0.05) was observed in the

Intact Acrosome Cap percentage. It dropped to 37.58% for the upper fraction and 33.27% for the lower fraction. Following treatment with the swim-up method, the decrease in the Intact Acrosome Cap percentage was relatively consistent; it reached 46.94% for the upper fraction and 45.74% for the lower fraction.

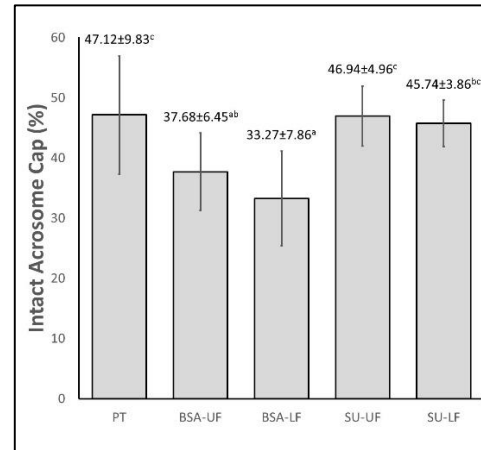


Figure 4. Intact acrosome cap of spermatozoa treated with BSA and SU after the freezing process. ^{a,b,c} Different superscripts in the same bar indicates differences in motility in each of treatment (P<0.05). PT: post-twaing; BSA-UF: bovine serum albumin – upper fraction; BSA-LF: bovine serum albumin – lower fraction; SU-UF: swim up – upper fraction; SU-LF: swim up – lower fraction.

Sperm DNA integrity in this study did not show significant differences among the treatments (Figure 5). The DNA integrity of buffalo spermatozoa in this study ranked sequentially from highest to lowest as follows: post-thawing (74.94%), upper BSA fraction (74.76%), lower BSA fraction (72.45%), upper swim-up fraction (70.57%), and bottom swim-up fraction (70.01%).

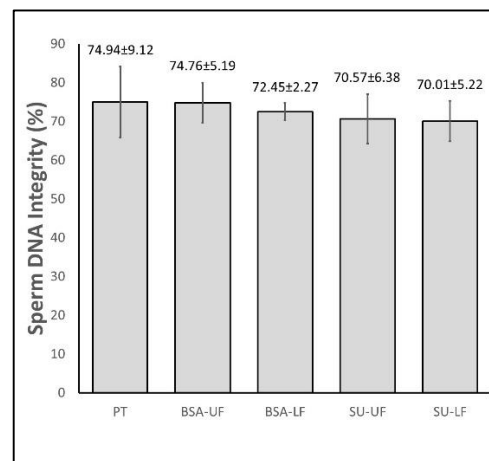


Figure 5. DNA integrity of spermatozoa treated with BSA and SU after the freezing process. PT: post-twaing; BSA-UF: bovine serum albumin – upper fraction; BSA-LF: bovine serum albumin – lower fraction; SU-UF: swim up – upper fraction; SU-LF: swim up – lower fraction.

The shape and permeability of the plasma membrane can be altered by cold shock induced by a drop in temperature (Sieme *et al.*, 2015). Changes in membrane function, particularly in membrane proteins that undergo structural alterations, lead to reduced cell function, including motility, viability, and fertilization ability. Additionally, modifying the ion channel function of membrane proteins and a transition from a double-layer structure to a hexagonal shape can cause membrane leakage, increasing membrane permeability and affecting intracellular activity. The tolerance of spermatozoa to cold shock is correlated with the membrane's hydraulic permeability. Furthermore, the extent to which spermatozoa can endure changes in the membrane phase and domain arrangement also impacts their ability to survive chilling. The temperature range within which the membrane lipid transition phase occurs is typically between 10 and 30°C (Oldenhof *et al.*, 2013).

According to Best (2015), hypertonic media did not significantly affect cell viability, suggesting that increasing cell viability may be achieved by reducing the duration of cell exposure to cryoprotectants. This implies that metabolic toxicity is the primary cause of cell damage during equilibration rather than osmotic stress. Moreover, the freezing procedure represents the most significant source of injury during cryopreservation. Complex cooling processes induce physiological changes in cells (Pegg, 2015).

The nucleation process in the extracellular space triggers the formation of ice crystals during freezing, with their size increasing as the temperature drops. Water molecules aggregate and bond together to create these ice crystals. The solute concentration rises because solutes in the extracellular fluid shift to the still-liquid component. This can result in a phenomenon known as the "solution effect," where the high concentration of dissolved ions in the extracellular fluid compromises the plasma membrane's integrity and structure. Additionally, this hyperosmotic state disrupts the osmotic balance between intracellular and extracellular tissues, leading to excessive shrinkage due to the removal of intracellular fluid. If this shrinkage surpasses the cell's tolerance limit, it can cause irreparable harm (Meryman, 2007).

Buffalo semen plasma contains the PDC-109 protein, which binds to the choline group of plasma membrane phosphatidylcholine (Harshan *et al.*, 2009). PDC-109 is crucial for inducing capacitation and acrosome reactions before

fertilization (Srivastava *et al.*, 2013). During the cryopreservation process, spermatozoa are exposed to semen plasma for an extended period, resulting in excessive phosphatidylcholine efflux and destabilization of the plasma membrane (Manjunath, 2012). Compared to cattle, the higher level of phosphatidylcholine in buffalo spermatozoa membranes (Andrabi, 2009) is believed to cause significant damage to buffalo spermatozoa during cryopreservation. Additionally, PDC-109 exhibits a strong affinity for low-density lipoprotein (LDL) from egg yolks (Manjunath, 2012), as well as for milk proteins, including both casein and whey (Lusignan *et al.*, 2011).

The sexing method using the BSA gradient has a long history of use and is generally considered efficient compared to other methods. It has been applied to various species, including sheep spermatozoa (Maxwell *et al.*, 1984), pigs (Dixon *et al.*, 1980), and hamsters (Dow and Bavister, 1989). Separating spermatozoa with a BSA gradient is believed to have a minimal impact on sperm manipulation. Additionally, spermatozoa exposed to a BSA medium are often added to semen diluent (before freezing) to prevent a decline in spermatozoa quality after separation.

However, in this study, the quality of buffalo spermatozoa was significantly reduced due to the use of BSA after the freezing procedure (post-thawing). According to Dixon *et al.* (1980), when BSA is used before the freezing process, the motility of spermatozoa from insemination can approach 70%. Afati (2004) suggested that the percentage of spermatozoa resulting from albumin gradient sexing was predicted to carry X chromosomes at 80.88% and Y chromosomes at 58.82%, with post-sexing motility reaching 75.00%. More over, Maxwell *et al.* (1984) and Kaiin *et al.* (2008) reported that the sexing process using BSA before freezing could maintain post-thawing motility of sexed spermatozoa by 49%.

The motility percentage still meets the requirements of the Indonesian National Standards for artificial insemination purposes, which stipulate that frozen semen used for artificial insemination must have post-thawing motility of at least 40%. In this study, the percentage of post-thawing motility was 41% (still meeting the requirements for artificial insemination), but it dropped to 35% after treatment with BSA after freezing (post-thawing). This does not meet the requirements set by the Indonesian National Standard (SNI) for artificial insemination (AI) and IVF. Therefore, sexing with a BSA column after freezing is not recommended.

Table 1. Buffalo frozen semen treated with BSA and SU after the freezing process

Parameters	PT	BSA		SU	
		UF	LF	UF	LF
Motility (%)	41±7.38 ^{bc}	34±8.43 ^{ab}	32±4.22 ^a	42±6.32 ^c	41±3.16 ^{bc}
Viability (%)	64.48±4.82 ^b	49.36±10.83 ^a	44.71±7.74 ^a	63.62±4.36 ^b	62.78±7.42 ^b
IPM (%)	55.42±7.36 ^b	44.78±8.29 ^a	37.13±7.56 ^a	54.42±4.47 ^b	54.74±4.06 ^b
IAC (%)	47.12±9.83 ^c	37.68±6.45 ^{ab}	33.27±7.86 ^a	46.94±4.96 ^c	45.74±3.86 ^{bc}
DNA integrity (%)	74.94±9.12 ^a	74.76±5.19 ^a	72.45±2.27 ^a	70.57±6.38 ^a	70.01±5.22 ^a

^{a,b,c} Different superscripts in the same row indicates differences in each of treatment (P<0.05).

BSA: bovine serum albumine; SU: swim up; PT: post-thawing; UF: upper fraction; LF: lower fraction; IPM: intact plasma membrane; IAC: intact acrosome cap.

Spermatozoa resulting from the BSA gradient sexing method are believed to exhibit poor motility and membrane integrity when applied after freezing. The low quality of post-thaw spermatozoa was the primary factor contributing to the significant decline in spermatozoa quality after BSA treatment. The integrity of the plasma membrane and acrosome of spermatozoa plays a crucial role in supporting the success of the fertilization process. Spermatozoa with damaged plasma membranes cannot bind to the zona pellucida (the binding zone). This loss occurs due to the absence of membrane receptors that typically bind the zona pellucida (Ducha *et al.*, 2012), affecting the embryo's ability to develop (Okabe, 2013). The integrity of the spermatozoa membrane is vital because various components, including proteins, phospholipids, cholesterol, and several others, function to protect and maintain the stability of the plasma membrane and prevent premature capacitation and acrosome reactions (Carvalho *et al.*, 2010). Assessing the post-thawing spermatozoa's motility is one of the factors that determine the success of fertilization.

In general, sexing spermatozoa using the BSA gradient method typically involves graded concentrations and is performed before the freezing process. This method is more straightforward compared to others, such as flow cytometry. The advantage lies in the minimal treatment of spermatozoa during the sexing process, allowing them to maintain motility and reducing damage to spermatozoa morphology (Dixon *et al.*, 1980). In this study, BSA was added after dilution of the spermatozoa. Consequently, the spermatozoa remained exposed to various cryoprotectant ingredients, further exacerbated by the addition of BSA.

Dow and Bavister (1989) reported concerns regarding the direct exposure of spermatozoa to BSA, fearing it might lead to capacitation and early acrosome reactions. This is because the protein in bovine serum albumin can bind to cholesterol and zinc ions in the spermatozoa's plasma membrane, resulting in cholesterol loss, membrane destabilization, and increased membrane fluidity (Abou-haila and Tulsiani, 2009; Visconti *et al.*, 1999). An unstable plasma membrane can increase its permeability to calcium ions (Ca²⁺), leading to the fusion of the outer acrosome membrane and triggering the acrosome reaction. High intracellular concentrations of Ca²⁺ ions are necessary for increasing tyrosine protein phosphorylation, which, in turn, triggers the movement of the spermatozoa flagellum. However, maintaining a high concentration of Ca²⁺ ions for an extended period before fertilization is undesirable because it can lead to the death of spermatozoa.

Motility is a critical parameter in the fertilization ability of spermatozoa and enhances their ability to penetrate the zona pellucida of oocytes (Suarez and Ho, 2003). In IVF, the sperm preparation method selects live, motile spermatozoa with normal morphology (Navarro-

Serna *et al.*, 2021). The swim-up method is one of the techniques used for preparing spermatozoa in IVF. It specifically selects spermatozoa with high motility that reach the surface of the media after incubation. The swim-up method relies on the active movement of spermatozoa from the pellets at the bottom of the medium to the surface (Henkel and Schill, 2003). In this study, the swim-up method proved to be the most effective for frozen semen. Notably, it did not show a significant difference in motility compared to post-thawing motility. Furthermore, the swim-up method offers practical advantages, saving on technical steps and minimizing the risk of damage to the spermatozoa's cytoplasmic membrane (Inaudi *et al.*, 2002). Therefore, the swim-up treatment is a viable option for use with buffalo semen after freezing, particularly in preparation for the IVF process.

The swim-up method's procedure can selectively choose spermatozoa with better chromatin quality and fewer morphological abnormalities, resulting in a higher division rate when used in IVF (Gillan *et al.*, 2008). Research by Shamsuddin *et al.* (1993) demonstrated high motility results after the swim-up process in modified Tyrode's lactate solution (TALP) media; however, there was a subsequent decrease shortly after centrifugation. The swim-up method is a technique for separating spermatozoa based on the movement of motile spermatozoa toward the surface of the media after incubation. Semen plasma separation techniques or cryoprotectants, along with the separation of motile from non-motile spermatozoa, can be performed to enhance the fertilization ability of spermatozoa (Henkel and Schill, 2003). The ability of spermatozoa to reach the media's surface is used to select motile spermatozoa.

Tanphaichitr *et al.* (1988) reported that using the swim-up method for sperm separation yielded a high fertilization rate when the semen sample was high quality. Park *et al.* (2009) stated that the swim-up method reduced the polyspermy rate in IVF of pigs. This is because the swim-up method can control the number of motile spermatozoa that reach the oocyte and limit the number of spermatozoa binding to the zona pellucida. Polyspermy at fertilization can occur in several species of mammals for various reasons, including oocyte aging, abnormalities in the zona pellucida, a high number of capacitated spermatozoa in the fertilization medium, and inappropriate culture media conditions both before and during IVF (Wang *et al.*, 2003).

Conclusions

Using the BSA column method on buffalo semen after the freezing process significantly reduces the quality of buffalo spermatozoa. Conversely, employing the swim-up method on buffalo semen after freezing is more effective in preserving the quality of buffalo spermatozoa.

Conflict of interest

All authors declared that there is no conflict of interest.

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Author's contribution

Masrizal and Ananda analyzed the data and wrote the manuscript. Masrizal and Tinda Afriani designed the concept, searched for funding, and compiled and reviewed the paper. Dwiki Wahyudi oversees field and laboratory work. Dwiki Wahyudi and Savira Saharani conducted field and laboratory work and data tabulation.

Ethics approval

The conducted research is not related to either human or animals use.

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