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The Quality of Buffalo Sperm Following Preservation Using Different Diluents and Sperm Concentrations

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ABSTRACT

Artificial Insemination (AI) success depends on the quality of the frozen semen. The quality of the frozen semen of swamp buffalo in Indonesia is still low. The study was conducted to determine the quality of buffalo sperm following freezing using three different diluents and three different doses. The study used buffalo semen from the Tuah Sakato Artificial Insemination Center, Payakumbuh (n = 3). The semen collecting was carried out once a week for 10 weeks (replication). The research method used was 3x3 factorial randomized block design. The first factor was diluent (Triladyl®, Andromed® and Tris egg- yolk) and the second factor was the dose of spermatozoa (10 and 15 and 20 x 10^6 sperm/ml). Data were analyzed using variant analysis, while the differences between treatments were tested by Duncan Multiple Range Test. The results showed that the plasma membrane integrity of buffalo sperm was found in Andromed® diluent, while tris egg-yolk diluent gave better motility, viability, plasma membrane integrity and recovery rate at a sperm concentration of 20 x10^6 sperm/mL compared to triladyl® diluent and a sperm concentration of 10 and 15 x10^6 sperm/mL. It was concluded that andromed® diluent and tris egg-yolk gave better motility, viability, plasma membrane integrity and recovery rate at a sperm concentration of 20 x10^6 sperm/mL compared to triladyl® diluent and a sperm concentration of 10 and 15 x10^6 sperm/mL. of Buffalo of sperm abnormalities not sicnificantly by the type of diluent but are influenced by sperm concentration.

Key words: Abnormality, Buffalo, Motility, Membrane plasma integrity, The recovery rate

Introduction

Artificial insemination (AI) is a reproductive technology that is proven to be effective and can be widely applied in the field (Singh and Balhara, 2016). The quality of semen is one of the factors that can succeed in the AI program. Freezing technique, type of diluent and sperm concentration are those the indicators of semen quality (Ariantie *et al.*, 2013). One of the purposes of the use of semen diluents is to maintain the quality of spermatozoa during preservation. Diluent of Tris egg-yolks, andromed®, and triladyl® have been used in semen cryopreservation of various animal species. However, the contradictory results related to the ability of these three diluents to maintain semen quality during freezing has been reported by some researchers. Dorado *et al.* (2010) and Alamaary *et al.* (2019) stated that tris egg-yolk is better in goat and horses semen preservation. While Optixell's diluent (Naz *et al.*, 2018) and andromed® diluents (Ansari *et al.*, 2017) were both better for Nili-Ravi Buffalo semen preservation. Researchers' reports on sperm concentration doses in diluents are also different. Alvarez *et al.* (2012) stated that the best concentration in Pigs is 800 x 10^6 sperm/mL. In contrast to Stuart *et al.* (2019), the best concentration of sperm on alpacas is 50 x 10^6 sperm/mL and Gaviraghi *et al.* (2013) reported that sperm concentrations of 4, 6 and 8 x 10^6 sperm/mL in 0.25 mL straw were not significantly associated with pregnancy in Mediterranian Italian buffalo.

Buffalo semen has 60% phosphatidylcholine that composes the spermatozoa membrane (Andrabi, 2009). This makes the chances of choline in inducing acrosomes higher, thus causing damage to the sperm membrane of buffalo (Manjunath, 2012). Cold shock due to the decrease in temperature during freezing requires a balanced diluent and sperm concentration so as not to cause structural changes, membrane damage and decreased

metabolic function (Holt, 2000). The purpose of the study was to find the right type of diluent and sperm concentration for the freezing of swamp buffalo.

Materials and Methods

Semen was collected using artificial vagina from three buffalo-bulls raising in Tuah Sakato Artificial Insemination Center. The age of bulls were 7 years old in averages and the weighs ranged between 450-500kg. The semen was collected in the morning (at 08.00 am) once a week for 10 weeks. Semen had motility >60% and >800 x 10^6 sperm/mL of sperm concentration were used for frozen processing.

Preparation of diluents

The Andromed® and *Tryladyl* (Minitub Germany) diluents were each mixed with aquabidest (20/80, v/v), homogenized and stored in the refrigerator. While Tris-egg yolk diluent were obtained by mixing 3,634g tris (hydroxymethyl) aminomethane, 0.5g of glucose and 1.99g of citrate acid, and diluted into 80mL aquabidest. The tris diluent were homogenized for 15 minutes, boiled and cooled to a temperature of 37^0C before mixed with 20mL of egg yolks. This tris-egg yolk diluent were then homogenized for 60 minutes, added by 6% glycerol, 1000IU/mL benzylpenicillin and 1000mg/mL streptomycin sulfate, homogenized and cooled at a temperature of 37^0C (Herbowo *et al.*, 2019). All chemicals purchased from Sigma-Aldrich Co, USA.

Dilution and freezing

Semen samples with different concentrations (10, 15, and 20 x 10^6 sperm/mL) were each diluted with andromed®, triladyl® and tris-egg yolk diluents. The diluted semen was loaded into 0.5mL straw (Minitub, Tiefenbach, Germany) and equilibrated at 4^0C for 5h. After equilibration, the straws were placed on 8cm above liquid nitrogen (LN_2) for 10 minutes for freezing process, and finally plunged into LN_2 (-196^oC) for storage.

Post thawed evaluation of frozen semen

After 24h of storage, the straw were thawed in the water at 37^0C for 5 seconds. Sperm motility evaluation were conducted by mixing one drop of semen with four drops of saline solution and put on the clean object-glass then covered with cover glass and evaluated under a light microscope at 400x magnification. While sperm viability and abnormality evaluation were observed by mixing one drop semen and 4 drops eosin-nigrosin on clean object-glass, homogenized, smeared, and dried above the heating plate. The smear was then evaluated using light microscope at 400x magnification. Sperm plasma membrane integrity (PMI) was evaluated by using the HOST (Jeyendran *et al.*, 1984 cit. Yendraliza *et al.*, 2019). The value of PMI was shown as a percentage of intact sperm (total 200 sperms counted). The recovery rate (RR) is comparison sperm motility after thawing to fresh semen was determined.

Statistical analysis

All data were shown as mean value ± SEM and analyzed using variant analysis. While the differences between treatments were analyzed using Duncan's multiple range tests. Differences were considered significant at $p<0.05$. All statistical analyses were performed with SPSS 16 package program for windows.

Results and Discussion

The quality of fresh semen

The quality of fresh semen buffalo was presented in Table 1. The average volume was 1.20±0.30 mL with pH 7±0.0 and the color of semen buffalo in this study was a cream color and watery consistency.

The frozen semen quality

The frozen semen quality of buffalo was presented in Table 2. The post thawing quality of buffalo sperm showed highest motility in andromed® diluents at sperm concentration of 20 x 10^6 sperm/mL ($P<0.05$). The sperm abnormalities not sicnificantly by the type of diluent but are influenced by sperm concentration ($P<0.05$). The observed viability of buffalo sperm had the highest values on tris egg-yolk diluents and sperm concentrations of 20 x10^6 sperm/mL compared to andromed® and triladyl® diluents ($P<0.05$). However, were the highest value of sperm PMI was obtained in the tris egg-yolk diluent and andromed® at a sperm concentration of 20 x10^6 sperm/mL compared to triladyl® diluents ($P<0.05$). The best recovery rates of buffalo sperm were obtained on andromed® diluents followed by tris egg-yolk compared to triladyl® diluents at sperm concentrations of 20 x 10^6 sperm/mL.

Table 1. Quality of fresh buffalo sperm

Variable	Mean ±SEM
Volume (mL)	1.20 ± 0.30
pH	7 ± 0.0.
The color of semen	cream
consistency	watery
Concentration (sperm/mL)	1.200 x 10^6 ± 4.75
mass activity	++
Motility (%)	75.5 ± 5.5
Viability (%)	90
Abnormality (%)	10 ± 1.0
Plasma membrane integrity (%)	70 ± 5.7

Table 2. Quality of buffalo sperm after thawing with difference diluents and sperm concentration (mean±SEM)

Variable	Diluents	Sperm concentration		
		10 x 10^6	15 x 10^6	20 x 10^6
Motility	Tris-eggyolk	48.00±9.64^{aB}	55.00±7.26^{aB}	62.83±3.69^{bB}
	Andromed®	50.50±0.39^{aB}	64.83±0.46^{bC}	68.50±0.31^{cC}
	Triladyl	33.67±4.48^{aA}	47.33±4.75^{bA}	53.00±4.77^{bA}
Abnormality	Tris egg-yolk	10.00±1.00^{A}	12.67±1.53^{A}	11.33±1.15^{A}
	Andromed®	11.00±0.39^{A}	10.33±0.46^{A}	9.67±0.31^{A}
	Triladyl	21.00±1.00^{B}	20.33±2.52^{B}	18.33±2.52^{B}
PMI	Tris egg-yolk	54.67±6.11^{aB}	50,33±2.52^{aB}	60.00±4.00^{bB}
	Andromed	49.33±0.39^{aB}	55.00±0.46^{bB}	60.33±0.31^{bcB}
	Triladyl	28.67±3.21^{aA}	30.67±3.06^{aA}	32.33±3.21^{aA}
Viability	Tris egg-yolk	57.33±2.52^{cB}	64.33±3.79^{cC}	66.67±3.21^{cC}
	Andromed®	58.00±0.39^{cB}	50,67±0.46^{bB}	57.00±0.31^{cB}
	Triladyl	35.33±5.03^{aA}	41.67±1.53^{bcA}	43.67±3.21^{bcA}
Recovery rate	Tris egg-yolk	63.58±7.70^{aB}	72.85±9.60^{bB}	83.22±4.80^{cB}
	Andromed®	66.89±1.39^{aB}	85.87±1.40^{bC}	90.51±1.31^{bcC}
	Triladyl	39.74±7.80^{aA}	62.69±6.29^{bA}	70.20±6,32^{cA}

Means in the same columns (uppercase) and rows (lowercase) with different superscripts differ significantly (P<0.05), PMI: plasma membrane integrity, mean ± stdev

The characteristics of fresh buffalo semen in this study have normal buffalo semen characteristics following the Indonesian Standardzation National (INS) year 2008 (Standardisasi Nasional Indonesia (SNI), 2008). The results of this study also support the findings of previous research Bhakat *et al.* (2011), Ghodasara *et al.* (2016) and Kaka *et al.* (2016).

The results of this study indicated that there was a significant interaction effect of diluent and sperm concentration on motility, viability, PMI and RR. But, the type of diluent did not have a significant effect on the concentration and abnormality of buffalo sperm. The results of this study differ from of Akhter *et al.* (2010), Ansari *et al.* (2017), Rakha *et al.* (2016) who stated that the motility and intact plasma membrane in Nili-Ravi buffalo were not significantly different in the diluents of bioexcel®, tris citrate, and andromed®.

The best post-thawing buffalo sperm quality in this study at a sperm concentration of 20 x10^6 sperm/mL was different from reports Morton *et al.* (2007) and Stuart *et al.* (2019) that the best sperm concentration is 50 x 10^6 sperm/mL. The effectiveness of cryopreservation depends on the number of spermatozoa viable to process (Naz *et al.*, 2018). Spermatozoa's resistance to cold shock is related to the permeability of hydraulic membranes, tolerance levels and domain order to membrane phase changes (Sieme *et al.*, 2016). Changes in membrane function due to decreased temperature will lead to dysfunction of ion canal regulation which will eventually result in decreased motility and viability function (Oldenhof *et al.*, 2013). This is seen in the decrease in the value of intact plasma membranes in each type of diluent and in each sperm concentration used in this study. Buffalo sperm on andromed® diluents has a higher motility than buffalo sperm in tris egg-yolk and triladyl® diluents. However, buffalo sperm on andromed diluents and tris egg-yolk at sperm concentrations of 20 x 10^6 sperm/mL have the same PMI but have different viability of sperm. Repairing the cell plasma membrane will have a positive impact on the motility and vitality of spermatozoa. This is because the motility of spermatozoa is highly dependent on the energy supply in the form of Adenosine Triphosphate (ATP) which is the result of metabolism (Manjunath, 2012).

The sperm motility of buffalo in this study (33.67%-68.50%) was different from that of the Nili-Ravi buffalo sperm in Pakistan (26.51-35.11%) (Naz *et al.*, 2018) and buffalo in Egypt (33.00-41.50%) (El-Sisy *et al.*, 2016). The death of spermatozoa is caused by the process of freezing and re-thawing. Khalil *et al.* (2018) states that cell damage due to freezing can occur due to dehydration, increased electrolyte concentrations, and the formation of intracellular ice crystals which can affect cell wall permeability and ultimately spermatozoa lose their motility. The loss of spermatozoa motility during the frozen process will affect the rate of recovery of sperm after thawing (Singh *et al.*, 2018). This can be seen from the low abnormality and high motility resulting in high recovery rate in this study.

The percentage of abnormalities in buffalo sperm in this study (9.67 - 21.00%) was lower than the abnormalities sperm in bulls in Egypt (20.33 - 33.00%) (El-Sheshtawy *et al.*, 2018). However, the abnormality value in this study was almost the same as the abnormality of buffalo sperm in tris-egg yolk diluent at different glycerol concentrations (7.80-16.30%) (El-Sisy *et al.*, 2016). The percentage of PMI of buffalo sperm in this study was higher than PMI of buffalo sperm in Pakistan (44.4-46.8%) that used andromed® and tris egg-yolk diluent (Ansari *et al.*, 2017) but not different from PMI of buffalo sperm in Ciawi that used tris egg-yolk diluent (51.38-62.41%) (Herbowo *et al.*, 2019).

The viability of buffalo sperm in this study (35.33 – 66.67%) was different from reports Herbowo *et al.* (2019) which used tris egg-yolk diluent (49.08- 61.52%) and reports Ansari *et al.* (2017) which used andromed® and tris egg-yolk diluents (61.5-67.5%). The difference in viability was caused by different types of buffalo, type of diluent and feed (Manjunath, 2012). In this study it was seen that egg yolk has the potential to overcome ROS-mediated loss of sperm integrity during freeze thawing process. The recovery rate of buffalo sperm after thawing in this study was

different from the recovery rate of FH bull sperm using tris egg-yolk diluents, andromed® and soyben lecithin (Arifiantini and Yusuf, 2010) and RR on Alpaca sperm in tris egg-yolk diluent with a sperm concentration of 50 $x10^6$ sperm/mL. The differences in abnormalities, plasma membrane, viability and RR values were due to different types of treatments, sperm and diluents (Holt, 2000).

Conclusions

Andromed® diluent and tris-egg yolk gave better motility, viability, plasma membrane integrity and recovery rate at a sperm concentration of 20 $x10^6$ sperm/mL. Buffalo sperm abnormalities were not different in all types of diluents, but the best sperm abnormality value was at a sperm concentration of 20 x 10^6 sperm/mL.

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