THE FERTILITY OF BUFFALO SPERM (*Bubalus bubalis*) ON THREE DILUENT TYPES AND THREE EQUILIBRATION TIMES

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ABSTRACT

This study objective was to determine the fertility of buffalo sperm in response to different types of diluent and equilibration times. The first research stage was arranged in completely randomized design with two factors, i.e., diluent types (tris egg yolk, andromed, and triladyl) and equilibration times (3 h, 4 h and 5 h). The second research stage was the fertility test of frozen semen that artificialy inseminated on 90 4-years-old buffalo-cows. The observed variables were the percentage of motility, abnormality, membrane plasma integrity, acrosome intact, recovery rate and pregnancy rate. The result showed that there was no interaction between diluent types and equilibration times on all observed variables. The best buffalo sperm fertility was produced from andromed and tris egg yolk compared to triladlil diluent. The best equilibration time for buffalo semen was 5 h rather than 4 h and 3 h.

Keywords: *Bubalus bubalis*, buffaloes, abnormality, acrosome intact, motility, membrane plasma integrity, pregnancy rate

INTRODUCTION

The quality of frozen semen is determined by freezing technique, type of diluent and type and concentration of cryoprotectant (Ariantie *et al*., 2013; Singh and Balharas, 2016). Contradictory reports found, i.e. (i) the ability of andromed with tris egg yolk to maintain the quality of buffalo semen (Ansari *et al*., 2017); (ii) the ability of bioexcel diluents with egg yolk-tris citrate to maintain the buffalo semen quality (Shahverdi *et al*., 2014); (iii) the ability of egg yolk diluents, egg-yolk citrate and egg-yolk fructose to maintain the quality of FH semen (Arifiantini and Purwantara, 2010); (iv) the ability of triladyl, egg-yolk diluents to maintain the buffalo semen quality (Naz *et al*., 2018).

Buffalo spermatozoa have phosphatidylcholine (60%) and phosphatidylethanolamine (23%) compared to cow sperm, so that buffalo sperm are susceptible to freezing and sensitive to cold shock (Andrabi, 2009). Spermatozoa have different adaptive abilities in integrating with diluents used at critical temperatures. Some studies report that a good

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equilibration time for buffalo is 5 h (Febriani *et al*., 2014), while other study stated 2 h (Shahverdi *et al*., 2014). In response to the variation of results produced, there is a need to optimize the equilibration times and diluent types specifically for mud buffalo semen in Riau. Therefore, this study was aimed to determine the fertility of buffalo sperm in term of motility, abnormalities, membrane plasma integrity, acromosom intact, recovery rate and pregnancy rate in response to different equilibration times and diluent types.

MATERIALS AND METHODS

Research materials

This study used fresh semen of 4-years-old bull buffalo that was maintained at the Tuah Sakato artificial insemination center in Payakumbuh. Several diluents used were Tris-egg yolk diluent (T-KT), commercial TKT concentrate diluent (Triladyl), and plant-based commercial diluent or soybean lecithin (Andromed). Other prepared materials were aquabides, antibiotics (streptomycin and penicillin), glycerol, eosin-nigrosin, KY jelly, physiological NaCl, 1% formalin, aquades, alcohol, and liquid nitrogen. The materials for the second reserach stage was 90 buffalo-cows that had already given birth with a bodyweight around 400 to 450 kg. Other materials used were 2.5 ml PGF2α (Dinoprost thromethamine 10 ml) for synchronization, syringe for injection, gun for artificial insemination and vaseline.

Research methods

The research was consisted of two stages. The first stage was arranged in completely randomized design with two factors. The first factor was diluent type, i.e tris egg-yolk, andromed and triladyl. The second factor was the equilibration time, i.e 3 h, 4 h and 5 h. In total, there were 6 treatments and every treatment was replicated 10 times. The second stage was the fertility test of frozen semen in the field on 90 buffalo-cows.

Research procedures

The research procedure for the first stage was initiated by the semen collection. Semen was collected using an artificial vagina once a week for about 10 weeks. The collected semen was evaluated macroscopically in term of color, odor, pH, consistency, volume, and also microscopically in term of mass movement, sperm concentration, motility, abnormality and membrane plasma integrity. The egg yolk-tris diluent consisted of tris aminomethane 3.634 g, citric acid 1.99 g, glucose 0.50 g, aquabides 100 ml, egg yolk 20 ml, glycerol 6.4 ml, penicillin 0.3 ml, and streptomycin 0.4 ml. Andromed diluents were diluted with 80% aquabides (1: 4) and homogenized. Triladyl diluents were diluted with 80% aquabides and homogenized. The diluted semen was packed into 0.5 ml straw and then the equilibration stage was carried out for 3 h, 4 h and 5 h in a temperature of 5^{o}C using refrigerator. The pre-freezing process was done by placing the straw on the surface of liquid nitrogen (-110^{o}C) as high as 3 cm using a styrofoam box for 10 minutes at -100^{o}C. The main freezing treatment was performed by inserting the straw into liquid nitrogen (-196^{o}C) for 24 h. The frozen straw was thawed in warm water (37^{o}C) for 30 seconds.

The research procedure for the second stage was started by grouping of the buffalo-cows into 3 groups, based on the bodyweight, i.e 400 kg, 425 kg and 450 kg. All bufalloes-cows were synchronized with 2.5 ml PGF2α (Dinoprost tromethamine 10 ml) intramuscularly (im). The

lust of buffaloes, indicated by the release of mucus from the vulva, were ready to artificially inseminate with the straw produced from the first research stage. The artificial insemination was performed by Kampar livestock services inseminators and veterinary health officer.

Research variables

Motility was indicated by the percentage of progressive moving spermatozoa. It was measured eight times under light microscope with a 400x magnification lens. The number of moving spermatozoa was determined in between 0 to 100% with a 5% scale. The percentage of live spermatozoa: There were 200 spermatozoa evaluated by using eosin-nigrosine staining method under a light microscope with 400x magnification lens. Living spermatozoa was indicated by a clear head, while the dead one was marked by a red head. Abnormality of spermatozoa were observed under microscope with a magnification lens of 450x. The sample was prepared from one drop of sperm mixed with one drop of eosin. Normal and abnormal spermatozoa were counted to 200 cells (Garner and Hafez, 2016). Acrosome intact (%) was indicated the acrosome hood of the spermatozoa and evaluated by using a phase-contrast microscope with a magnification lens of 100x. The semen was mixed with NaCl plus 1% formalin in order to kill and fixate spermatozoa. The number of spermatozoa was evaluated by 200. The evaluation was carried out with a scoring system of 0% to 100%. Membrane plasma integrity (%) was marked by circular spermatozoa tail after being inserted into a hypoosmotic medium 0.032 M NaCl (NaCl 0.179 g in 100 aquabides). Incubation was carried out at 37^{o}C for 1 h. The evaluation was carried out under a light microscope magnification lens of 40x. The results were rated in scoring system of 0% to 100%. All variables were measured after thawing. The recovery rate was calculated by reducing fresh sperm motility with sperm motility after freezing. The pregnancy rate was calculated by dividing the number of pregnant buffaloes by the total of observed buffaloes.

Data analysis

Data were pooled and processed by using analysis of variance (F test). Any significant differences between treatments were further tested by Duncan Multiple Range Test at α 5% (Steel *et al*., 1991).

RESULTS

The quality of fresh buffalo semen was stated in Table 1. The mean values of motility, abnormality, membrane plasma integrity and acrosome intact of buffalo semen were significantly affected ($P<0.01$) by different types of diluents both after equilibration at 5^{o}C and after thawing at 37^{o}C. The use of andromed diluents produced the highest motility, abnormality, membrane plasma integrity and acromosome intact compared to egg yolk-tris and triladyl diluent (Table 2).

The equilibration times for about 5 h and 4 h showed the higher motility than 3 h equilibration time. There was no interaction effect between the diluent types and the equilibration times on the motility, abnormality, membrane plasma integrity and acrosome intact (Table 3). In addition, the recovery rate and pregnancy rate in treatment of andromed, tris egg-yolk higher than tryladlil diluent (Table 4).

DISCUSSIONS

In general, the quality of buffalo semen was categorized as a good quality and feasible to be diluted (National Standardization Agency of Indonesias, 2008). This study results was not so much different from previous study with Thailand buffalo semen (Koonjaenak *et al*., 2007) and buffalo semen in India (Das *et al*., 2017). The highest buffalo semen motility was recorded in the use of new Andromed diluents followed by tris egg yolk diluents and triladyl diluent. It was probably due to the different chemical composition among three tested diluents (Manjunath, 2012). The motility of buffalo semen in present study was differed from spotted buffalo motility, i.e 70.11% vs 41.67%, respectively (Yulnawati *et al*., 2010). Previous study on Iranian buffalo showed that there was no significant different on the semen motility in response to different diluents used egg yolk-tris compared to bioxcell diluents (Shahverdi *et al*., 2014). The difference on mentioned results was likely due to the variation on livestock type, livestock age and semen storage technique (Jainudeen and Hafez, 2016).

The equilibration time of 4 h and 5 h equilibration time showed the performance of diluents to inhibit the formation of ice crystals that could kill spermatozoa. In addition, previous study by Febriani *et al.* (2014) also obtained the best equilibration time at 5 hours. Similar results was also found for cattle (Leite *et al*., 2010). However, the best equilibration time for Aceh buffalo was 4 h (Eriani *et al*., 2017) and for Iranian buffalo was 2 h (Shahverdi *et al*., 2014).

The high rate of membrane plasma integrity in post-thawing buffalo semen showed that the metabolism took place normally so that the transporting process of substrate and electrolyte in and out of the membrane occurred so well (Manjunath, 2012). The membrane plasma integrity in this study was also differed from previous study on Aceh buffalo semen due to the different of diluent used (Eriani *et al*., 2017). In addition, the difference of membrane plasma integrity was also caused by the variation of semen quality and livestock age (Garner and Hafez, 2016). The best equilibration time in this study was 5 h and it was different from previous studies result, i.e. 4 h (Shah *et al*., 2016; Kumar *et al*., 2015). This showed that 5 h equilibration time could help buffalo spermatozoa in adapting and interacting with diluents so that the membrane integrity of spermatozoa could be well protected by diluents and the chemical potential balance of intracellular and extracellular water (Benson *et al*., 2012). Preservation in this study carried out with three types of diluents and three equilibration times resulted in the similar rate of spermatozoa abnormality (Ariantie *et al*., 2013). In opposite, 3 h and 4 h equilibration time were not sufficient to achieve that balance so that they damaged the cell lead to the death of spermatozoa after thawing (Febriani *et al*., 2014).

In general, the rate of acrosome intact in present experiment was still above the standard for buffalo sperm (National Standardization Agency of Indonesia, 2008). The acmosome intact of buffalo sperm in present study was differed from Pakistan buffalo (Shah *et al*., 2016). The difference of acmosome intact could be caused by the difference of livestock types, diluent types and preservation techniques (Manjunath, 2012).

The best equilibration time in this study was obtained at 5 h (67.72%), similar with Febriani *et al.* (2014) but different to Shahverdi *et al.* (2014) with the best equilibration time by about 2 h and Eriani *et al.* (2017) who got the best time of 4 h for Aceh buffalo. The enzyme system of sperm

Table 1. The macroscopic and microscopic characteristics of buffalo sperm.

Characteristics	Value
Macroscopic semen variables	
Volume (ml)	1.23±0.30
Smell	Spesific of swamp buffalo
pH	7±0.0
Color	Cream
Consistency	Thick
Microscopic semen variables	
Concentration (million)	1.233±47.25
Mass motion	++ (good)
Individual motion	2±0.0
Motility (%)	76.3±5.50
Viability (%)	73.21±0.65
Abnormality (%)	11±1.00
The membrane plasma integrity (%)	68.3±5.77

Table 2. The effect of different diluent types on motility, abnormality, membrane plasma integrity and acrosome intact of buffalo sperm at 5^{o}C and 37^{o}C.

Variables	Diluent types	Equilibration at 5^{o}C	After thawing at 37˚C
Motility	Tris-egg yolk	70.55^{a}	47.83^{a}
	Andromed	70.11^{a}	58.43^{a}
	Triladyl	63.34^{b}	35.01^{b}
Abnormality	Tris-egg yolk	11.22^{a}	12.32^{a}
	Andromed	10.17^{a}	11.83^{a}
	Triladyl	15.50^{b}	16.5^{b}
Membrane plasma integrity	Tris-egg yolk	64.33^{a}	53.2^{a}
	Andromed	64.42^{a}	56.95^{a}
	Triladyl	56.00^{b}	43.41^{b}
Acrosome intact	Tris-egg yolk	73.66^{a}	67^{a}
	Andromed	75.39^{a}	67.72^{a}
	Triladyl	65.09^{b}	56^{b}

Note: mean values followed by different alphabet superscripts in the same column were significantly different based on DMRT at α 5%.

Table 3. The effect different equilibration times on motility, abnormality, membrane plasma integrity and acrosome intact of buffalo sperm at 5°C and 37°C.

Variables	Equilibration times	Equilibration at 5°C	After Thawing at 37˚C
Motility	3	65.33[a]	42.67[a]
	4	72.67[b]	49.29[b]
	5	65.94[a]	49.32[b]
Abnormal	3	11.95[a]	13.07
	4	12.17[b]	13.39
	5	12.78[b]	13.05
Membrane plasma integrity	3	61.72[a]	47.39[a]
	4	60.50[a]	47.06[a]
	5	62.61[b]	59.28[b]
Acrosome intact	3	71.72[a]	61.72[a]
	4	70.50[a]	60.50[a]
	5	72.73[b]	68.61[b]

Note: mean values followed by different alphabet superscripts in the same column were significantly different based on DMRT at α 5%.

Table 4. The rate of recovery and preganncy rate of buffalo-cows in response to different diluent types with equilibration time of 5 h.

Diluent types	Recovery rate (%)	Pregnancy rate (%)
Tris-egg yolk	28.47[b]	83.33 (25 per 30)
Andromed	17.87[b]	86.67 (26 per 30)
Triladyl	41.29[a]	56.67 (17 per 30)

Note: mean values followed by different alphabet superscripts in the same column were significantly different based on DMRT at α 5%.

to ensure certain specific amino acids normally present in egg yolk and hydrogen peroxide yield which is toxic to sperm during storage under aerobic conditions (Hezavehei *et al*., 2018).

The difference recovery rate and the pregnancy rate of buffalo-cows was caused by the differences in motility, viability, membrane plasma integrity and acromosome intact from the straw used (Das, 1985). Previous study by Garner and Hafez (2016) showed that the motility affected the ability of sperm to fertilize an egg. In addition, the buffaloes used also had a different bodyweights resulting in different individual responses to the success of artificial insemination (Gordon, 2017).

CONCLUSION

The best buffalo sperm fertility was produced from andromed and tris egg yolk diluents compared to triladlil diluent with an equilibration time of 5 h compared to 4 h and 3 h.

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