



Effect of Three Diluent Types and Equilibration Times on the Quality and Fertility of Buffalo (*Bubalus bubalis*) Semen

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Abstract | The present research was conducted to determine the effects of three types of diluents and three equilibration times on the quality of buffalo semen. The experiment was arranged in a randomized block design having two factors, i.e., diluent types (tris egg-yolk, Andromed, and Triladyl) and equilibration times (3, 4, and 5 hours). In first stage of experiment, semen samples (n= 60) from two bulls were analyzed for motility (%), abnormality (%), membrane plasma integrity (%), acrosome intact (%), and recovery rate (%). In second stage, semen fertility was tested on buffalo-cows (n=90) by calculating the pregnancy rate (%). Results showed that there was an interaction between diluent types and equilibration times on motility, membrane plasma integrity, acrosome intact, and recovery rate. The motility, membrane plasma integrity, acrosome intact, and recovery rate of buffalo sperm was higher ($p<0.05$) with Andromed diluent than with tris egg-yolk and Triladyl diluents. Furthermore, the best equilibration time for buffalo semen was 5 hours rather than 3 and 4 hours. The pregnancy rate (86.67%) was higher in buffaloes inseminated with Andromed diluent semen compared to tris egg-yolk (83.33%) and Triladyl (56.67%) diluents. In conclusion, Andromed diluent with 5 hours equilibration time have better effect on the characteristics of post-thaw semen and increase the pregnancy rate in swamp buffalo.

Keywords | Tris egg-yolk, Acrosome intact, Motility, Diluent, Pregnancy rate

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INTRODUCTION

The swamp buffalo (*Bubalus bubalis*) is one of the major part of livestock in Indonesia that had significant role in animal protein requirement of country. According to the Indonesian Statistics Department, the buffalo population in Indonesia has decreased by 42.5% during 2004 to 2018 (BPS, 2018). One of the efforts that can be made to increase the population of buffalo in Indonesia is by optimizing reproductive technology. Artificial insemination (AI) is a reproductive technology that is proven to be effective and can be widely applied in the field (Singh and Balharas, 2016). One of the factors for the success of the AI program is the quality of the frozen semen used.

The freezing technique, the type of diluent, as well as the type and concentration of cryoprotectants, have been known to determine the quality of frozen semen in livestock (Ariantje et al., 2013; Singh and Balharas, 2016). Contradictory reports were found regarding, i.e. (i) the ability of Andromed diluent and tris egg-yolk diluent to maintain the quality of bull sperm (Ansari et al., 2017); (ii) the ability of bioexcel diluents and tris citrate egg-yolk to maintain the quality of buffalo sperm (Shahverdi et al., 2014); (iii) the use of tris egg-yolk diluent, citrate egg-yolk, and fructose egg-yolk to maintain the quality of Friesian Holstein sperm (Arifiantini and Purwantara, 2010); (iv) the ability of Triladyl and egg yolk diluents to maintain the fertility of buffalo semen (Naz et al., 2018). All these

mentioned studies indicates the further research on semen diluents.

Buffalo sperm freezes quickly, and it is more sensitive to cold shock compared to cow sperm because buffalo spermatozoa contain phosphatidylcholine (60%) and phosphatidylethanolamine (23%) (Andrabi, 2009). At a critical temperature, any spermatozoa have different adaptability in integrating with the diluents used. Several studies have reported that a good equilibration time for buffalo sperm is 5 hours (Febriani et al., 2014), while other studies suggested two hours (Shahverdi et al., 2014). In response to the variety of results produced, there is a need to optimize the equilibration time and to determine the most suitable diluent type specifically for mud buffalo semen. Therefore, this study aimed to compare the effect of various diluents (tris egg-yolk, Andromed, and Triladyl) with different equilibration times (3, 4, and 5 hours) at 5 °C on the quality of buffalo sperm. The study further investigated the resultant sperms for the pregnancy rate in buffaloes.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

All the animal handling procedures in this experiment were carried out in compliance with the Animal Ethics and were approved by the Ethics Committee of the Faculty of Agriculture and Animal Science, UIN Suska Riau. The sample used in this study was the semen of a 5-years-old buffalo bulls (n=2) that were maintained at the Tuah Saka-to frozen semen station in Payakumbuh, Indonesia. Inseminations were then conducted on 90 buffalo-cows that had already given birth to Kampar, Riau, Indonesia. The temperature during the study was 22°C with 96% humidity. The semen was collected during January to March and insemination was carried out in April. The rectal palpation was conducted in July.

DILUENT PREPARATION

The tris egg-yolk diluent was obtained from the Tuah Saka-to frozen semen station. Tris egg-yolk diluent (YET) consists of 3.63 g tris (hydroxymethyl-aminomethane), 1.99 g citric acid, 0.50 g glucose, 74 mL distilled water, 20 mL egg yolk, 6.4 mL glycerol, 1000 IU / mL of penicillin (Pharmacia & Upjohn, Belgium), and 1000 µg / mL of streptomycin (Pharmacia & Upjohn, Belgium). All chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA). Both Andromed® (AND) and Triladyl® (TRI) diluents were diluted with 80% distilled water (1:4) and homogenized.

SEMEN PROCESSING

Semen collection was carried out once a week for ten weeks per buffalo bull using an artificial vagina (IVM, France) at

42°C. The only ejaculation collected had a sperm concentration greater than 800 x 10⁶ sperm/ml, >70% motility. After the collection, semen was observed microscopically (x400, Olympus B x 20, Tokyo, Japan) for sperm progressive motility, and sperm concentration was determined using a digital photometer (IVM, France). After the evaluation, the semen was divided into three aliquots and diluted with YET, AND or TRI at 37 °C according to freezing standards. After the dilution, the semen was infilled to straw 0.5 mL with a sperm concentration of 50 x 10⁶ sperm/mL. For the sake of stability, the semen was maintained in a water bath for 10 min at 35°C and then cooled from 35 °C to 25°C in one hour at room temperature. The straw was cooled from 25 °C to 4 °C in a cold cabinet and equilibrated for 3, 4 and 5 hours. The pre-freezing process was carried out by placing the straw on a liquid nitrogen surface as high as 3 cm using a Styrofoam box for 10 minutes at a temperature of -100°C. The straw was dipped and store in liquid nitrogen at -196 °C for 24 hours.

EVALUATION OF SEMEN QUALITY CHARACTERISTICS

The frozen semen thawing was carried out in a water bath for 30 second at 37°C to evaluate sperm motility, abnormality, viability, plasma membrane integrity, and acrosome integrity (Sarıözkan et al., 2014). Motility was measured eight times under a light microscope with a 400x magnification lens. The number of moving spermatozoa was determined between 0 to 100% with a 5% scale. About 200 spermatozoa were observed by means of eosin-nigrosine staining under a light microscope with a 400x magnification lens. Live spermatozoa were marked with a clear head, while a red one marked those that died. Abnormality of spermatozoa was observed under a microscope with a magnification lens of 450x. The sample was prepared by mixing one drop of semen with one drop of eosin. The 200 spermatozoa cells were counted to determine the normal and abnormal ratio (Garner and Hafez, 2016). Acrosome intact (%) indicated the acrosome hood of the spermatozoa and was evaluated through a phase-contrast microscope with a magnification lens of 100x by killing and fixation of spermatozoa mixed with NaCl plus 1% formalin. The evaluation was carried out using a 0 to 100% rating system for 200 spermatozoa. Plasma membrane integrity (%) was characterized by coiled spermatozoa tails after being put into 0.032 M NaCl hypoosmotic medium (0.17g NaCl in 100 mL distilled water). Afterward, incubation was carried out at 37°C for one hour. The evaluation was performed under a light microscope with 40x magnification lens. The results were rated in the scoring system of 0% to 100%, while the recovery rate was calculated by subtracting fresh sperm motility with sperm motility after freezing.

ARTIFICIAL INSEMINATION

In second stage, the semen was used for AI on 90 buffalo-

Table 1: The characteristics of fresh buffalo semen.

Characteristics	Average with standard deviation
Macroscopic characteristics	
Volume (ml)	1.23±0.30
pH	7.0±0.0
Color	Cream
Consistency	Thick
Microscopic characteristics	
Concentration (million/ml/ejaculation)	1.23±7.25
Mass activity (0-5 scale)	3.0 ± 7.8
Individual activity (0-5 scale)	2.0±0.0
Motility (%)	76.3±5.50
Viability (%)	73.21 ± 0.65
Abnormality (%)	8.0 ±1.0
Plasma membrane integrity (%)	68.3±5.77

Table 2: Effect of different diluents and equilibration time on characteristics of buffalo semen samples after thawing at 37°C.

Variables (%)	Diluents	Equilibration times (hours)		
		3	4	5
Motility	Tris-egg yolk	42.33±2.53 ^{bb}	45.00±7.26 ^{bb}	56.17±8.41 ^{aA}
	Andromed	50.00±0.39 ^{ab}	59.76±0.46 ^{aA}	65.55±0.31 ^{aA}
	Triladyl	35.67±8.7 ^{cb}	37.33±3.4 ^{cb}	32.03±7.9 ^{bb}
Abnormality	Tris-egg yolk	12.76±4.0	12.50±4.2	11.38±2.5
	Andromed	11.00±1.3	11.01±2.4	10.11±1.2
	Triladyl	15.45±5.4	16.67±5.5	17.65±3.2
Plasma membrane integrity	Tris-egg yolk	49.27±15.5 ^{bb}	45.50±13.5 ^{bb}	65.00±8.9 ^{aA}
	Andromed	52.33±2.39 ^{bb}	53.67±3.46 ^{ab}	65.17±3.40 ^{aA}
	Triladyl	40.56±7.06 ^{bA}	42.00±8.90 ^{bA}	47.67±3.65 ^{bA}
Acrosome integrity	Tris-egg yolk	65.17±15.29 ^{ab}	60.83±11.27 ^{ab}	75.00±9.01 ^{aA}
	Andromed	62.33±0.39 ^{ab}	63.67±0.46 ^{ab}	77.17±0.31 ^{aA}
	Triladyl	57.67±5.06 ^{aA}	57.00±3.91 ^{aA}	53.67±7.65 ^{bA}

Note: means with different uppercase superscripts (A, B) in a row showing significant difference (at p<0.05) between the equilibration times, while means with different lowercase superscripts (a,b,c) in a column showing significant difference (at p<0.05) between the diluents.

Table 3: The rate of recovery and pregnancy rate of buffalo-cows in response to different diluent types with an equilibration time of 5 hours.

Diluent types	Recovery rate (%)*	Pregnancy rate (%)
Tris-egg yolk	73.61 ^b	83.33 (25/30)
Andromed	85.91 ^c	86.67 (26/30)
Triladyl	41.97 ^a	56.67 (17/30)

* means with different alphabet superscripts in the same column are significantly different at p<0.05. Recovery rate was calculated by subtracting sperm motility after freezing from fresh sperm motility.

cows that had already given birth with a bodyweight of 400-450 kg. All of the selected buffalo-cows were synchronized using 3 mL/animal (tail) gonadotropin-releasing hormone (GnRH; Fertagyl®) on the first day and 2.5

ml prostaglandin F2 alpha (PGF2α) in the form of Dinoprost tromethamine (10 mL; a synthetic analogue of the naturally occurring PGF2α) on the 7th day after GnRH injection. The buffalo showed estrus after the injection of

PGF2 α were inseminated with frozen semen from the first stage of the research. A total of 90 inseminations (30 inseminations/extenders) were tested on buffalo-cows in Kampar, Riau, Indonesia. Pregnancy was detected 60 days after artificial insemination by rectal palpation. The pregnancy rate was calculated by dividing the number of pregnant buffaloes by the number of buffaloes inseminated.

DATA ANALYSIS

Data were presented as mean \pm standard deviation. Data were statistically analyzed using Minitab 17 for Windows with a Randomized Block Design of two factors with three equilibration times, three types of diluents, and ten replications, except for the analysis of pregnancy rates with descriptions. The Duncan Multiple Range test was performed to compare the differences between groups. The difference in treatments was considered significant at $P < 0.05$ (Steel et al., 1991).

RESULTS

The quality of fresh buffalo semen was presented in Table 1. The average semen volume per ejaculation was 1.23 ± 0.30 ml with 7 pH. The mean motility, plasma membrane integrity, and acrosome integrity of buffalo semen were significantly affected ($P < 0.01$) except for the mean abnormalities by different types of diluent and equilibration time after the thawing at 37°C (Table 2). The use of Andromed diluents produced the highest motility, abnormality, membrane plasma integrity, and acrosome intact compared to egg yolk-tris and Triladyl diluent. The equilibration times of about 5 hours and 4 hours showed higher motility than that of 3 hours. Furthermore, the recovery rate and pregnancy rate from the use of Andromed and tris egg-yolk were higher than those produced from Triladyl diluent (Table 3).

DISCUSSION

The quality of fresh buffalo semen was good and feasible to be diluted (National Standardization Agency of Indonesia 2008). The average volume of semen in the study was 1.23 ± 0.30 ml, which was similar to Das et al. (2017) who reported that the volume of fresh semen in swamp buffalo of Assam ranged between 1.08 ± 0.06 to 1.78 ± 0.21 ml. However, Koonjaenak et al. (2007) reported higher volumes of 3.2 to 3.8 mL in swamp buffalo in Thailand. The mass activity in this study (3 ± 7.8) was lower than that of swamp buffaloes in India (3.51 ± 0.12 to 3.78 ± 0.06) (Das et al., 2017). The sperm motility found in this study ($76.3 \pm 5.50\%$) was similar to the one found in Thai swamp buffaloes (72.8 to 75.2%) (Koonjaenak et al., 2007), but was in contrast to that in Indian swamp buffaloes $78.94 \pm 0.49\%$ (Das et al., 2017). The viability of sperms of swamp buf-

faloes in this study ($73.21 \pm 0.65\%$) was lower than that of swamp buffaloes in India 89.36 ± 0.85 to 90.21 ± 0.8 (Das et al., 2017). However, the sperm concentration of swamp buffaloes found in this study (1.233 ± 7.25 million/mL) was higher than that of swamp buffaloes in India ($1.057.08 \pm 29.07$ million/ml) (Das et al., 2017) and in Thailand (1.1 to 1.2 billion/ml) (Koonjaenak et al., 2007). The differences in motility, viability, and concentration between swamp buffaloes are attributable to difference in environment, feed, age, shelter, and season (Garner and Hafez, 2016).

The current study found best motility and viability of buffalo sperm in Andromed diluent with an equilibration time of 5 hours (Table 2). It was caused by different chemical composition among the three tested diluents (Manjunath, 2012). The lecithin content in Andromed diluent can protect the plasma membrane and safeguard the cholesterol and phospholipids of the spermatozoa membrane. High concentration of lecithin can help absorb protein in the seminal plasma and protect sperm from cold (Barbonetti et al., 2010). The composition in the Andromed diluent stimulates the production of ATP so that sperm motility and vitality can be maintained (Gordon, 2017). The motility of buffalo semen in the present study differed from the motility of spotted buffalo (70.11% vs 41.67%) (Yulnawati et al., 2010), Nili Ravi in optixell diluent with 4 h equilibration ($35.11 \pm 0.8\%$) (Naz et al., 2018), Nili-Ravi buffalo in Pakistan in Andromed diluent with 4 h equilibration ($75 \pm 2.0\%$) (Ansari et al., 2017), and the motility of buffalo study in Iran in tris egg-yolk and bioexell diluent with 2 h equilibration (Shahverdi et al., 2014). The difference in the mentioned results seemed due to the variation in livestock type, age, and storage conditions of semen (Jainudeen and Hafez, 2016).

In this study, no change was observed in the abnormality of buffalo sperm between various types of diluents and equilibration times which is in agreement to previous report (Ariantie et al., 2013). In agreement to our study Febriani et al. (2014) also reported that compared to 5 hours, 3 and 4 hours equilibration time are deleterious for sperms viability as these equilibration durations leading to the death of spermatozoa after thawing.

The plasma membrane integrity was positively correlated with viability and acrosomal integrity. The plasma membrane plays an important role in regulating the whole process inside the cell (Gordon, 2017). The good plasma membrane integrity shows that the sperm can adapt to the diluent at an equilibration so that the transport of the substrate and electrolytes out of the membrane runs well and cell metabolism runs normally (Manjunath, 2012). Membrane integrity is needed to support sperm motility in the process of fertilization, capacitation, and adsorption

(Rastegarnia et al., 2013). We found higher supravital plasma membrane integrity, spermatozoa viability, and intact acrosome by Andromed diluent with 5 h equilibration at post-thawing. The protective agent in the Andromed is believed to be the low-density lipoprotein (LDL) fraction (Singh et al., 2018). The plasma membrane integrity in this study also differed from the plasma membrane integrity found in similar study on Aceh buffalo semen (Eriani et al., 2017), Nili-Ravi buffalo in tris egg-yolk diluent with 4 h equilibration (80%) (Qadeer et al., 2015), and Indonesian swamp buffalo in skim egg-yolk diluent with 4 hours equilibration (51.38 to 62.41 %), probably due to the different diluents used (Herbowo et al., 2019). The variety of semen quality and livestock age also made a difference in the plasma membrane integrity (Garner and Hafez, 2016). In general, the rate of acrosome intact in the present experiment was still above the standard for buffalo sperm (National Standardization Agency of Indonesia, 2008). We found a higher sperm percentage of acrosome intact in Andromed diluent with 5 hours equilibration than in the tris egg-yolk and Triladyl. The acrosome intact of buffalo sperm in the present study was different from that of Pakistani buffalo (Shah et al., 2016) and Nili-Ravi buffalo in Optixell with 4 hours equilibration (Naz et al., 2018). The difference of acrosome intact was probably caused by the difference in animal species, diluent types, and preservation techniques (Manjunath, 2012). In this study, the best equilibration time was obtained at 5 hours (67.72%), similar to Febriani et al. (2014). However, it was different from Shahverdi et al. (2014) and Leite et al. (2010) which found the best equilibration time at about 2 hours and Eriani et al. (2017); Shah et al. (2016) and Kumar et al. (2015) at 4 hours for swamp buffalo. When stored under aerobic conditions, the amino acids in egg yolks produce hydrogen peroxide, which is toxic to sperm (Hezavehei et al., 2018). This shows that the equilibration time of 5 hours help buffalo spermatozoa in adaptation and its' interaction with the diluent so that the spermatozoa membrane's integrity and the balance of intracellular and extracellular water well protected by the diluent (Benson et al., 2012).

The results of the recovery rate and the pregnancy rate of buffalo-cows with Andromed diluent with 5 hours equilibration were higher than with tris egg-yolk and Triladyl with 3 and 4 hours equilibration, which was probably caused by the differences in motility, viability, membrane plasma integrity, and acromosome intact of the semen used (Das, 1985). The pregnancy rate in this study was higher than reported in previous studies in buffaloes (Naz et al., 2018; Ansari et al., 2017; Kumar et al., 2015). Garner and Hafez (2016) reported that the motility affects the ability of sperm to fertilize an egg. Besides, the buffaloes used also had different body weights, resulting in different individual responses to the success of artificial insemination (Gordon,

2017).

CONCLUSION

The best buffalo semen characteristics were exhibited by the Andromed diluent with an equilibration time of 5 hours compared to tris egg yolk and Triladyl diluent with 4 hours and 3 hours equilibration time. Meanwhile, the highest pregnancy rate (86.67%) was produced with the use of Andromed diluent.

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CONFLICT OF INTEREST

We declare there is no conflict of interest with personal, financial, or other relationships with any person or organization related to the materials discussed in the manuscript.

AUTHOR CONTRIBUTION

Yendraliza and Zumarni designed, conducted research activities and revised manuscript. Muhamad Rodiallah and Sadarman analyzed data and wrote manuscript. The all authors contributed to conducting research and writing this manuscript.

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