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Preservation of spotted buffalo epididymal spermatozoa in andromed with raffinose addition

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Abstract

Raffinose is an external cryoprotectant that would protect cell membrane from cold shock effect during preservation process. This study was perform to observe the effect of raffinose on the quality of spotted buffalo epididymal spermatozoa during storage at 4 °C for 24 h. Three different extenders, i.e. commercial tris-egg yolk free extender (Andromed) as control (AM), Andromed Andromed plus 0.2% w/v raffinose (AMR0.2), and Andromed plus 0.4% w/v raffinose (AMR0.4), were used in this study. The results showed that the progressive motility and viability of spermatozoa reduced significantly during storage period. At 12 h of storage, the progressive motility was 60.33% in AM versus 60% in both AMR0.2 and AMR0.4 (P>0.05). Meanwhile, after 24 h of storage, the progressive motility in both AM and AMR0.4 extenders was 55% versus 55.33% in AMR0.2 (P>0.05). Spermatozoa viability in AM, AMR0.2, and AMR0.4 were 70.30%, 73%, 72.33% after 12 h of storage and 66.33%, 68.67% and 69.67% after 24 h of storage, respectively (P>0.05). This results suggested that Andromed without an external cryoprotectant addition could maintain the quality of epididymal spermatozoa as good as the one with 0.2% and 0.4% raffinose addition.

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Introduction

The spotted buffalo (Fig. 1) is mostly exists in Tana Toraja District, South Sulawesi Province, Indonesia. Although it is known that spotted buffalo is also found in a very small number in other region such as: Central Sulawesi, Sumba, Flores, Roti, and Timor (Bo'do',http://sulawesi.cseas.kyotou.ac.jp/final_reports2007/article/212-stephanus.pdf). In Tana Toraja, male spotted buffalo is very important for funeral ceremony tradition, called *rambu solo*.

In general, spotted buffalo population is very limited. The delivery rate of spotted buffalo is much lower than the slaughter rate during *rambu solo* ceremony. The limitation of delivery rate is due to the misperception in Toraja tradition, which believe that natural mating activity would affect the performance and wildness of a male spotted buffalo. As consequence, the price of a male spotted buffalo is ten times above the normal buffalo.

Artificial insemination (AI) technology using epididymal spermatozoa is one method to could be performed in order to avoid worst situation and extinction of spotted buffalo. Epididymal spermatozoa have been showed to be equally effective as ejaculated spermatozoa in promoting conception in AI programs in cattle and buffaloes (Lambrechts *et al.*, 1999; Herold *et al.*, 2004; Herrick *et al.*, 2004; Harshan *et al.*, 2006; Herold *et al.*, 2006). Previous studies showed that epididymal spermatozoa of African buffalo has good quality as the one from ejaculation (Herold *et al.*, 2004; Herold *et al.*, 2006).

Epididymal spermatozoa is collected directly from cauda epididymis, without ejaculation process. Compare to ejaculated spermatozoa, epididymal spermatozoa has no interaction with seminal plasma that is produced in accessories glands as vesicularis, prostate, and bulbourethralis. Due to that, it is hypothesized that epididymal spermatozoa has slightly different membrane composition compare to ejaculated spermatozoa (Senger, 1999). This might cause the different needs of extender composition for liquid storage at 4 °C. One of the factors on spermatozoa preserving is additives which use to dilute semen. Extenders are named for substances which can protect spermatozoa from temperature changes and provide nutritional needs of spermatozoa while freezing (Patra *et al.,* 2001; Rasul *et al.,* 2000). It is reported that sugars can protect freeze spermatozoa by their interaction with membrane phospholipids. Raffinose ($C_{18}H_{22}O_{16}.5H_2O$) is a high molecular three saccharide which consist of glucose, fructose, and galactose (Bansal *et al.,* 2011).

The available commercial extender usually used for ejaculated spermatozoa. Moreover, they were produced for cattle, goat, sheep, boar or horse. There is no buffalo spermatozoa extender available commercially in the market yet. In this study, we used Andromed that usually used for cattle spermatozoa. Furthermore, we also addition of 0.2% and 0.4% w/v raffinose as an external cryoprotectant into that Andromed, in order to give more protection to the spermatozoa membrane during storage at 4 °C for 24 h.

Materials and methods

Spermatozoa collection and processing

Epididymal spermatozoa was collected from five pairs of cauda epididymis within 2 h after slaughtered. Spermatozoa was collected by slicing and flushing the cauda epididymis tissue (Lone *et al.*, 2011) using Andromed. The collected-spermatozoa was centrifuged at 3,000 rpm for 20 menit. Spermatozoa pellet was diluted up to 60.10^6 cells/ml using three different extenders, i.e. Andromed as control (AM), Andromed plus 0.2% w/v raffinose (AMR0.2), and Andromed plus 0.4% w/v raffinose (AMR0.4). Moreover, the diluted-spermatozoa was placed in the refrigerator (4 °C) for 24 h.

Spermatozoa evaluation

Spermatozoa quality parameters, i.e. progressive motility and viability were evaluated after 0 h, 12 h, and 24 h of storage. A drop of spermatozoa sample was placed on object glass and covered by a thin cover glass. The progressive motility was evaluated under

40x objective magnifications light microscope to observe the spermatozoa motility randomly in ten different fields (Rasul *et al.*, 2001).

The viability parameter was evaluated by placing a mix of spermatozoa sample and eosin B stain. Living cells did not absorb the eosin B. In the opposite, damage and dead spermatozoa absorbed the stain on its head part (Rasul *et al.*, 2001). About 200 cells were counted to evaluate spermatozoa viability under 40x objective magnifications light microscope.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) by the linear model using SAS statistical software (SAS 9.1, 2001). The comparative analysis of Mean was performed using least significant difference (LSD) test with 0.05 significant level. The results were presented as the means ± standard error mean (SEM) on the table.

Results and discussion

The results of this study showed that epididymal spermatozoa quality in three different group of extenders were slowly decreasing during storage at 4 °C for 12 h and 24 h. Our results showed that Andromed could maintain the quality of spotted buffalo epididymal spermatozoa during storage at 4 °C. There was no significant different of spermatozoa progressive motility and viability after storage for 12 h and 24 h among three groups (Table 1 and Table 2). The raffinose addition in skim milk and dimitropoulos extenders was not enhancing progressive motility and viability of stallion spermatozoa after 12 h and 24 h of storage at 5 °C (Arifiantini *et al.*, 2013). In contrast, other study showed that raffinose addition in Ham's F10 extender can increase the maintenance ability and progressive motility of rat epididymal spermatozoa after storage for 12 h at 4 °C (Sariozkan *et al.*, 2012).

Table 1. Percentage of spermatozoa progressive motility of spotted buffalo epididymal spermatozoa, fresh and after 12 h and 24 h of storage at 4 °C (mean ± SEM).

Treatment	Spermatozoa progressive motility (%)		
	0 h (fresh)	12 h	24 h
AM	70.00 ± 0.00	60.33 ± 2.36	55.00 ± 3.18
AMR0.2	70.00 ± 0.00	60.00 ± 3.08	55.33 ± 2.36
AMR0.4	70.00 ± 0.00	60.00 ± 2.58	55.00 ± 2.08

Note: AM = Andromed, AMR0.2 = Andromed plus 0.2% raffinose, AMR0.4 = Andromed plus 0.4% raffinose.

In general, good spermatozoa extenders should consist of sugar as energy source, cryoprotectant and antibiotics (Sansone *et al.*, 2000; Sztein *et al.*, 2001). Cryoprotectant is needed as anti-cold shock and plasma membrane protection. Andromed consist of tris hydroxy aminomethane, fructose, lechitin, and antibiotics (Minitübe, Germany, 2001). This tris-egg yolk free commercial extender has components that needed during spermatozoa storage at low temperature.

Table 2. Percentage of spermatozoa viability of spotted buffalo epididymal spermatozoa, fresh and after 12 h and 24 h of storage at 4 °C (mean ± SEM).

Treatment	Spermatozoa viability (%)		
	0 h (fresh)	12 h	24 h
AM	76.00 ± 2.83	70.30 ± 0.47	66.33 ± 1.25
AMR0.2	79.33 ± 1.70	73.00 ± 1.41	68.67 ± 1.89
AMR0.4	76.00 ± 1.63	72.33 ± 1.25	69.67 ± 1.25

Note: AM = Andromed, AMR0.2 = Andromed plus 0.2% raffinose, AMR0.4 = Andromed plus 0.4% raffinose.

The results of this study showed that there was no significantly difference of epididymal spermatozoa quality in Andromed with or without an external cryoprotectant addition. This suggested that the

component in Andromed alone is enough for protecting the spermatozoa from damage during storage at 4 °C up to 24 h. It suspected that an external cryoprotectant is needed to protect the spermatozoa plasma membrane when there is ice crystal formating inside the cell or in the extender (Storey *et al.*, 1998). Ice crystal formation usually happen during cryopreservation process, while cells were store at -196°C. A previous study showed that the addition of fructose and sucrose into milk-based extender would protect spermatozoa cells much better compared to lactose, glucose, xylose, and raffinose (Kumar *et al.*, 1992). Together with phospholipid, sugars also acts to reorganize spermatozoa plasma membrane (Mollinia *et al.*, 1994; Rasul *et al.*, 2000; Yildiz *et al.*, 2000; Aisen *et al.*, 2002).

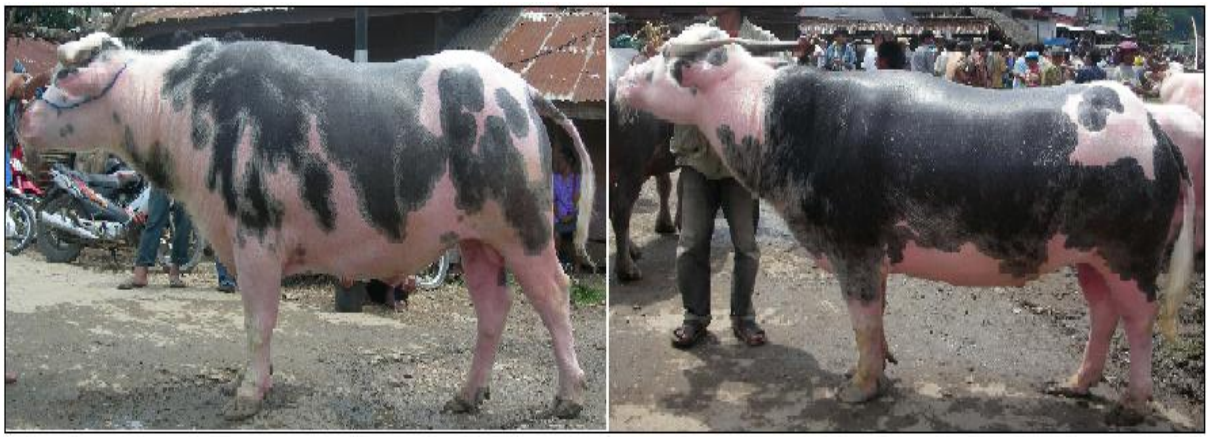

Fig. 1. Spotted buffalo bulls which slaughter during cultural ceremony in Tana Toraja District, Indonesia.

Raffinose is a trisaccharide (familiy of oligosaccharide) that composed of galactose, glucose, and fructose (Storey *et al.*, 1998; Bansal *et al.,* 2011). It is known that carbohydrate is useful to protect the plasma membrane externally from cold-shock damage effect. Besides, carbohydrate is also useful as an energy source for the cells during storage. When the energy level is decreasing, anabolism process of carbohydrate in the extender will be performed. The availability of carbohydrate as an energy source and an external cryoprotectant is important to maintain the spermatozoa progressive motility and viability during storage in low temperature. Raffinose play a cryoprotective role against spermatozoa CASA motility, acrosome abnormality and DNA damage (Tuncer *et al.*, 2010) and provided a better protection of Merino ram spermatozoa parameters against cryopreservation injury (Bucak *et al.*, 2013). In conclusion, Andromed without an external cryoprotectant addition could maintain the quality of epididymal spermatozoa as good as the one with 0.2% and 0.4% raffinose addition.

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