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Preservation of epididymal spermatozoa of garut ram in soybean lechitin-based extender

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Abstract

Objective of the research was to obtain the best ratio between Andomed® and bidestilled water in maintaining quality of garut ram epididymal spermatozoa, preserved at 5 °C. Spermatozoa was collected by the combination of slicing, flushing, and pressure of cauda epididymis tissue using physiological saline (0.9% NaCl). The collected-spermatozoa was divided in equal volume into four tubes and centrifuged at 3,000 rpm for 20 min. Spermatozoa pellet in each tubes were diluted up to 200.10^6 cells/ml with Tris-egg yolk (control extender), 15% of Andromed® (Andro15), 20% of Andromed® (Andro20), and 25% of Andromed® (Andro25), respectively. The diluted-spermatozoa was placed in the refrigerator (5 °C) for three days. The quality of diluted-spermatozoa including percentages of progressive motility (PM), viability (VB), and intact plasma membrane (IPM) were evaluated every day for three days. The results showed that the PM, VB, and IPM of spermatozoa were reduced significantly during storage. At day-4 of storage, mean percentages of PM, VB, and IPM for Andro20 (26%, 42%, and 37,8%) was significantly ($P<0.05$) higher than control (21%, 34.8%, and 33.6%), Andro15 (11%, 25.8%, and 24.2%), and Andro25 (10%, 23.4%, and 26.4%). In conclusion, 20% Andromed® concentration in 80% bidestilled water is the best option of extender in term of the ability in maintain the quality of garut ram epididymal spermatozoa during storage at 5 °C.

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Introduction

The garut ram (Fig. 1) is mostly exists in West Java Province, Indonesia. This breed is an outcome of crossbreeding of merino and kaapstadt from Africa and Indonesia native breed. It has been established since 1800s.

Cauda epididymal could be used as an alternative source of gamete in the application of various reproductive technologies such as artificial insemination (AI). Spermatozoa cells from cauda epididymal tissue have the ability to fertilize the oocyte (Hafez and Hafez, 2000). Utilization of epididymal spermatozoa in the application of artificial insemination and in vitro fertilization has been reported in some animals, including: in monkey (Tollner *et al*., 1990; Sankai *et al*., 1994; Feradis *et al*., 2001), sheep (Graham, 1994; Mir *et al*., 2012), bull (Graham, 1994; Herrick *et al*., 2004; Bertol *et al*., 2013; Strand *et al*., 2016), rhinoceros (Lubbe *et al*., 1999), boar (Kikuchi *et al*., 1998), bear (Anel *et al*., 1999), stallion (Squires *et al*., 2000), llama and alpaca (Bravo *et al*., 2000), deer (Garde *et al*., 2000; Soler *et al*., 2003; Fernandez Santos *et al*., 2008), goat (Hossein *et al*., 2012; Hossein *et al*., 2013; Perera and Ariyaratne, 2013), buffalo (Herold *et al*., 2004; Herold *et al*., 2006; Barati *et al*., 2009; Rezaei *et al*., 2013), and spotted buffalo (Yulnawati *et al*., 2013).

Andromed® in egg-yolk free soybean lechitin-based extender which contains Tris, sugars, phospholipids, citric acid, glycerol, antioxidants, buffers, antibiotics (tylosin, gentamycin, spectinomycin, lincomycin), and purest water. It has various benefits, *i.e.* animal free content, origin, free risk of microbiological contamination, efficient production protocols, broad application range, and high fertility rates (Minitube, 2014). The objective of this research was to obtain the best ratio between Andomed® and bidestilled water in maintaining quality of garut ram epididymal spermatozoa preserved at 5 °C.

Materials and methods

Spermatozoa collection and processing

Epididymal spermatozoa was collected from six pairs of cauda epididymis within 2 h after the animals were slaughtered.

Spermatozoa was collected by the combination of slicing, flushing, and pressure techniques on the cauda epididymis tissue (Lone *et al*., 2011), using physiological saline (0.9% NaCl). The collected-spermatozoa was divided in an equal volume into four tubes and centrifuged at 3,000 rpm for 20 min. The pellet in four tubes were diluted up to 200.10^6 cells/ml with four different extenders, *i.e.* Tris extender containing 20% egg yolk as control, 15% Andromed® plus 85% bidestilled water (Andro15), 20% Andromed® plus 80% bidestilled water (Andro20), and 25% Andromed® plus 75% bidestilled water (Andro25), respectively. Moreover, the diluted-spermatozoa was preserved in the refrigerator (at 5 °C) for three days. Tris (control) extender consists of 3.32 g Tris(hydroxymethyl)aminomethane, 1.86 g citrate acid, 1.37 g fructose, 100,000 IU streptomycin, and 100,000 IU penicilin in 100 mL bidestilled water.

Spermatozoa evaluation

Epididymal spermatozoa qualities that would be evaluated in this study were the percentages of progressive motility, viability, and membrane integrity of the spermatozoa cells during preservation at 5 °C every day during three days. Spermatozoa concentration and abnormal morphology was also observed in fresh spermatozoa, before its dilute. Spermatozoa concentration was counted using Neubauer chamber (Hafez and Hafez, 2000). The percentage of spermatozoa abnormal morphology was the ratio between abnormal and total counted cells under microscope observation. The abnormality of morphology was referred to general classification in different species (Hafez and Hafez, 2000).

The progressive motility was evaluated a drop spermatozoa sample on object glass and covered by a thin cover glass under 40x objective magnifications of light microscope in ten random fields (Rasul *et al*., 2001). The percentage of progressive motility is the number of the progressive spermatozoa divided with dead, silent, vibrate and unprogressive motile spermatozoa. The percentage of viability cells was observed using eosin nigrosin staining (Felipe-Perez *et al*., 2008).

The percentage of live cells was counted by divided the number of live cells with the total number of the cells in several sites of observation. Dead cells would reserve the staining head part, while live cells would release the stain and let the head unstained. At least 200 cells were counted from the different 10 site of observation under 40x objective magnifications of light microscope.

The percentage of membrane integrity was observed using osmotic resistance test (ORT) method (Revell and Mrode, 1994). The hypo-osmotic swelling (HOS) solution contained 0.9 g fructose and 0.49 g sodium citrate in 100 mL bidestilled water. About 20 µl of spermatozoa sample in 200 µl of HOS solution was incubated at 37 °C for 45 min. Spermatozoa with intact plasma membrane would be swollen on the tail site, while the spermatozoa with damage plasma membrane would have the linear tail.

At least 200 cells were counted from the different 10 sites of observation 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 levels. The results were presented as the means ± standard error mean (SEM) on the table.

Results and discussions

Characteristics of fresh epididymal spermatozoa

The results showed that the Mean concentration, percentage of progressive motility, viability, abnormality, and intact plasma membrane of fresh epididymal spermatozoa were 11,660.10^6/mL, 70%, 81.5%, 7.5%, and 82.75%, respectively (Table 1).

Table 1. Characteristics of garut ram fresh epididymal spermatozoa.

Parameters	Means ± SEM
Concentration (10^6 spermatozoa/mL)	11,660.00 ± 1106.93
Progressive motility (%)	70.00 ± 0.00
Viability (%)	81.50 ± 1.00
Abnormality (%)	7.50 ± 1.29
Membrane integrity (%)	82.75 ± 1.50

Table 2. Mean percentage of progressive motility of garut ram epididymal spermatozoa during preservation at 5 °C

Treatments	Day of storage			
	1	2	3	4
Control	65.00 ± 0.00	50.00 ± 3.54^b	43.00 ± 2.74^c	21.00 ± 2.24^b
Andro15	65.00 ± 0.00	54.00 ± 2.24^{bc}	38.00 ± 2.73^b	11.00 ± 2.24^a
Andro20	65.00 ± 0.00	55.00 ± 3.54^c	46.00 ± 2.23^c	26.00 ± 2.24^c
Andro25	65.00 ± 0.00	45.00 ± 3.53^a	34.00 ± 2.24^a	10.00 ± 3.54^a

^{a,b,c} Superscript in the same column showed that significantly different (P<0.05).

Those described that the epididymal spermatozoa was suitable for further process of preservation at 5 °C or cryopreservation at -196 °C in liquid nitrogen container. The minimum standard of quality of fresh semen should have the percentage of progressive motility more than 70% (Sharafi *et al.*, 2009; Swellum *et al.*, 2011), less than 10% of spermatozoa with abnormal morphology (Delgadillo, 1992), percentage of normal spermatozoa more than 80% (Swellum *et al.*, 2011), and more than 60% of spermatozoa with intact plasma membrane (Revell and Mrode, 1994).

The results of several other studies reported that the percentage of motile progressive of fresh cauda epididymis spermatozoa was 64% (cynomolgus monkeys; Feradis *et al*., 2001), 70% (white rhinocerus; Lubbe *et al*., 1999), 66% (stallion; Squires *et al*., 2000), and 65% (spotted buffalo; Yulnawati *et al*., 2013).

Table 3. Mean percentage of viablity of garut ram epididymal spermatozoa during preservation at 5 °C.

Treatments	Day of storage			
	1	2	3	4
Control	75.80 ± 0.84	61.80 ± 3.70^{b}	60.00 ± 1.58^{bc}	34.80 ± 3.49^{b}
Andro15	76.60 ± 2.51	63.80 ± 4.55^{b}	59.20 ± 5.40^{b}	25.80 ± 3.11^{a}
Andro20	76.00 ± 1.00	64.80 ± 3.83^{b}	63.60 ± 1.67^{c}	42.00 ± 1.22^{c}
Andro25	76.60 ± 1.52	56.40 ± 3.13^{a}	48.80 ± 2.39^{a}	23.40 ± 2.19^{a}

^{a,b,c} Superscript in the same column showed that significantly different (P<0.05).

Quality of spermatozoa during storage at 5 °C

The results of this research showed that epididymal spermatozoa quality after diluted in four different group of extenders were similar. In general, the quality of epididymal spermatozoa was slowly decreasing during storage for three days. At day-4 of storage, the Mean percentages of progressive motility, viability, and intact plasma membrane for Andro20 (26%, 42%, and 37,8%) was significantly (P<0.05) higher than control (21%, 34.8%, and 33.6%), Andro15 (11%, 25.8%, and 24.2%), and Andro25 (10%, 23.4%, and 26.4%) (Table 2, 3, and 4).

Table 4. Mean percentage of intact plasma membrane of garut ram epididymal spermatozoa during preservation at 5 °C.

Treatments	Day of storage			
	1	2	3	4
Control	77.00 ± 2.00	66.00 ± 1.87^{ab}	52.60 ± 2.70	33.60 ± 2.51^{b}
Andro15	77.60 ± 0.89	66.40 ± 3.71^{ab}	53.40 ± 2.61	24.20 ± 2.39^{a}
Andro20	77.80 ± 0.45	68.40 ± 2.51^{b}	53.40 ± 1.52	37.80 ± 2.17^{c}
Andro25	76.60 ± 2.19	62.40 ± 5.45^{a}	51.80 ± 1.79	26.40 ± 1.82^{a}

^{a,b,c} Superscript in the same column showed that significantly different (P<0.05).

These results showed that ratio between 20% Andromed® with 80% bidestilled water was the best combination than others, in maintain the quality of garut ram epididymal spermatozoa during storage at 5 °C for three days, and no difference significant with the control. It is clear that 15% of Andromed® concentration in the extender was too low to maintain the spermatozoa quality. In contrast, other extender that contain 25% Andromed have high osmotic pressure, that negatively affect spermatozoa quality during preservation. According to Soylu *et al*. (2007) the addition of solutes such as carbohydrates in large quantities into extender will increase the osmotic pressure. High osmotic pressure contributes to some changes of physiological conditions, and can even cause death of the cells. The reported optimal concentrations of soybean lecithin in extender used for cryopreservation in the literature were ranged from 0.8% in canine (Kmenta *et al*., 2011) to 1% in ram (Forouzanfar *et al*., 2010), human (Reed *et al*., 2009), and cat (Vick *et al*., 2010), and 1.5% in goat (Tasdemir *et al*., 2013; Masoudi *et al*., 2016).

In general, good spermatozoa extenders should consist of sugar as an energy source, cryoprotectant, and antibiotics (Sansone *et al*., 2000; Sztein *et al*., 2001).

Andromed® as a lechitin-based commercial extender has components that needed during the preservation of spermatozoa. Andromed® in egg-yolk free soybean lechitin-based extender which contains Tris, sugars, phospholipids, citric acid, antioxidants, buffers, antibiotics (tylosin, gentamycin, spectinomycin, lincomycin), purest water, and cryoprotectant (glycerol). Glycerol is needed as anti-cold shock and plasma membrane protection, especially in cryopreservation of semen. Although glycerol protects the spermatozoa from cryoinjury by removing the water found within the cell and by increasing extracellular osmolality (tonicity) (Sayre *et al.*, 1997), the presence of glycerol in semen extender reduces motility (at 30 ^{o}C and 5 ^{o}C) fertilizing capability following intracervical insemination, and acrosomal integrity, along with accelerates acrosome reaction (Abdelhakeam *et al.*, 1991a; Abdelhakeam *et al.*, 1991b). In conclusion, 20% Andromed® concentration in 80% bidestilled water is the best option of extender in term of the ability in maintain the quality of garut ram epididymal spermatozoa during storage at 5 °C.

Fig. 1. The garut ram is mostly exists in West Java Province, Indonesia.

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