

## VIABILITY AND PLASMA MEMBRANE INTEGRITY OF THE SPOTTED BUFFALO EPIDIDYMAL SPERMATOZOA AFTER THAWING WITH THE ADDITION OF DEXTROSE INTO THE EXTENDER

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### ABSTRACT

The objective of this study was to obtain the viability and plasma membrane integrity of the spotted buffalo epididymal sperm after addition of dextrose into Andromed<sup>®</sup> extender. Spermatozoa that have been collected from cauda epididymis were diluted with Andromed<sup>®</sup> extender as control (K) and Andromed<sup>®</sup> + 0.2% dextrose (P1) and Andromed<sup>®</sup> + 0.4% dextrose (P2) as treatments. The results showed that the quality of epididymal spermatozoa decreased during cryopreservation process. The percentage of motility after thawing in P1 (46%) and P2 (46.67%) were significantly higher ( $P < 0.05$ ) compared to K (41%) as well as the percentage of live sperm in P1 (58.8%) and P2 (60%) compared to K (52.2%). The percentage of membrane integrity in P1, P2 and K were 67.4; 66.8 and 68 %, respectively. In conclusion, the addition of 0.2 and 0.4% of dextrose into Andromed<sup>®</sup> acted as an extra cellular cryoprotectant and could maintain the viability and membrane integrity of the spotted buffalo epididymal spermatozoa after thawing.

**Key words:** epididymal sperm, cryopreservation, dextrose, spotted buffalo

### INTRODUCTION

Indonesia is worldwide known to have high biodiversity that need to be considered for the conservation of the flora and fauna specific to this country. Spotted buffalo is one of the Indonesian biodiversity that need a particular attention as its population tends to decrease every year. This animal is thought to bring fortune and is usually used for the veneration in traditional ceremony in Tana Toraja, South Sulawesi. According to the local people believe, spotted buffalo can only survive in its natural habitat, which is in Tana Toraja. The farmer put a special treatment for the spotted buffalo; particularly the male is maintained separated from the female

to prevent it from reproduction activities. This condition is, therefore, thought to make the conservation effort of this animal become more difficult.

One of the alternative attempts to prevent this animal from extinction is to utilize the gonad, which are the testis and the epididymis of the male that is sacrificed in the traditional ceremony. The epididymis can be used as a source of potential spermatozoa that can conserve the genetic material. The fertility of the spermatozoa taken from the cauda epididymis is more or less the same as the spermatozoa from the ejaculate (Hafez and Hafez 2000). Collection of the epididymal spermatozoa has been done in various species such as primate (Feradis *et al.* 2001), sheep (Rizal 2006), cattle (Graham 1994), cat (Tsutsui *et al.* 2003, Yulnawati and Setiadi 2005), pig (Kikuchi *et al.* 1998), rhinoceros (Lubbe 1999), equine (Squires *et al.* 2000; Papa *et al.* 2008), deer (Soler *et al.* 2003), dog (Setiadi *et al.* 2007, Hori *et al.* 2004) and African buffalo (Herold *et al.* 2004, Herold *et al.* 2006). Spermatozoa collected from cauda epididymis is then stored in the form of liquid and/or frozen sperm. Furthermore, epididymal sperm could be used for assisted reproductive technology such as artificial insemination (AI), *in vitro* embryo production (IVEP) or intra cytoplasmic sperm injection (ICSI).

In order to maintain the quality of the spermatozoa during storage and after thawing for further process, the extender with a certain composition is needed. During the freezing process, the important material in the extender is the cryoprotectant that can protect sperm from coldshock. Cryoprotectant can directly prevent the formation of ice crystal in the cell, as well as working extracellularly as a membrane protector. Both the intracellular and extracellular cryoprotectants can be added at the same time in a certain ratio into the extender. Sugar is used in the research as one of the extracellular cryoprotectant that should be added to the glycerol-as intracellular cryoprotectant-contained Andromed<sup>®</sup> extender. It is hoped that the addition of dextrose sugar can maintain the viability dan integrity of plasma membrane of the spotted buffalo's epididymal spermatozoa after thawing.

## MATERIALS AND METHODS

Cauda epididymis of the spotted buffalo was collected during the Rambu Solo' funeral ceremony in the Pangli village, Sesean, North Toraja. The cauda epididymis was flushed and stored in 0.9% of NaCl before the collection of spermatozoa. Epididymal spermatozoa was collected using combination technique, slicing/ flushing and pressing all parts of the cauda's tissues (Rizal *et al.* 2004) using Andromed<sup>®</sup> solution as an extender. Evaluation of the quality of fresh spermatozoa observed was percentage of progressive motility, percentage of live spermatozoa, concentration, percentage of abnormality and percentage of plasma membrane integrity (MPU). Collected spermatozoa was centrifuged at 500 G for 20 min. at room temperature. The supernatant was discharged and the pellet that contained spermatozoa was re-diluted with Andromed<sup>®</sup> (Minitub, Germany) extender. The volume of the extender used is based on the concentration previously calculated.

Basic extender used in this research was Andromed<sup>®</sup>. Andromed<sup>®</sup> is a commercial extender that contains glycerol as an intracellular cryoprotectant. Andromed<sup>®</sup> without

any addition of substance was grouped as control, whereas the addition of 0.2 and 0.4% w/v of dextrose (Merck, Germany) into Andromed® were grouped as treatments.

The diluted epididymal spermatozoa were placed in 0.25 ml of mini straw with 60 million of motile spermatozoa per straw and equilibrated in the refrigerator at 5°C for 3 hours. Freezing of the epididymal spermatozoa was started by arranging the straw 10 cm above the surface of the liquid nitrogen with the temperature of -130°C for 15 min. Afterwards, the straw was plugged into the liquid nitrogen with the temperature of -196°C and stored in the container. Later, each frozen epididymal spermatozoa sample was thawed for quality evaluation. Thawing process was carried out by placing the straw into the water bath at 37°C for 30 seconds.

The percentage of sperm motility, live sperm, and plasma membrane integrity of post diluted, equilibrated and thawed epididymal spermatozoa were recorded. The percentage of progressive motility (move forward) of the sperm was counted subjectively at eight different visual fields, using light microscope with the magnitude of 400x. The percentage of live sperm was counted using eosin staining. Live spermatozoa were characterized by white colour of the head, whereas the dead sperm had red head (Toelihere 1993). Plasma membrane integrity (MPU) was characterized by circular tail or inflated, whereas the broken one was characterized by by straight tail when the sperm was exposed to the hypoosmotic solution and incubated at 37°C for 60 min (Revell and Mrode 1994). For each parameter observed, a sum of minimum 200 sperma was evaluated using light microscope with the magnitude of 400x.

Data obtained were analyzed using random Analysis of Variance (ANOVA) with three treatments and five replications. Difference between treatments was tested using Duncan multiple range test (Steel and Torrie 1993).

## RESULTS AND DISCUSSIONS

The quality of fresh epididymal spermatozoa of the spotted buffalo obtained from this research is shown in Table 1. The concentration of spermatozoa collected from the cauda epididymis using flushing technique and pressure, was  $10445 \times 10^6$  spermatozoa/ml. Theoretically, this was reliable because cauda epididymis functions as a spermatozoa storage before ejaculation and in the cauda and the spermatozoa has not have seminal plasma addition (Garner and Hafez 2000). The sperm concentration in bull cauda epididymal was 3593 - 4406,7 x 10<sup>6</sup> sperm/ml (Suhendra 2002) and 6,26 x 10<sup>9</sup> sperm/ml in buffalo cauda epididymal tissue (Toelihere 1993).

Table 1. The avarage quality of fresh spotted buffalo epididymal spermatozoa

Parameters	Mean ± st.dev
Concentration (x 10 <sup>6</sup> sperma/ml)	10445 ± 43.62
Volume (ml)	0.45
Progressive motility (%)	65.0 ± 0.00
Live (%)	79.3 ± 1.30
Abnormality (%)	15.0 ± 2.24
Plasma Membrane Integrity (%)	80.8 ± 0.43

The percentage of the epididymal spermatozoa motility obtained from the research was 65 % and this value is still in the range of the requirements for using it in the artificial insemination (AI) application. The percentage of the abnormality of this epididymal spermatozoa was also high (15 %), however this value is still in the normal range and reliable to be used for fertilization process, since the normal fertile semen contains not more than 20 % abnormal spermatozoa (Ax *et al.* 2000). The percentage of abnormal morphology of 8-10 % did not give any significant effect to the fertility, however, if the abnormality form one ejaculate which is more than 25 %, the decrease of the fertility could not be anticipated (Bearden and Fuquay 1997).

Generally, after undergoing three steps of freezing process, the research indicated that the percentage of the motility, viability and plasma membrane integrity of the treatment groups were better compared to the control group. The percentage of motility after thawing in the control group, dextrose 0.2 and 0.4 %, were 41, 46 and 46.67 %, respectively. There was a significant difference ( $P < 0.05$ ) between treatment groups which had dextrose addition and the control group. On the other hand, there was no significant difference ( $P > 0.05$ ) between treatment groups and the control group of the percentage of plasma membrane integrity (Table 2). It could be concluded that the value of post thawing epididymal sperm still met the requirements for AI, IVF or ICSI application.

Table 2. The mean ( $\pm$ stdev) percentage of motility and viability of epididymal spermatozoa of spotted buffalo at different stages of freezing processes

Frozen Stage	Parameters	Control	Dextrose 0,2%	Dextrose 0,4%
Dilution	% M	65,00 $\pm$ 0,00 <sup>a</sup>	65,00 $\pm$ 0,00 <sup>a</sup>	65,00 $\pm$ 0,00 <sup>a</sup>
	% Live	76,00 $\pm$ 2,83 <sup>a</sup>	82,00 $\pm$ 1,41 <sup>b</sup>	82,67 $\pm$ 1,25 <sup>b</sup>
	% MPU	78,67 $\pm$ 0,47 <sup>a</sup>	77,33 $\pm$ 2,87 <sup>a</sup>	80,00 $\pm$ 0,82 <sup>a</sup>
Equilibration	% M	50,00 $\pm$ 0,00 <sup>a</sup>	56,67 $\pm$ 4,71 <sup>a</sup>	56,67 $\pm$ 4,71 <sup>a</sup>
	% Live	70,33 $\pm$ 0,47 <sup>a</sup>	73,00 $\pm$ 1,89 <sup>a</sup>	72,67 $\pm$ 1,25 <sup>a</sup>
	% MPU	72,00 $\pm$ 0,82 <sup>a</sup>	71,33 $\pm$ 0,94 <sup>a</sup>	73,00 $\pm$ 0,82 <sup>a</sup>
Thawing	% M	41,00 $\pm$ 2,00 <sup>a</sup>	46,00 $\pm$ 2,00 <sup>b</sup>	46,67 $\pm$ 2,36 <sup>b</sup>
	% Live	52,20 $\pm$ 2,48 <sup>a</sup>	58,80 $\pm$ 1,83 <sup>b</sup>	60,00 $\pm$ 0,82 <sup>b</sup>
	% MPU	68,00 $\pm$ 1,10 <sup>a</sup>	67,40 $\pm$ 1,36 <sup>a</sup>	66,80 $\pm$ 1,47 <sup>a</sup>

Note:

M: motility, MPU: plasma membrane integrity, <sup>a,b</sup> Different superscript in the different column showed significantly different ( $P < 0,05$ ).

Dextrose is produced by the conversion enzyme found in cornstarch and undergoes demineralization ion exchange process. Dextrose is the form of D-glucose that can be metabolized by the cell through biochemical process called glycolysis to be an energy source. In general, the addition of the sugar into the spermatozoa extender can affect the motility, viability and plasma membrane integrity of the spermatozoa. The addition of sugar into the extender can diminish the damage of the spermatozoa acrosome (Yildiz *et al.* 2000). Sugar is known as an extracellular cryoprotectant. The mechanism of how dextrose works in this research is assumed as a protector for the plasma membrane rather than to prevent the formation of the ice crystal in the cell.

Whereas, glycerol already contained in the Andromed®, play a role as intracellular cryoprotectant, to prevent the formation of ice crystal during freezing process.

There are no reports of experiments on the epididymal sperm of spotted buffalo freezing and thawing process. Therefore, none of the publications reported on the use of sugar that was added into the basic extenders in spotted buffalo sperm cryopreservation process. For the references, the use of sugar as cryoprotectant in different animal species were reported, such as sheep (Aisen *et al.* 2002; Molinia *et al.* 1994), dog (Yildiz *et al.* 2000; Rigau *et al.* 2001), pig (de los Reyes 2000) and bull (Woelders *et al.* 1997). Generally, sugars play a role as the capacity agent of cryoprotectant and works as the spermatozoa plasma membrane protector during freezing process. It was also known that the sugars have dehydration activity and be able to interact with the cell membrane (Aisen *et al.* 2000).

## CONCLUSIONS

In conclusion, recovered spermatozoa from cauda epididymis of spotted buffalos that undergo freezing processes using Andromed with the addition of 0.2 or 0.4% dextrose are suitable for AI.

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