

## ***In Vitro* Fertility of Post-thawed Epididymal Ram Spermatozoa after Storage at 5 °C before Cryopreservation**

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### **ABSTRACT**

This study addressed the effects of storage duration of epididymides at 5 °C before sperm collection and their fertility after cryopreservation *in vitro*. Spermatozoa from one of the testes pairs were immediately collected, evaluated and frozen (control group). The remaining epididymides were cooled to 5 °C and stored for 24, 48, 72, and 96 h (experimental groups), after which spermatozoa were collected and frozen as in the control group. Before and after thawing, sperm motility, sperm viability and plasma membrane integrity were assessed. The fertilizing ability of frozen-thawed spermatozoa of each group was evaluated by *in vitro* fertilization of matured sheep oocytes. Sperm quality (sperm motility, viability, and plasma membrane integrity) at collection and after cryopreservation decreased as the duration of the epididymal storage interval increase ( $P < 0.05$ ). The motility decreased steadily along the studied time periods. Although, the fertilizing ability of post-thawed epididymal spermatozoa gradually decreased as the storage period was prolonged, the spermatozoa collected from the cauda epididymides stored at 5 °C for up to 96 h were able to fertilize 16%-65% of oocytes *in vitro*. Results of the present study showed that ram epididymal spermatozoa survive in storage at 5 °C for up to 96 h. These spermatozoa maintain their fertilizing ability and may be suitable for use in IVF and other assisted reproductive procedures.

*Key words: epididymis, sheep, spermatozoa, in vitro fertilization, cryopreservation*

### **ABSTRAK**

Penelitian ini bertujuan untuk mengetahui daya fertilisasi *post-thawed* spermatozoa yang dikoleksi dari epididymis setelah disimpan pada suhu 5 °C selama 96 jam *post mortem* secara *in vitro*. Epididymis disimpan pada suhu 5 °C selama 0-96 jam sebelum dibekukan. Kualitas spermatozoa (motilitas, viabilitas, dan integritas membran plasma) dievaluasi sebelum dan setelah dibekukan dengan interval waktu 24 jam. Kemampuan spermatozoa setelah dibekukan untuk memfertilisasi oosit secara *in vitro* dievaluasi dengan fertilisasi *in vitro*. Walaupun terjadi penurunan kualitas spermatozoa seiring dengan waktu penyimpanan epididymis ( $P < 0,05$ ) tetapi sperma yang hidup dan motil masih dapat dikoleksi setelah 96 jam *post mortem*. Berdasarkan pembentukan pronukleus setelah fertilisasi *in vitro*, 16%-65% oosit terfertilisasi secara *in vitro* oleh *post-thawed* spermatozoa yang dikoleksi dari epididymis setelah disimpan 0-96 jam. Dapat disimpulkan bahwa walaupun kualitas spermatozoa yang dikoleksi dari epididymis pasca penyimpanan dan pembekuan menunjukkan penurunan tetapi spermatozoa tersebut masih mempunyai kemampuan untuk membuahi oosit secara *in vitro*.

*Kata kunci: epididymis, domba, spermatozoa, fertilisasi in vitro, kriopreservasi*

### **INTRODUCTION**

The recovery and cryopreservation of viable sperm from the epididymis of dead animals (post-mortem re-

covery) from genetically valuable animals or endangered species is an important technique for preserving male gametes and thus for maintaining germ-plasm banks. Sperm stored in the cauda epididymis have usually good quality and a high level of maturation, being able to fertilize oocytes (Matas *et al.*, 2010; Pamungkas *et al.*, 2012). After an animal dies its germ cells remain alive for a certain period of time and it may be possible to pro-

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duce progeny of an animal after its death (Hishinuma *et al.*, 2003). However, in order to get good quality samples, sperm collection and processing should be carried out immediately after the death of the animal since the conditions (time and temperature) under which epididymis is handled could cause important changes in the viability of spermatozoa (Martinez-Pastora *et al.*, 2005). Shakeri *et al.* (2008) reported that the better quality of ram spermatozoa preserved at room temperature for 48 h was found when it was collected in the first 24 h after death. Garde *et al.* (2005) reported that viability and *in vitro* fertility of sperm decreased when the time between the animal's death and the moment of semen collection increased (up to 40 h at ambient temperature). Thus, storing method is required for temporarily preserving spermatozoa viability when spermatozoa could not be collected and/or frozen immediately. If longer preservation of sperm was possible, it would provide greater flexibility and more options for producing embryos from the sperm of dead animals.

To date, many studies on influence of prolonged cold storage of epididymis of several species to the quality and fertility of the spermatozoa with various results were reported. It is possible to obtain viable gamete and even fertile sperm from refrigerated epididymides for many hours or days such as in bovine (Martins *et al.*, 2009), equine (Vieira *et al.*, 2012), domestic cat (Ganan *et al.*, 2009; Thuwanut *et al.*, 2010), dog (Suppawiwat *et al.*, 2006), ram (Mir *et al.*, 2012), wild ruminants (Martinez-Pastora *et al.*, 2005), camel (Waheed *et al.*, 2012), buffalo (Barati *et al.*, 2009), and red deer (Soler *et al.*, 2003; Malcotti *et al.*, 2012). These works showed that sperm quality deteriorates depending on time postmortem and that storage of the epididymis at around 5 °C prolongs the survival of spermatozoa in the epididymides allows extra time for sperm recovery and processing. However there are dissimilarities between species, possibly due to differences on cold shock endurance of epididymal sperm (Martinez-Pastora *et al.*, 2005). There are few reports on effect of prolonged storage of epididymis prior freezing on the *in vitro* fertility of post-thawed epididymal spermatozoa in ram. Therefore, the purpose of this study was to determine viability and *in vitro* fertilizing ability of cryopreserved epididymal ram spermatozoa after storage of epididymis at 5 °C for up to 96 h prior cryopreservation. The fertility of post-thawed epididymal ram spermatozoa was measured by their penetration ability to sheep matured oocytes *in vitro*.

## MATERIALS AND METHODS

### Ram Epididymides and Spermatozoa Collection

Twelve pairs of testes with attached epididymides from adult rams were obtained at local slaughterhouse. They were transported to laboratory at room temperature. The testes were washed with isotonic saline supplemented with 100 IU/ml penicillin (Sigma, St. Louis, MO, USA) and 0.1 mg/ml streptomycin (Sigma, St. Louis, MO, USA). The epididymis from one testes of each pairs was dissected free and spermatozoa were recovered

from it in a culture dish containing 1 mL NSF-I extender to serve as a control group. Immediately after semen collection, the motility of sperm was measured. Only semen samples with an initial sperm motility > 65% to 70% were used for freezing. The remaining testes of the pair were put into plastic bags and stored in refrigerator of around 5 °C for 24 (24 h group), 48 (48 h group), 72 (72 h group), and 96 h (96 h group), and afterwards the spermatozoa were recovered, evaluated, and frozen.

### Extenders for Ram Epididymal Sperm Freezing

Niwa and Sasaki freezing (NSF) extender was prepared according to a method described by Pamungkas *et al.* (2012) as follow: Niwa and Sasaki Freezing (NSF)-I extender consisted of a mixture of 8.8% (wt/vol) lactose (Merck, Germany), 20% (v:v) egg yolk, and 100 µg/mL penicillin and streptomycin in distilled water. The NSF-II extender consisted of 92.5% (v/v) of NSF-I, 1.48% (v/v) of orvus ES paste (Miyazaki Kagaku, Tokyo, Japan) and 6% (v/v) glycerol (Merck, Germany).

### Freezing of Ram Epididymal Sperm Freezing

Freezing of spermatozoa was performed according to the method described by Karja *et al.* (2010). After collection, the spermatozoa (about 100 µL) was diluted with 400 µL of NSF-I extender and were equilibrated at 4 °C for 2 h. Thereafter 250 µL of NSF-II extender was added and then equilibrated at 5 °C for an additional 5 min. At the end of the equilibration period, the same volume (250 µL) of the NSF-II extender was added at 4 °C until a final volume of 1 mL was reached, resulting in a final concentration of 3% glycerol. The spermatozoa were then immediately loaded into a 0.25 mL French straw (I.V.M., France), which were placed in liquid nitrogen vapor for 20 min (4 cm above the surface of liquid nitrogen), and subsequently stored in liquid nitrogen.

### Evaluation of Sperm Quality

Sperm samples of each group were evaluated for sperm motility, viability and membrane integrity before and after freezing. The percentage of motile was determined subjectively on a warmed glass-slide immediately after collection and after freezing by a phase-contrast microscope at magnification of x 400. Number of viable spermatozoa was assessed by eosin-nigrosin staining. The functional integrity of the sperm plasma membrane was evaluated using the hypoosmotic swelling test (HOS test). HOS-test was assessed with fructose and sodium citrate. The results are expressed in percentage.

### Penetration Assay by *in Vitro* Fertilization

Sheep ovaries were collected at local slaughterhouse. Each ovary was sliced repeatedly with a scalpel blade to release cumulus-oocyte complexes (COCs) in a 60 mm culture dish containing m-PBS (Gibco, Grand Island, NY, USA) at 37 °C. Only COCs exhibiting uniform, dark-pigmented ooplasm and an intact cumulus cell investment were used for further culture. Cumulus-

oocyte complexes were cultured separately in a 100  $\mu$ L drop of maturation medium under mineral oil (Sigma, St. Louis, MO, USA) for 28 h. The maturation medium consisted of tissue culture medium (TCM) 199 with Earle's salts (Gibco, Grand Island, NY, USA), supplemented with 5% bovine serum albumin (BSA, Sigma), 0.1 IU/mL follicle stimulating hormone (Teikokuzoki, Tokyo, Japan), 10 IU/mL human chorionic gonadotropin (Chorulon, Intervet International B.V. Boxmeer-Holland), and 50  $\mu$ g/mL gentamycin (Sigma, St. Louis, MO, USA). All cultures were performed in a 38.5 °C humidified incubator containing 5% CO<sub>2</sub> in air.

Matured oocytes were then fertilized by frozen-thawed sperm of each group experiment. Briefly, after thawing at 37 °C, the spermatozoa were washed in fertilization medium by centrifugation at 1800 rpm for 5 min. *In vitro* fertilization medium consisted of 90 mM NaCl, 12 mM KCl, 25 mM NaHCO<sub>3</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgSO<sub>4</sub> 7H<sub>2</sub>O, 10 mM Sodium lactate, 10 mM hepes, 8 mM CaCl<sub>2</sub> 2H<sub>2</sub>O, 2 mM sodium pyruvate, 2 mM caffeine, and 5 mg/ml BSA (Sigma, USA). The supernatant was removed and the sperm pellet was diluted in 500  $\mu$ L of the IVF medium. The spermatozoa concentration was adjusted to  $5 \times 10^6$  spermatozoa/mL in the IVF medium. Matured oocytes were then fertilized separately into 100  $\mu$ L of the sperm microdrops in concentration of  $5 \times 10^6$  spermatozoa/mL for 14 h. Thereafter, presumptive zygotes were fixed and stained for the assessment of fertilization. Zygotes were fixed with acetic acid: ethanol (1:3 v:v) for 48 to 72 h. The fixed oocytes were stained with acetic-orcein (1% orcein in 45% acetic acid) and examined under a phase-contrast microscope with 200x of magnification. Oocytes containing both female and male pronuclei were considered to be fertilized and were categorized as normal or polyspermic, according to the number of pronuclei in the cytoplasm.

### Statistical Analysis

The percentages of sperm motility, sperm membrane integrity, and the oocytes fertilized were subjected to arc sin transformation before analysis. Transformed data were tested by ANOVA followed by a post hoc, Fisher's protected least significant difference (PLSD) test using the Statview program (Abacus Concepts Inc., Berkeley, CA, USA). Differences  $P < 0.05$  were considered significant.

## RESULTS AND DISCUSSION

This study addressed the effects of duration of storage of epididymides at 5 °C before sperm collection and their fertility after cryopreservation. The motility decreased steadily along the studied time periods (Figure 1). After 24 h of storage ( $67\% \pm 2.0\%$ ) no change in motility was observed compared to control group ( $74\% \pm 1.0\%$ ), but the motility decreased significantly around 48 h of storage ( $P < 0.05$ ). The same pattern was observed for spermatozoa from epididymis after cryopreservation.

Figure 2 showed a significant decrease in viability of spermatozoa after 72 h of storage. Nevertheless, even at 96 h postmortem,  $77.5\% \pm 6.1\%$  of spermatozoa were

alive, as assessed by live-dead sperm staining. However, after freezing, the decrease of viability has already observed in spermatozoa retrieved from epididymis 24 h after storage.

The plasma membrane is of crucial importance to the freeze-thaw survival of spermatozoa and is regarded as the primary site of freezing injury. In our study, the percentage of membrane integrity of spermatozoa in the epididymis for up to 96 h did not decrease significantly (Figure 3). A slower decrease in sperm membrane integrities after cryopreservation was seen as storage time progressed. A significantly decrease ( $P < 0.05$ ) was observed after 96 h of storage.

In general, the results in this study showed that sperm quality at collection decreased as the duration of the epididymal storage interval increase, this reduction was also seen after cryopreservation. The decrease of sperm quality along with post mortem due not only to sperm aging, but also to the processes inherent to tissue decomposition after death. Ram epididymal spermato-

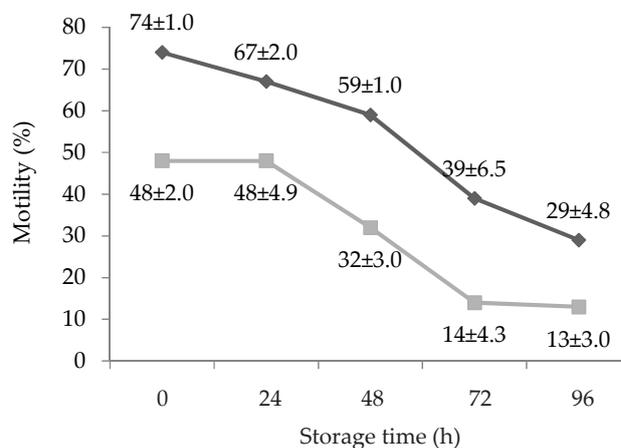


Figure 1. Number of motile epididymal spermatozoa collected from ram epididymis stored at 4 °C for up to 96 h at 5 °C before (◆) and after (■) freezing

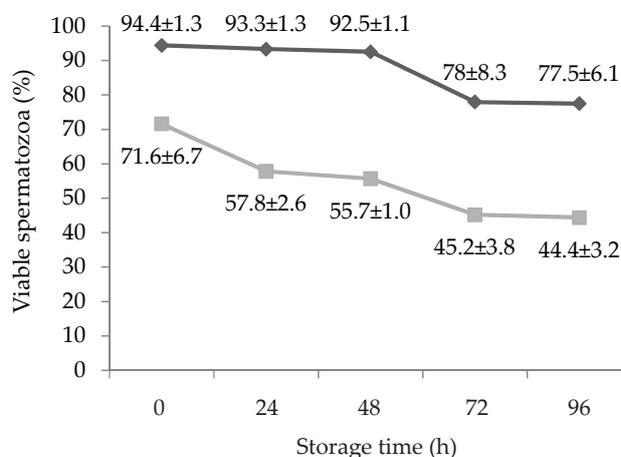


Figure 2. Number of viable spermatozoa collected from ram epididymis stored at 4 °C for up to 96 h at 5 °C before (◆) and after (■) freezing

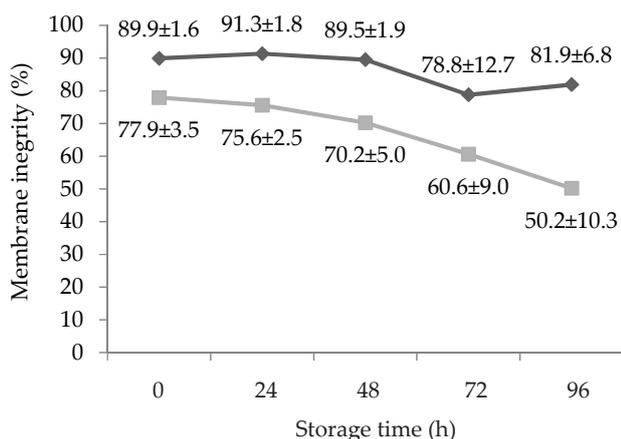


Figure 3. Number of membrane integrity of spermatozoa collected from ram epididymis stored at 4 °C for up to 96 at 5 °C before (◆) and after (■) freezing

zoa in this study, and possibly the spermatozoa of all other mammalian species, begin to degenerate within the testis and male genital tract after death. Hishinuma *et al.* (2003) working with sika deer epididymis, described the degeneration of the epididymal tubules, showing that histological changes several days postmortem.

Nevertheless, results of our study suggested that cold storage of epididymed at 5 °C prevented a quick decrease in motility of epididymal spermatozoa, which coincides with other findings in many species (Kaabia *et al.*, 2003; Soler *et al.*, 2003; Wani, 2009). The beneficial effect of refrigeration on various parameters of sperm quality, especially motility, may be explained by the reduced metabolic rate of sperm cells when they are at 5 °C (Salomon & Maxwell, 2002). In this sense, Sankai *et al.* (2001) find that motility of mouse epididymal spermatozoa decreases when the storage temperature is increased, suggesting that this effect is related to changes in spermatozoa metabolic activity. Whereas, Reyes-Moreno *et al.* (2002) reported in bovine that the survival of sperm stored in epididymis related to the secretory epididymal proteins sustain sperm motility during storage *in vitro*. Bovine epididymal epithelium fluid from cauda epididymides was able to protect sperm against oxidative damage and to enhance frozen-thawed sperm motility and survival *in vitro*. Little is known about the epididymal secretions and the molecular mechanisms that allow sperm survival and preservation of sperm function during cauda epididymal storage. To date, the proposed mechanisms regulating sperm protection during epididymal storage could implicate potent inhibitors of complement mediated cell lysis (Reyes-Moreno *et al.*, 2002), the enzymes involved in glutathione conjugation and metabolism that provide protection against oxidative damage (Agarwal *et al.*, 2003; Zubkova & Robaire, 2004), and potential proteolysis activity regulators such as serine- and cysteine-protease inhibitors that may protect membrane-sperm from inappropriate proteolytic degradation (Fouchecourt *et al.*, 2000). Therefore cauda epididymal fluid can be considered as a complex mixture of survival or sperm-maintaining factors that

Table 1. Fertility of post-thawed spermatozoa collected from ram epididymis stored at 4 °C for up to 96 at 5 °C before cryopreservation

Storage time (h)	Oocytes inseminated (n)	Oocytes fertilized (%) ± SD (n)		
		Total	Normal	Polyspermic
0	49	65.3±4.0 (32) <sup>a</sup>	51.0±4.0 (25) <sup>a</sup>	14.3±0.6 (7) <sup>a</sup>
24	52	51.9±1.5 (27) <sup>ab</sup>	50.0±2.1 (26) <sup>ab</sup>	1.9±0.6 (1) <sup>b</sup>
48	58	32.8±0.0 (19) <sup>bc</sup>	29.3±0.5 (17) <sup>bc</sup>	3.4±0.6 (2) <sup>b</sup>
72	44	27.3±0.6 (12) <sup>c</sup>	22.7±0.0 (10) <sup>bc</sup>	4.5±0.6 (2) <sup>b</sup>
96	49	16.3±4.7 (8) <sup>c</sup>	12.3±4.0 (6) <sup>c</sup>	4.1±1.2 (2) <sup>b</sup>

Note: Means in the same column with different superscript differ significantly ( $P < 0.05$ ).

can be retained *in vitro*. In addition, cryopreservation of spermatozoa in this study caused a decreased in quality of sperm samples (considering progressive motility, number of viable spermatozoa and plasma membrane integrity of spermatozoa), in which the 72 h and 96 h groups were affected to a greater extent with less post-thaw quality than other groups ( $P < 0.05$ ). It is well known during the freezing and thawing process, the spermatozoa may be affected by several factors that can cause loss of plasma membrane integrity, acrosomal damage and mitochondrial dysfunctions, resulting in irreversible loss of motility and fertilizing capacity (Pukazhenthil *et al.*, 2002).

The fertilizing ability of post-thawed epididymal spermatozoa gradually decreased ( $P < 0.05$ ) as the storage period was prolonged in this study, however, the spermatozoa collected from the cauda epididymides stored at 5 °C for up to 96 h were able to fertilize 16%-65% of oocytes *in vitro* (Table 1). This result coincides with other previous reports. Fertilizing ability of bull epididymal spermatozoa that were cryopreserved shortly after death of the animals was demonstrated by Martins *et al.* (2007). In cats, epididymal spermatozoa have similar fertilization rates as fresh spermatozoa until 2 days of storage, but reduced by day 7 (Bogliolo *et al.*, 2004). Similarly, fertility rates of the mouse epididymal spermatozoa have been reported to decrease on storage for up to 5 d at 22 °C (Sato & Ishikawa, 2004). Kaabi *et al.* (2003) reported that spermatozoa collected from the cauda epididymis of ram were able to fertilize oocytes *in vitro* and similar cleavage rates for oocytes inseminated with ram sperm from epididymes stored at room temperature or refrigerated, both at 24 and 48 h postmortem. Moreover, even the samples have lost its motility because of a long storage, normal live fetuses were even obtained using immotile spermatozoa retrieved 20 d when the spermatozoa were injected into oocytes (Kishikawa *et al.* 1999). In the present study, it was observed that spermatozoa retained their motility and fertilizing ability for a greater duration within the epididymis.

## CONCLUSION

Ram epididymal spermatozoa survive in storage at 5 °C for up to 96 h. These spermatozoa maintain their

fertilizing ability and may be suitable for use in IVF and other assisted reproductive procedures. These techniques are important tools for animal preservation when sperm cells could not be cryopreserved immediately after an animal's death.

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