Epididymal sperm quality of Kacang goat preserved in low temperature for genetic material utilization in assisted reproductive technologies

S Prastowo¹,²,*, A F Nugroho¹ and R Widyastuti³

¹Department of Animal Science, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Indonesia
²Animal Breeding, Reproduction and Biostatistics Research Group, Universitas Sebelas Maret, Surakarta, Indonesia
³Laboratory of Animal Reproduction and Artificial Insemination, Department of Animal Production, Animal Husbandry Faculty, Universitas Padjajaran, Jatinangor, Indonesia

Corresponding author: prastowo@staff.uns.ac.id

Abstract. Postmortem epidydimal preservation at low temperature (3 - 4°C), is a way to preserve and recover male genetic material. This effort aims for prolonging male function as sperm source, followed with its utilization in assisted reproductive technologies. This study aimed to observe the quality of sperm form cauda epididymis which preserved at low temperature for consecutive days. Sperm were retrieved from twelve cauda epididymis of Kacang Goat and its qualities namely motility, intact membrane, life/dead, and abnormality (all in %) were evaluated in every 2 days until 0% motility. Data were compared using analysis of variance at α = 0.05. Result shows significant (P<0.05) decrease in motility, intact membrane, and life/dead, but increase in abnormality during observation at day 0, day 2, day 4 and day 6, respectively. At the respective days, motility was 91.33±1.25%; 74.67±3.88%; 28.17±2.25% and 0.33±0.57%, intact membrane was 54.83±1.04%; 39±3.77%; 25.1±3.32% and 14.83±2.75%, life/dead was 55.17±4.01%; 36±3.5%; 24.3±3.25% and 12±2.78%, abnormality was 3.16±0.76%; 4.16±0.76%; 6.16±2.25% and 11±2.17%. According to the study, it is concluded that preserved sperm from cauda epididymis at low temperature shows decrease in quality and its utilization should rely on the quality status to select the most appropriate assisted reproductive technology.

1. Introduction

Dead in animal could be the end of reproduction process. In this regard, however, testis and ovary can be collected, preserved then its genetic material could be utilized to produce offspring. The use of that genetic material, combined with the advance of assisted reproduction technologies (ART) will prolonging animal reproductive process with limitless possibilities. For example, sperm from collected epididymis can be froze and store in liquid nitrogen. Sperm from cauda epididymis has same capabilities in fertilizing ova as same as with ejaculated sperm. Therefore, sperm from cauda epididymis could be collected, preserved, and then utilized for reproduction purpose.

Collecting epididymis from dead animal is a simple procedure that can be followed by simple preservation in low temperatures (3 - 4°C) with minimal equipment. Even though, the most critical
factor influencing the success rate of epididymal sperm preservation is time interval between animal's death and sperm collection. Numerous studies on epididymal sperm preservation have been done, primarily for animals with high genetic value and wild animals to prevent the genetic losses. Previous research has described techniques for sperm epididymal collection [1–3], transportation to the laboratory [4,5], and procedure for preservation [6,7]. The process of recover spermatozoa from epididymal has been described as a possible method in ram [3], goat [8], European read deer [9], stallion [10] and also rat [11].

Following preservation sperm can be utilized in ART to prolong reproductive process, such as for artificial insemination [12,13], in vitro fertilization (IVF) [14] and intracytoplasmic sperm injection (ICSI) [15,16]. Recently, the development of ART brings many opportunities to use preserved genetic material of male to produce offspring. For that, this study aimed to know whether simple preservation of cauda epididymis in low temperature could be use as model for male genetic material preservation, by observing its sperm quality. Next according to the sperm quality parameters, best ART choice can be suggested for higher successful of fertilization rate.

2. Material and method

2.1. Testis and epididymis collection
Kacang goat testis were collected from nearby local slaughterhouse. The goats were slaughtered using Islamic method by experienced butcher which employed by local authority. Once the skinning and evisceration process were done, testis then collected and whole epididymis (caput to cauda) were dissected and stored in warm (37°C) NaCl 0.9% following transportation to the laboratory. Once arrived, epididymis then cleaned up from blood or other material attached, then only the cauda epididymis was stored individually (left of right selected randomly) in plastic container at 3-4°C.

2.2. Retrieval of epididymal sperm
On the day of sperm quality parameter observation, sperm from three cauda epididymis were collected by dissecting the cauda and firmly press the sperm in 1.5 ml collection tube filled with 1 ml pre warmed NaCl 0.9%. Sperm then let sit in the tube for 10 minutes and incubated at 37°C using heating block. Later, sperm are ready to be access for its quality.

2.3. Sperm quality observation

2.3.1. Motility. Sperm motility was observed under microscope. A drop of retrieved epididymal sperm was diluted in warm (37°C) NaCl 0.9% and then placed in pre warmed slide glass and covered. Motility was subjectively evaluated, and the percentage of moving forward sperm were calculated in scale 0-100%.

2.3.2. Intact membrane. Sperm membrane was evaluated using hypo-osmotic swelling test (HOST-test). Retrieved epididymal sperm was diluted in NaCl 0.9% then incubated in hypo-osmotic solution according to published protocol [17]. Following incubation, 2µl of sample then mixed with 8µl of eosin-nigrosine staining solution then prepared for smear on the slide glass. This test was based on the swelling ability when functional sperm membrane submitted to hypo-osmotic solution. Intact membrane is indicated by the present of swell tails, and in the contrary for the non-intact. Using 1000x magnification, minimum 200 sperm were observed and then the ratio between intact vs non-intact was calculated in percentage.

2.3.3. Life/dead. The sperm viability or life dead ratio is tested using Eosin-Nigrosine staining test. Two µl of sperm sample were mixed with 8µl eosin-nigrosine staining solution then prepared for smear on the slide glass. White or light pink head of sperm indicated the sperm is viable (life), while sperm with
red or dark head indicate non-viable (dead) one. Minimum 200 sperm are observed under microscope, and the ration between life and dead sperm was calculated in percentage.

2.3.4. Abnormality. Along with the life/dead sperm observation, the abnormality of sperm also observed. The sperm normality was evaluated in the head and tail. Macro and micro size of sperm head shape, wrinkle and broken tails are considered as abnormal sperm. Minimum 200 sperm were evaluated, and the ratio between abnormal and normal then calculated in percentage.

2.4. Data analysis
The sperm quality between days of observation were compared using analysis of variance at $\alpha = 0.05$. All the data analysis were prepared accordingly in R programing language [18].

3. Result and discussion
In total 12 pair of testes were collected from Kacang goat, obtained from local slaughterhouse. The physical condition of collected testis was shown in Table 1. All sampled goats were in the same age (1-1.5 y.o.), mature and have fully function on its reproduction activity. The scrotal circumference (SC) of the Kacang goat in this study, is ranged from 19-25 cm. This range was lower compared to the mean of SC in mature Boer goat which around 26-32 cm [19]. Testis weight in this study was weighed for one testicular, and all were in normal condition, none of them has cryptorchid. The weight of testis is indicating the capabilities of male to produce sperm. The more size of testis, in weight and SC, representing the ability of male to produce more sperm and hormone. Later this would be a mirror of male fertility, due to male hormone function for maintain male secondary reproductive characteristic.

| Parameter                          | Min | Max | Mean ± sd |
|-----------------------------------|-----|-----|-----------|
| Scrotal circumference (cm)        | 19  | 25  | 21 ± 1.46 |
| Testis weight (gram)              | 147.8 | 310.6 | 218.3 ± 35.42 |

The quality of sperm retrieved from cauda epididymis during preservation was shown in Table 2. Motility, intact membrane, life/dead were significantly decline ($p<0.05$) from day 0 to day 2, 4 and 6. However, abnormal sperm were found significant different in day 0 vs day 2, 4 and 6. Moreover, the abnormality between day 2, 4 and 6 were remain not significantly differ. According to the quality data, we see a fast decline in motility was found in day 2 to 4 from 74.67% to 28.17%. As the quality standard sperm motility must be in around of 60-70% to be use for AI.

| Sperm quality parameter (%)       | 0   | 2   | 4   | 6   |
|-----------------------------------|-----|-----|-----|-----|
| Motility                          | 91.33±1.25$^a$ | 74.67±3.88$^b$ | 28.17±2.25$^c$ | 0.33±0.57$^d$ |
| Intact membrane                   | 54.83±1.04$^a$ | 39±3.77$^b$ | 25.1±3.32$^c$ | 14.83±2.75$^d$ |
| Life/dead                         | 55.17±4.01$^a$ | 36±3.50$^b$ | 24.3±3.25$^c$ | 12±2.78$^d$ |
| Abnormality                        | 3.16±0.76$^a$ | 4.16±0.76$^b$ | 6.16±2.25$^b$ | 11±2.17$^b$ |

$^a, b$ different superscript in the same row shows significant different ($p<0.05$)

At day 4 of preservation, sperm shows motility less than 30%. This due to less life sperm (Table 2 in life/dead parameter) and more dead sperm were found. The pattern also applied to day 6, when the motility is nearly 0%. Along with more dead sperm, less intact membrane and more abnormal sperm were observed. At this point, we can say that simple preservation using NaCl 0.9% in low temperature cannot maintain the sperm quality no longer than 2 days. Preserved sperm at day 4 can be utilized in reproduction purpose by using IVF technology. In the IVF, smaller number of sperm is possible. To select best sperm for IVF in low gross motility of sperm, a swim up methods can be applied. The use of preserved sperm from day 6, 0% motility, can be help with ICSI method as best option. ICSI has
advantage to use single sperm for fertilization [20] by directly injecting into oocyte cytoplasm. In here ICSI don’t need motile sperm but require intact membrane [21]. At day 6, we can see around 14.83±2.75% sperm still had intact membrane although it’s not motile.

The decrease in sperm motility, intact membrane, and percentage of viable sperm may be associated with oxidative stress caused by excessive ROS formation during preservation. The oxidative stress caused a lesion in the ATP utilization or the contractile apparatus of the flagellum sperm, which had a negative effect on sperm motility [22]. Furthermore, the sperm plasma membrane's main component is polyunsaturated fatty acid, and it lacks antioxidative protection, making it extremely vulnerable to oxidative stress [23]. As the result, excessive ROS production causes lipid peroxidation and membrane disruption in the sperm plasma membrane, then lowering sperm quality. This disruption has a direct impact on sperm viability as well as a negative impact on the transport membrane process which required for sperm metabolism and fertility [24].

To overcome excess production of ROS, preserve sperm with the addition of semen extender which contain energy, buffer, antioxidant, and other beneficial ingredients for sperm during preservation can be applied [25]. The use of 0.9% NaCl as preservation media in this study, is not sufficient to support sperm nutrition requirements during the preservation period. For long term preservation of genetic material, it is preferable if the sperm from epididymal were diluted in complex extender media supplemented with cryoprotectant and then frozen as frozen straw. Considering the above result, the use of genetic material for ART purpose must rely on the sperm quality parameter. We suggest using of preserved sperm at day 2 for AI, at day 4 for IVF with special sperm selection treatment, and at day 6 for ICSI but need to consider the intact membrane. The option to preserve epididymal sperm in form of frozen straw could be the best choice to keep the genetic material for long term rather than use simple preservation media which only able to protect sperm for short period.

4. Conclusions
Preserving sperm form cauda epididymis using simple preservation method in low temperature 3-4°C is able to maintain the sperm quality parameter until day 2. More than 2 days, sperm quality will be decline in fast and almost showed no motility at day 6. The utilization of the preserved sperm from cauda epididymis needs to be rely on the quality parameter, to have better fertilization rate.

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