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PRESERVATION OF GARUT RAMS SPERMATOZOON AS A SOURCE OF MALE GERM PLASM

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ABSTRACT

This study was conducted to examine the quality of ejaculated sperm by Garut rams to be used for artificial insemination (AI) and viability of sperm that were collected from prescrved *cauda epididymis* (4°C up to 12 days) for assisted reproductive technology. The semen was collected by artificial vagina, with the sperm motility, live sperm, acrosomal intact, and intact plasma membrane observed. Sperm motility was 75%, while for the live sperm, intact plasma membrane and sperm abnormality were 91.5%, 90.0%, and 1.8%, respectively. In the other study, sperm was collected from *cauda epididymis* by aspiration method and diluted in different media: 1) Brackett Oliphant (BO) media and 2) modified Phosphate Buffer Saline (mPBS). Evaluation of sperm motility and intact plasma membrane were conducted after washing, counting and dilution of the sperm. The results of this study showed that the sperm motility and intact plasma membrane could be maintained better in BO rather than PBS medium although they were not statistically different ($P>0.05$). At day 12 of preservation, the motility and intact plasma membrane of sperm collected from *cauda epididymis* were 0.7% and 1.33% for motility and plasma membrane intact, respectively. These findings showed that the Garut rams semen was qualified for AI and frozen processing; *in vitro* embryo production by introducing the assisted reproductive technology such as intracytoplasmic sperm injection (ICSI) could be applied by using the sperm collected from preserved *cauda epididymis* until 12 days of preservation at 4°C.

Keywords : Reproduction/spermatogenesis/insemination/Garut rams/small ruminant

INTRODUCTION

Indonesia is a country with tremendous amounts of genetic resources and biodiversity. In Indonesia, the percentage of meat and wool produced by sheep as a small ruminant in West Java Province is approximately 46.2% (Anonymous 1999). One of the ruminants is Garut sheep, endemic to Indonesia, and potential for meat production. Moreover, they have a good performance for sheep contest. The population of Garut sheep decreased from 7.6 million in 1997 to 7.2 million in 2001.

In an attempt to increase the Garut sheep population, several efforts should be done to maintain the population. Application of reproduction biotechnology such as artificial insemination (AI) and *in vitro* embryo production are the solutions. Artificial insemination has been routinely done in large ruminants such as cattle and

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buffaloes. However, in small ruminants like sheep and goat this technique should be modified according to the small size of the reproductive tract. Sperm usually was collected from ejaculation for artificial insemination procedure. Collected sperms were then diluted for fresh sperm insemination, sperm preservation or sperm cryopreservation. In the case of dead ram, sperm was collected from *cauda epididymis.*

Epididymal sperm may be the last chance to ensure preservation of genetic materials after injury or death of a valuable male. Studies have been conducted to determine if epididymal sperm can be used to produce viable embryos and offsprings. Iwamatsu and Chang (1971) have reported successful fertilization of mouse oocytes using epididymal sperm. Fuller and Whittingham (1996) reported the production of normal mouse fetuses after IVF using epididymal sperm that had been cooled at 4°C. Sperm collected from the caudal epididymides has been used for both IVF and intracytoplasmic sperm injection (ICSI) in domestic cats (Pope *et al.* 1998). Caprine epididymal sperm has also been reported for use in the production of IVF embryos to the blastocyst stage (Song and Iritani 1988), and production of an IVF goat offspring born from artificial insemination (Blash *et al.* 2000).

From recent studies, viable epididymal sperm can be harvested post-mortem for possible use in assisted reproductive technologies (ART). Most of the species evaluated so far, produce more viable sperm when the testicles are stored at 4°C, prior to harvesting the epididymal sperm. The objective of this study was to identify an alternative to collect ovine ejaculate and epididymal sperm for subsequent fertilization.

MATERIALS AND METHODS

Sperm Collection

Ejaculated sperm. Sperm ejaculate was collected from 5 healthy Garut rams by using artificial vagina. The artificial vagina consists of a hose 20 to 25 cm in length and 5 to 7 cm in diameter, with a rubber liner. The temperature of artificial vagina was set between 42 to 46°C, and it is necessary to lubricate it with K-Y jelly. At the time of the ram mounts to the ewe, his penis was gently guided inside the artificial vagina connected with a warm tube (37°C) to avoid the cold shock. After ejaculation, the tube containing the semen was removed and placed in a water bath at 30°C.

Testicle Preservation

Ovine testicles were collected as pairs from mature rams from a slaughter-house. One pair of testicle (control testicle) was processed shortly after the rams were slaughtered (day-0), while the other testicle of the pair was processed and stored at 4°C up to 12 days of preservation. Collected sperms were evaluated daily.

Epididymal sperm. After storage at 4°C, each testicle was allowed to warm at room temperature (25°C) for not more than 30 minutes. During this warming period, the testicle was dissected away from the connecting tissues. Sperm dilution media (Brackett and Oliphant, BO and modified phosphate buffer saline, mPBS) were warmed in water bath to 37°C prior to exposure to the epididymal sperm. Sperm was collected from the *cauda epididymis* region by aspiration method. Briefly, sperm was collected by aspiration using 21G needle connected to 3 ml spuit already filled with 1 ml warmed dilution media. Collected sperm was then washed by centrifugation at 500G for 3 minutes to create sperm pellet. The supernatant was discarded and the pellet was resuspended in sperm dilution media.

Evaluation

The volume of ejaculated sperm was recorded in each collection. Microscopic evaluation of the sperm was done including the sperm concentration, motility, percentage of live sperm, intact plasma membrane, and morphology. For epididymal sperm, analysis was done on percentage of sperm motility and intact plasma membrane.

An evaluation of live and dead sperm was done by eosin-negrosin staining. The appearing red sperms were considered dead, otherwise they are alive. The sperm which had an intact plasma membrane was shown by coiled sperm tail after incubation in hypo-osmotic solution at 37°C for 30 min (Correa and Zavos 1994). Hypo-osmotic solution contained 0.032 M NaCl in destilled water.

Data were analyzed using the simple one-way ANOVA to make comparisons between testicle pairs and Duncan's test to identify the difference in means between the treatment groups.

RESULTS AND DISCUSSION

Ejaculated sperm was successfully collected from 5 rams. The mean number of Garut ram ejaculated sperm was 3463 x 10^6 sperm mL^{-1}, with the average volume of semen per ejaculation of 0.7 mL (Table 1). By comparison, the volume of semen per ejaculation was 1.7 mL in St. Croix sheep (Feradis 1999), and 1.05 mL in Suffolk sheep (Boland *et.al.* 1985). The difference had correlation with individual factors such as age, body weight, and breed (Hafez and Hafez 2000).

The motility and percentage of live sperm of Garut ram ejaculation were 75.0% and 91.5%, respectively. These results were higher than that reported previously in St. Croix (64.0% and 76.0% for motility and percentage of live sperm, respectively) (Sirman and Situmorang 1987).

Table 1. Sperm quality of ejaculated semen collected from Garut ram by artificial vagina.

No.	Parameter	Results
1.	Volume (mL/ejaculation)	0.7 ± 0.1
2.	Concentration (10^6 sperm mL^{-1})	3463 ± 65
3.	Motility (%)	75.0 ± 3.2
4.	Live (%)	91.5 ± 3.1
5.	Intact plasma membrane (%)	90.0 ± 6.6
6.	Abnormality	1.8 ± 0.8

The percentage of intact plasma membrane of Garut ram ejaculated sperm was 90.0%. This was higher than the percentage of intact plasma membrane of St. Croix sheep ejaculated sperm i.e. 86.3% (Feradis 1999). When sperm was exposed to hypo-osmotic solution, the spermatozoa that had intact plasma membrane would be swollen. If the sperm have intact plasma membrane, the water that influx into the cell could not come out from the cell. The percentage of intact plasma membrane had positive correlation with sperm motility.

No single test accurately predicted fertility of a sperm sample; however, examining various physical characteristics of semen could determine greater fertility potential. Processing semen for artificial insemination or sperm freezing depends on semen quality. Analysis of ram semen involves obtaining maximum information about the physiology status of testicular and epididymis functions by examining one or several ejaculations. In general, the minimal requirements for semen processing (fresh or frozen semen) would include: at least 65% motility, concentration 700 x 10^6 sperm mL^{-1}, and less than 20% morphological abnormalities (Hafez and Hafez 2000).

Motility and intact plasma membrane of sperm collected from *cauda epididymis* could be found up to 24 hours of incubation *in vitro* both in mPBS or BO medium. At the time of collection sperm motility was 58.0% in mPBS medium and 55.7% in BO medium. They decreased gradually during the *in vitro* incubation up to 24 hours (0.3% in mPBS and 0.6% in BO medium) (Figure 1). Intact plasma membrane of sperm collected from *cauda epididymis* was 60.0% in mPBS and 56.7% in BO medium at the time of sperm collection. The intact plasma membrane in both media during *in vitro* incubation exhibited a declining pattern, as with sperm motility. However, the intact plasma membrane was higher than sperms motility after 18 hours of incubation. This was shown by some of the immotile sperm that had intact plasma membrane. In this case, spermatozoa may be used for *in vitro* fertilization by injection of single sperm directly into the oocyte cytoplasm called intracytoplasmic sperm injection (ICSI) .

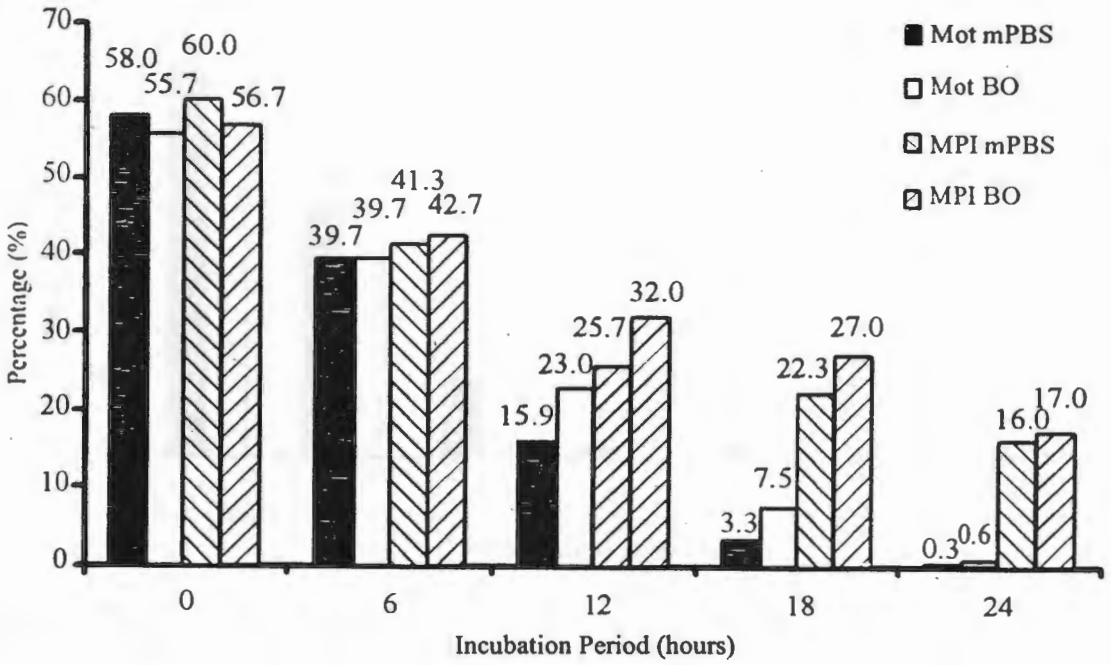

Figure 1. Motility and intact plasma membrane of sperm collected from *cauda epididymis* after incubation *in vitro* in different medium. Mot mPBS: sperm motility in mPBS medium, Mot BO: sperm motility in BO medium; MPI mPBS: intact plasma membranc of sperm in mPBS medium, MPI BO: intact plasma membrane in BO medium.

The sperm motility and intact plasma membrane of sperm collected from *cauda epididymis* decreased significantly after 2 days of preservation at 4°C. However, 0.7% motile sperm and 1.3% sperm with intact plasma membrane were found after 12 days of preservation (Figure 2).

According to motility and live sperm, Rizal *et al.* (2004) reported that sperm collected from testicles after 3 days of preservation at 5°C could be used for artificial insemination or *in vitro* embryo production of sheep. Epididymal sperm was used in a goat *in vitro* embryo production that resulted in cleavage and development of blastocyst, although none of the blastocysts were transfered to the recipients (Song and Iritani 1988). Further work is needed to optimize the *in vitro* embryo production, through micro-fertilization procedure. The valuable sperm has the ability to fertilize the oocytes and, therefore, could increase the chances of propagating valuable genetics.

Intracytoplasmic sperm injection (ICSI) could initiate the early embryo development to cleavage in goat oocyte (Boediono 2001). Moreover, Cochran *et al.* (1998) reported that live IVF offsprings from oocyte donor mares have been produced by using ICSI procedure. Although there has been some success using assisted reproductive technologies, more researches are needed to improve the efficiency of the procedures.

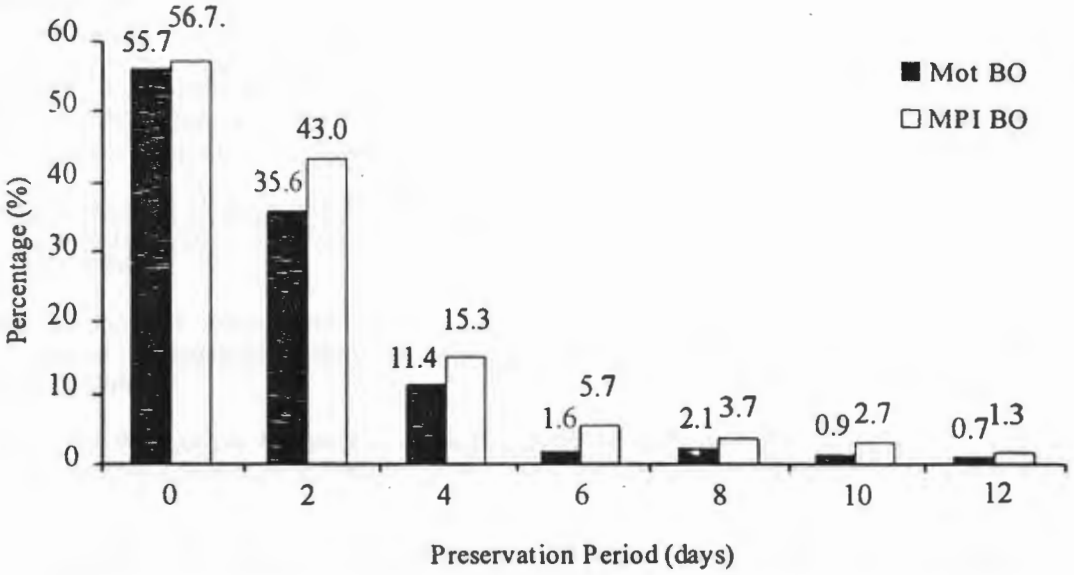

Figure 2. Motility and intact plasma membrane of sperm collected from *cauda epididymis* after storage at 4°C and incubation *in vitro* in BO medium. Mot BO: sperm motility in BO medium; MPI BO: intact plasma membrane in BO medium.

CONCLUSIONS

These results indicate that Garut rams semen was qualified for AI and frozen processing, and motile sperm can still be collected from *cauda epididymis* after being stored at 4°C up to 12 days. Sperm cooled in the testicle at 4°C could be used in assisted reproductive technologies (intracytoplasmic sperm injection) for the untimely death of a valuable sheep and an effective tool to conserve important genetic resources. This study investigated the standard parameter of sperm analysis. It would stand to reason that further investigations with particular emphasis on chromatin damage and/or fertility are suggested.

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