INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 10, No. 2, p. 6-11, 2017

RESEARCH PAPER OPEN ACCESS

Sex ratio of calves generated from artificial insemination using frozen-thawed epididymal spermatozoa of spotted buffalo

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Key words: Epididymal spermatozoa, Sex ratio, Spotted buffalo

http://dx.doi.org/10.12692/ijb/10.2.6-11 Article published on February 20, 2017

Abstract

Utilization of the cauda epididymis as an alternative spermatozoa source for assisted reproductive technology (ART) has been adopted in many species. This technique is useful to preserve genetic diversity in endangered species, and from animals experiencing sudden death or pathology resulting in an inability to ejaculate. This technique has been used for several decades, but data about the distribution of the sex ratio of the offspring produced is limited. In a preliminary artificial insemination (AI) study concerning using frozen-thawed epididymal spermatozoa from the spotted buffalo, a skewed sex ratio was observed. Results of this study showed that mean progressive motility, viability, and membrane integrity of fresh epididymal spermatozoa were 73.3%, 85.4%, and 86.2%, decreasing to 43.3%, 66.6%, and 66.9% after thawing, respectively. We got high and with a significantly higher number of female (12; 85.7%) compare to male (2; 14.3%) offspring ($P<0.05$). These interesting preliminary results indicate that a further study is needed.

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Introduction

Spermatozoa collected from cauda epididymis tissues were proved to be of suitable quality to fertilize oocytes both fresh and frozen-thawed (Martinez-Pastor *et al*., 2005; Hermansson and Axnér, 2007; Papa *et al*., 2008; Gañán *et al*., 2009; Gloria *et al*., 2011; Waheed *et al*., 2011; Lone *et al*., 2012; Turri *et al*., 2012). This method is useful to conserve gametes from animals with pathology that obstructs ejaculation, or in sudden death or illness in wild animals that are rare or endangered.

One of the many species that is becoming extinct in Indonesia nowadays is the spotted buffalo (*Bubalus bubalis carabanensis*). This species is important to the Toraja people in South Sulawesi, both economically and socially. The spotted buffalo has a unique coat pattern and iris color that distinguish them from the normal swamp buffalo. The spotted buffalo has been domesticated for approximately one thousand years in the Toraja area. In Toraja tradition, the spotted bull is slaughtered during funeral ceremony, called *Rambu Solo*, to show respect to a departed colleague and as a parting gift before that person is buried. This is a social event that has been going on for centuries, the effect of which has been a dramatic decrease in spotted buffalo population since the birth rate is much lower than the slaughter rate. Furthermore, the value of the spotted bull is much greater ~ 10 times more expensive than the swamp buffalo with normal coat and iris color. Moreover, farmers do not allow them to mate naturally because of a belief that the bull would lose weight and thus its economic value would be reduced. So far, the birth of spotted buffalo bulls arises only from the mating of spotted females.

In an effort to prevent the spotted buffalo from becoming extinct, we attempted to collect the epididymis tissues from spotted bulls after their slaughter during funeral ceremony, for use as the spermatozoa source for an artificial insemination (AI) program to produce spotted offspring. This study was performed to determine the fertilizing ability of epididymal spermatozoa; a secondary objective was to determine the gender ratio of the offspring generated, since the demand is higher for male than female spotted offspring.

Materials and methods

Experimental design

Cauda epididymis tissues were collected from three different spotted bulls during funeral ceremony for use as the spermatozoa source. The spermatozoa were collected using incision and flushing method of cauda epididymis tissue (Lone *et al*., 2011) using soya lechitin based extender (AndroMed®, Minitube, Germany). The same extender was also used to collect and suspend the epididymal spermatozoa before and after quality assessments.

Good quality epididymal spermatozoa was extended, packed in 0.25 ml plastic straws and equilibrated at 4 °C for 3 hours to allow the spermatozoa to adjust to the extender and temperature before freezing. The freezing process was started by placing the plastic straws 10 cm above liquid nitrogen (-120 °C) for 15 minutes, followed by plunging the straw into liquid nitrogen for cryopreservation, and stored in liquid nitrogen container (-196 °C) for 7 days. The frozen spermatozoa were thawed at 37 °C for 30 seconds before AI was performed.

Straw samples were analyzed randomly to monitor spermatozoa quality, including: progressive motility, viability, and membrane integrity being evaluated for both fresh and frozen-thawed epididymal spermatozoa. Progressive motility was observed subjectively using a phase contrast microscope (Nikon, Japan) in 10 fields of view (Rasul *et al*., 2001). Spermatozoa viability was analyzed using the eosin-nigrosin staining method (Rasul *et al*., 2001). Eosin can not penetrate through an intact spermatozoa membrane, with the result that living spermatozoa do not stain red, whereas dead or dying spermatozoa appear red against the blue background. Furthermore, membrane integrity was observed using hypo-osmotic swelling (HOS) test (Revell and Mrode, 1994). Spermatozoa samples were incubated for 45 minutes in hypo-osmotic medium. Spermatozoa with an intact membrane have a swollen tail, while spermatozoa with a damaged membrane have a straight tail when observed by phase contrast microscope.

Experimental animals

Two doses of $PGF_{2\alpha}$ injection 11 days apart were administered to 40 female buffaloes to synchronize the estrous cycles. These recipients were inseminated twice with an 8h-interval using 60.10^6 cell/straw frozen-thawed epididymal spermatozoa. Each female received spermatozoa from the same bull. Pregnancy was detected by rectal palpation after week-10 of AI. Sex ratio of calves was determined base on calving data of recipients.

Statistical analysis

Data about sex distribution of the offspring was analyzed using the Chi-squared test.

Results and discussion

The mean progressive motility of fresh epididymal spermatozoa was 73.3%, decreasing into 43.3% after thawing.

Decreasing quality was also seen in the viability and membrane integrity parameters. The mean viability and membrane integrity of fresh epididymal spermatozoa were 85.4% and 86.2%, decreasing into 66.6% and 66.9% after thawing (Table 1).

In general, the quality of fresh and frozen-thawed spotted buffalo epididymal spermatozoa was similar to the ejaculated spermatozoa of water buffalo (Akhter *et al*., 2012; Singh *et al*., 2012; Waheed *et al*., 2012). The results of this study showed that spotted buffalo epididymal spermatozoa could be used in artificial insemination program. Presumably they could also be used in other assisted reproductive technology applications, e.g. *in vitro* embryo production (IVEP) and intra-cytoplasmic sperm injection (ICSI), as shown for other species (Blash *et al*., 2000; Morris *et al*., 2004; Martins *et al*., 2007; Santiago-Moreno *et al*., 2008; Kozdrowski *et al*., 2011; Álvarez *et al*., 2012).

Table 1. Quality of fresh and frozen-thawed epididymal spermatozoa of spotted buffalo (mean ± SD).

Parameters	Fresh	Post-thawed
Progressive motility (%)	73.3 ± 2.4	43.3 ± 2.4
Viability (%)	85.4 ± 1.4	66.6 ± 2.3
Membrane integrity (%)	86.2 ± 2.3	66.9 ± 1.4

Fourteen of forty (35%) females were pregnant as the result of the artificial insemination program using frozen-thawed epididymal spermatozoa. This result was lower than 44 – 47% (Akhter *et al*., 2010) and 41.5 – 56% (Akhter *et al*., 2012) in river buffalo using ejaculate spermatozoa. However, the sex ratios of the offspring were different to the expected 50:50 ratio, since the number of female offspring (12 of 14; 85.7%) was significantly higher ($P<0.05$) than the number of male calves (2 of 14; 14.3%) (Tabel 2).

The pregnancy (35%) rate for the spotted buffalo using epididymal spermatozoa was consistent with those of other species, which vary from 16.7% in Spanish ibex (Santiago-Moreno *et al*., 2006), 22.2% in European bison (Kozdrowski *et al*., 2011), 28% in equine (Heise *et al*., 2010), 50% in bovine (Guerrero *et al*., 2008), 55.8% in ovine (Álvarez *et al*., 2012), 56% in red deer (Soler *et al*., 2003), and 66.6% in equine (Papa *et al*., 2008). However, there are few reports about the sex distribution of the offspring generated from AI using epididymal spermatozoa.

Most of the previous studies aimed only at showing that epididymal spermatozoa could produce offspring without mentioning their gender. Data about sex distribution of the offspring was showed that the result of AI using epididymal spermatozoa produced 2 males and 3 females offspring in white-tail deer (Saenz, 2007) and 1 male and 1 female calf in European bison (Kozdrowski *et al*., 2011).

It is known that in the normal ejaculation, the ratio of X and Y-bearing spermatozoa are approximately equal (Checa *et al*., 2002; Madrid-Bury *et al*., 2003; Parati *et al*., 2006). Thus, it is expected that the ratio of male to female offspring are also equal.

Table 2. Sex ratio of the offspring result artificial insemination using frozen-thawed epididymal spermatozoa of spotted buffalo (n = 14).

Sex of the offspring	Number of the offspring (%)
Female	12 (85.7)[a]
Male	2 (14.3)[b]

Different letters in the same column indicate significant difference by chi-squared test at P<0.05.

The results of IVEP programs indicate that the sex ratio can be changed due to the influence of treatments during spermatozoa preparation before in vitro fertilization or the culture conditions, or a combination of both factors (Kochhar *et al*., 2001; Kochhar *et al*., 2003; Lechniak *et al*., 2003).

In our study, we used a simple collection method with the same extender as for cryopreservation. The spermatozoa were not subjected to any procedures before artificial insemination, in contrast to IVEP. The skew in the sex distribution obtained in this AI program is interesting and deserves further study since it might be that the proportion of X and Y-bearing spermatozoa in spotted buffalo epididymis is not equal. To prove this, we suggest a further study comparing the proportion of X and Y-bearing spermatozoa in spotted buffalo cauda epididymal and ejaculated spermatozoa using fluorescence in situ hybridization (FISH). We expect that the result would show us whether this method should be investigated in other species where production of female offspring is desired. The effect of extender on sex ratio should also be investigated as a possibility for a simple sex selection procedure. In spotted buffalo case, study about sex ratio between female and male of the offspring in AI program using cauda epididymal spermatozoa was different compare to the other animals, e.g white-tail deer (Saenz, 2007) and European bison (Álvarez *et al*., 2012). Sex distribution of the offspring generated from AI using epididymal spermatozoa in white-tail deer and European bison are normal (50:50).

Acknowledgement

The authors wish to thankfull the head and staff of the Animal Division of North Toraja Government for supporting this study and collecting samples.

Finally, we wish to thanks all of the spotted buffalo owners that have provided samples; without their help, this study would not have been possible.

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