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The fertility of South Kalimantan buffalo spermatozoa after cold preservation and cryopreservation

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Abstract

Sugar palm juice can be an alternative extender of buffalo semen because it contains nutrients needed by spermatozoa during preservation. The objective of this study was to examine the fertility of South Kalimantan buffalo spermatozoa after the preservation and cryopreservation process. Semen was collected by the artificial vagina. Fresh semen was divided into two tubes in equal volume and then diluted with sugar palm juice extender with a different composition. Semen in the first tube was diluted with 80% sugar palm juice + 20% egg yolk, and then stored in a refrigerator at 5°C (chilled-semen) for three days. Semen in the second tube was diluted with 73% sugar palm juice + 20% egg yolk + 7% glycerol. Diluted-semen was loaded in a mini straw and then stored in a liquid nitrogen container (-196°C) for seven days. A total of 22 adult female buffaloes aged 3–5 years were selected as acceptors and estrous synchronization by PGF2α. Thirteen females were inseminated with chilled-semen, and nine females were inseminated with frozen semen The results of this study showed that motility of spermatozoa after preservation for three days was an average of 45.83% and 38.33% for frozen-thawed semen. The percentage of pregnancy and birth for chilled-semen was 69.23% and 61.54%, higher than for frozen-thawed semen (66.67% and 55.55%), but there was no significant difference between treatment. It can be concluded that sugar palm juice is suitable as an alternative extender for South Kalimantan buffalo semen because it is able to maintain the quality and fertility of spermatozoa after preservation and cryopreservation.

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Introduction

Swamp buffalo (*Bubalus bubalis*) in South Kalimantan Province through the Minister of Agriculture's decree in 2012 has been designated as a separate family and named the South Kalimantan buffalo. The decree also emphasized that the South Kalimantan buffalo is a wealth of genetic resources of Indonesian local livestock that must be protected and preserved. One effective form of conserving buffaloes in South Kalimantan is to increase the population, so as to improve the welfare of buffalo breeders.

The application of artificial insemination (AI) technology is one alternative method to accelerate population increase and improve buffalo genetic quality (Morrell, 2006). Preservation and cryopreservation of semen are an integral and inseparable part of AI technology (Sansone *et al.*, 2000), and it is an essential method for conservation of germ cells and is suitable for the proper genetic management (Singh *et al.*, 2013). Semen cryopreservation is an effective method in the preservation of superior male genetic resources directly related to AI technology so that its application will enhance the genetic quality improvement of livestock (Martinez *et al.*, 2007). According to Hafez and Hafez (2000), the main purpose of processing semen is to increase the capacity of semen to serve more female. To achieve this goal, semen is diluted with certain extenders, which meet conditions such as energy source, buffer, non-toxic, prevent damage to spermatozoa, do not reduce spermatozoa fertility, do not impede the evaluation of spermatozoa after dilution, inexpensive, and easily obtained.

Extracts of natural ingredients from vegetables and fruits and their seeds contain an important compounds for health, so it is able to maintain life (Aviram *et al.*, 2000; Aviram *et al.*, 2004). These natural extracts and infusions are used in semen extenders for preserving animal sperm (Sansone *et al.*, 2000). Some natural ingredients commonly used as semen extender contains strong antioxidant compounds, thus protecting spermatozoa from oxidative damage during the cryopreservation process (Seeram *et al.*, 2005; Tezcan *et al.*, 2009). Utilization of natural materials such as coconut water in semen cryopreservation has been reported in buck (Daramola *et al.*, 2016) and cattle (El-Sheshtawy, 2017; Tarig *et al.*, 2017), with good results.

Sugar palm juice can be an alternative extender of semen because it contains nutrients needed by spermatozoa during preservation (Rizal and Riyadhi, 2016), but do not have protective compounds of spermatozoa during the process of semen preservation at 5°C and cryopreservation at -196°C. In the process of preservation and cryopreservation of semen, extender must be added a special compound in the form of cryoprotectant which functions to protect spermatozoa from damage (Andrabi *et al.*, 2008). Egg yolk is one component that is commonly used in the preservation and cryopreservation of semen. Egg yolk lipoprotein, cholesterol, and lecithin are the function to maintain the integrity of the plasma membrane lipoprotein of spermatozoa during preservation at low temperatures (Kumar *et al.*, 1992). Specifically, in the cryopreservation process of semen, other than egg yolk, extender should also be added to intracellular cryoprotectant compounds such as glycerol which functions to minimize cell damage during freezing (Leboeuf *et al.*, 2000). Through this treatment, it is expected that the quality of spermatozoa during preservation and cryopreservation of semen can be maintained, and does not reduce fertility when applied in AI technology. The objective of this study was to examine the fertility of South Kalimantan buffalo spermatozoa after the preservation and cryopreservation process.

Materials and methods

Collection, evaluation, preservation, and cryopreservation of semen

Semen was collected from two adult South Kalimantan buffaloes using artificial vagina at The Center for Artificial Insemination, South Kalimantan Province, Banjarbaru. Semen was collected 12 times as a replication. Quality of fresh semen was evaluated,

including volume, consistency, pH, mass movement, spermatozoa concentration, spermatozoa motility, and spermatozoa abnormality. Fresh semen that meets the quality requirements was diluted according to treatment. The good fresh semen must have spermatozoa motility > 70% (Barati *et al.*, 2009; Akhter *et al.*, 2011; Wadood *et al.*, 2016; Almadaly *et al.*, 2019), mass movements > ++, spermatozoa concentration > 1,000 million cells/mL, and spermatozoa abnormality < 15% (Almadaly *et al.*, 2019). Preservation, cryopreservation, and evaluation of spermatozoa quality were carried out at The Animal Production Laboratory, Department of Animal Science, Lambung Mangkurat University, Banjarbaru.

Fresh semen was divided into two tubes in equal volume and then diluted with sugar palm juice extender with a different composition. Semen in the first tube was diluted with 80% sugar palm juice + 20% egg yolk, and then stored in a refrigerator at 5°C (chilled-semen) for three days. Chilled-semen was diluted to a concentration of 10 million motile per mini straw (0.25 mL). The extender was added with an antibiotic, penicillin 1,000 µg/mL and streptomycin 1,000 IU/mL extender. Sugar palm juice was prepared by heating to boiling and then filtered with filter paper.

Semen in the second tube was diluted with 73% sugar palm juice + 20% egg yolk + 7% glycerol. Diluted-semen was loaded in a mini straw (0.25 mL), and then equilibrated in a refrigerator at 5°C for four hours. Semen was frozen by placing straw 10 cm above the surface of liquid nitrogen in closed styrofoam (temperature around -130°C) for 15 minutes. Straw was put into liquid nitrogen (temperature around -196°C) and then stored in a liquid nitrogen container for seven days. Frozen semen was diluted to a concentration of 30 million motile spermatozoa per mini straw.

Estrous synchronization, artificial insemination, and pregnancy evaluation

A total of 22 adult female buffaloes aged 3–5 years were selected as acceptors in the Maju Jaya 1 Farmers Group in Benua Raya Village, Tanah Laut District. Acceptors were estrous synchronization by injecting 2 ml (0.5 mg cloprostenol sodium) prostaglandin hormone (PGF2α, BoVet). Each acceptor was injected with PGF2α twice with an injection period of 12 days. The symptom of estrous was observed 2 days after the second injection of PGF2α.

Artificial insemination was carried out on females who show symptoms of estrous 3 days (72 hours) after the second injection of PGF2α. Thirteen females were inseminated with chilled-semen, and nine females were inseminated with frozen semen. Artificial insemination was using rectovaginal methods. Semen was deposited in the cervical lumen and the uterine corpus. Each buffalo was inseminated with one straw. Motility of spermatozoa was evaluated before using an AI application. Thawing of frozen semen was by putting a straw into 37°C water for 30 seconds.

Pregnancy was evaluated by observation of estrus 20-22 days (one estrous cycle) and 41-43 days (two estrous cycles) after insemination. Females who showed no symptoms of estrus were diagnosed pregnant, and others were not pregnant. The percentage of birth was obtained after the buffaloes give birth.

Statistical analysis

Data about the percentage of pregnancy and birth were analyzed using the Chi-squared test.

Results and discussion

Characteristics of fresh semen and semen quality after preservation and cryopreservation

The results obtained that fresh semen characteristics of South Kalimantan buffalo were volume 1.97 mL, concentration of spermatozoa 1,066.5 million/mL, and spermatozoa motility 71.67% (Table 1). In previous studies, it was reported that the volume of swamp buffalo semen was 2.02 mL (Amin *et al.*, 1999), 0.225 mL (Yulnawati *et al.*, 2010), and 1.44–1.76 mL (Sianturi *et al.*, 2012).

Table 1. Characteristics of South Kalimantan buffalo fresh semen.

Semen attributes	Mean ± SD
Volume (mL)	1.97 ± 0.35
Consistency	Moderate
Degree of acidity (pH)	6.9 ± 0.42
Mass movement (1 – 3)	2 ± 0.14
Concentration of spermatozoa (million/mL)	1,066.5 ± 98.23
Spermatozoa motility (%)	71.67 ± 3.27
Spermatozoa abnormality (%)	8.67 ± 0.49

The consistency and mass movement of swamp buffalo semen were thick and 2–3 (Amin *et al*., 1999), thin to thick and 2–3 (Sianturi *et al*., 2012), respectively. Spermatozoa concentrations of swamp buffalo was 1,447.14 million/mL (Amin *et al*., 1999), 2,695 million/mL (Yulnawati *et al*., 2010), 1,070–1,983 million/mL (Sianturi *et al*., 2012), and 1,600 million/mL (Almadaly *et al*., 2019). The percentage of spermatozoa motility of swamp buffalo was 76.43% (Rizal *et al*., 1999), 70% (Yulnawati *et al*., 2010), and 78.7% (Almadaly *et al*., 2019). The percentage of spermatozoa abnormality of swamp buffalo was 12.14% (Rizal *et al*., 1999) and 78.7% (Almadaly *et al*., 2019). According to Mahmoud *et al*. (2013) that semen volume, spermatozoa concentration, and spermatozoa motility of buffalo in Iran were 2.9 mL, 1,079.2 million/mL, and 65.8%, respectively.

Table 2. Percentage of pregnancy and birth of South Kalimantan buffalo.

Treatment	Pregnancy (%)	Birth (%)
Chilled-semen (5°C)	69.23 (9/13)	61.54 (8/13)
Frozen-thawed semen (-196°C)	66.67 (6/9)	55.55 (5/9)

The results of this study showed that there was a decrease in spermatozoa motility with increasing storage time of semen at 5°C. The motility of spermatozoa after preservation for three days was an average of 45.83%. Decreased spermatozoa motility caused by the decreasing quality of the extender which becomes more acidic. The acidic condition of semen extender is caused by a buildup of metabolic lactic acid. The end result of the metabolic process of spermatozoa that preserved in the refrigerator is lactic acid because the storage takes place anaerobically (without oxygen). According to Stryer (1995), the end result of cell metabolism under anaerobic conditions is lactic acid.

The main principle of preservation of semen associated with efforts to extend the life of spermatozoa is to reduce the degree of metabolism through storage at low temperatures (Lemma, 2011). Preservation of semen at 5°C causes changes in the spermatozoa cell membrane from liquid to gel. Metabolism of the cell is maximum at body temperature, and optimal when stored at room temperature (24–29°C). McKinnon (1999) reported that every drop in temperature of 10°C, resulting from a decrease in metabolism up to 50% so that preservation at 5°C metabolism of spermatozoa only about 10%.

Spermatozoa motility after thawing was obtained an average of 38.33%. The results showed that a combination of 20% egg yolk and 7% glycerol in sugar palm juice extender is able to maintain the spermatozoa motility of South Kalimantan buffalo during the cryopreservation process. Egg yolk contains lecithin and cholesterol which functions to protect the cell plasma membrane due to cold shock during freezing (Hermansson and Linde-Forsberg, 2006). While, glycerol is functions to protect the cell by minimizing the formation of ice crystal in the cell

(Mazur, 1984), preventing cell dehydration, maintain the balance of electrolytes osmotic pressure inside and outside of the cell (Watson, 2000), and maintain the stability of protein and glycoprotein of the cell plasma membrane (Park and Graham, 1992). Almadaly *et al.* (2019) reported the percentage of buffalo spermatozoa motility after thawing cryopreserved with 7% glycerol is 41.3%.

Fig. 1. Pedets results from AI chilled-semen.

El-Sisy *et al.* (2016) reported that the glycerol concentration was 7% better compared to 3 and 5% in the cryopreservation of buffalo semen. According to Abbas and Andrabi (2002), the cryopreservation process of Nili-Ravi buffalo semen that glycerol concentration was 7% better compared to 5, 6, 8, and 10%. The quality of frozen semen of Saanen goats containing 7% glycerol is better compared to 5 and 9% (Kulaksiz *et al.*, 2013). The opposite occurs at a concentration of 8%, which is thought to be too much so that it increases the osmotic pressure of the extender, and is bad for spermatozoa. Willoughby *et al.* (1996) reported that cells will experience death if they are in an osmotic pressure of medium that exceeds the tolerance limit. According to Mughal *et al.* (2018), the osmotic pressure of the solution used in extender preparation plays an important role in the post-thaw quality of cryopreserved buffalo bull semen.

Estrous response and percentages of pregnancy and birth from AI

The result of this study was showed that all (100%) acceptors showing estrous symptoms 2–3 days after the second injection of PGF2α. This shows that all buffaloes have good reproductive conditions because the occurs cyclically and regularly, typically in buffaloes around 20–22 days. According to Brito *et al.* (2002), responses to the administration of PGF2α to cattle will be effective if the estrous cycle is regular and there is a corpus luteum (CL) in the luteal phase (about 17 days from the estrous cycle for 20–22 days). The results also confirm that the estrous synchronization method applied can initiate various physiological mechanisms such as folliculogenesis, ovulation, spermatozoa and oocyte transport, fertilization, and the synthesis and secretion of hormones directly involved in regulating various reproductive activities. Sianturi *et al.* (2012) reported that female swamp buffaloes injected with the PGF2α or a combination of the PGF2α and gonadotropin-releasing hormone (GnRH) as well as a combination of the PGF2α and human chorionic gonadotropin (hCG) produce a percentage of estrous is 100%, and estrous symptoms began to appear on two days after the second injection of the PGF2α.

The percentage of pregnancy for chilled-semen was 69.23%, higher than for frozen-thawed semen (66.67%). The percentage of birth for chilled-semen was 61.54%, while for frozen-thawed semen was 55.55%. There was no significant difference between treatment on the percentage of pregnancy and birth (Table 2). There was an embryo death in each treatment. According to (Hunter, 1981), the mortality of embryo in the early stage of pregnancy in livestock can reach 30%.

Fig. 2. Pedets results from AI using frozen-thawed semen.

The pregnancy rate of chilled-semen was higher than frozen-thawed semen. This is presumably because the quality of spermatozoa in chilled-semen was better than that of spermatozoa in frozen-thawed semen, so it also affects the fertility of spermatozoa. Generally, 40–50% of sperm cells die after cryopreservation even with an optimized methodology (Sa*et al.*, 2007). According to Singh *et al.* (2012), freezing-thawing semen procedure is results in more damage to the spermatozoa of bull semen than chilling semen, so that leads to reduced conception rate. Watson (2000) also reported that one of the factors that can reduce spermatozoa fertility is the process of semen cryopreservation. According to Ansari *et al.* (2012), buffalo spermatozoa are more vulnerable to freezing-thawing damage compared with other mammalian species.

The results of this research explain that sugar palm juice was feasible as an alternative extender for semen of the South Kalimantan buffalo because it was able to maintain the fertility of spermatozoa after the cold preservation (Fig. 1) and cryopreservation (Fig. 2). This is confirmed that the combination of sugar palm juice with egg yolk and glycerol can minimize spermatozoa damage during cryopreservation. According to Barile (2005), the success of artificial insemination is influenced by several factors, i.e., the main thing is the reproductive condition of females, the quality of inseminated semen, the accuracy of estrous detection, and the correct skills and handling of semen.

The percentage of pregnancy obtained in this study was confirmed by the results of several previous researchs. Sianturi *et al.* (2012) reported that the percentage of pregnancy of swamp buffalo in Banten Indonesia was 66.7–75%, which inseminated with frozen-thawed semen. The pregnancy rate of buffaloes which inseminated with frozen-thawed semen was 56.5% (Berber *et al.*, 2002), 40–60% Barile (2005), 34.6–46.2% (Swelum *et al.*, 2011) and 52% (Almadaly *et al.*, 2019) of buffalo in Egypt, 44.45% of buffalo in Iran (Mahmoud *et al.*, 2013),

40% in Bangladeshi buffaloes (Hamid, 2018), and 68.5% of swamp buffalo in Ngawi Indonesia (Budiarto *et al.*, 2019).

Yulnawati *et al.* (2013) reported the pregnancy rate of swamp buffalo inseminated with frozen-thawed epididymal spermatozoa of spotted buffalo was 37.5–40%. In water buffalo, the pregnancy rate is reported as 36% in Italian Mediterranean buffaloes (Neglia *et al.*, 2003), 33% in Murrah buffaloes (Paul and Prakash, 2005), 36% (Warriach *et al.*, 2008) and 37% (Naseer *et al.*, 2011) in Nili-Ravi buffaloes, and 45–62.7% (Gutiérrez-Añez *et al.*, 2018). Based on the results of the study it can be concluded that fresh semen of South Kalimantan buffalo has a good quantity and quality, and is eligible to be processed into chilled-semen or frozen semen for AI purposes. Sugar palm juice is suitable as an alternative extender for South Kalimantan buffalo semen because it is able to maintain the quality and fertility of spermatozoa after preservation and cryopreservation.

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