FROZEN-THAWED QUALITY OF SWAMP BUFFALO SPERMATOZOA CRYOPRESERVED WITH SUGAR PALM JUICE EXTENDER

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ABSTRACT

The objective of this research was to examine the effectivity of sugar palm juice as an alternative extender to cryopreserve buffalo. Moreover, we expected to obtain the best composition of sugar palm juice that can be used widely as an alternative extender for buffalo semen cryopreservation. Semen was collected from swamp buffalo bull using artificial vagina. Fresh, diluted, equilibrated, and frozen-thawed was evaluated for general parameters, *i.e* spermatozoa progressive motility (M), viability (V) and membrane integrity (MI). Fresh semen that had good quality was diluted up to 1.10^8 sperm/ml in four different compositions of extenders, *i.e* lactose extender containing 20% egg yolk as control (C), 74% sugar palm juice + 20% egg yolk + 6% glycerol (SPG6), 73% sugar palm juice + 20% egg yolk + 7% glycerol (SPG7), and 72% sugar palm juice + 20% egg yolk + 8% glycerol (SPG8), respectively. Diluted-semen was equilibrated at 5°C for 4 h. It followed by frozen and stored in liquid nitrogen (-196°C). The results showed that the percentages of M, V, and MI of frozen-thawed spermatozoa in control (C) were 45.83, 59.37, and 57.83%), respectively. These results were significantly ($P<0.05$) higher than those parameters in SPG7 (30.83, 38.83, and 48.83%), SPG6 (18.33, 38.83, and 40.33%), and SPG8 (18.33, 39.17, and 39.33%). In conclusion, sugar palm juice might not be as ideal as control group in maintaining frozen-thawed swamp buffalo semen quality. But this inform us that in a very remote area where chemical or commercial extenders are not easy to find, sugar palm juice can be an alternative due to the fact that it did not affect the pregnancy rate of AI program. Moreover, the cryoprotectant (glycerol) concentration play important role in order to maintain the spermatozoa post thawed quality.

Keywords: *Bubalus bubalis*, buffaloes, sugar palm juice, cryopreservation, artificial insemination, swamp buffalo

INTRODUCTION

Artificial insemination (AI) is most common reproductive technologies that is very useful to increase farm animals population. It is also useful for genetic improvement from the male side (Harshan *et al*., 2006). AI technology is an integrated process of semen processing

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(preservation or cryopreservation) and the artificial insemination itself. In most effective end eficient way, AI has been done using frozen-thawed semen. In the cryopreservation process, ice crystal formation can be harmful for sperm viability. Ice crystal formation occurs during the frozen stage, where the spermatozoa cells exposes to very low temperature (-196°C) of liquid nitrogen.

Intracellular ice crystallization during cryopreservation is the main cause of damage to the cells. Due to ice crystal formation, normally about 50% of spermatozoa are damaged during cryopreservation (Watson, 2000). Therefore, extender composition and suitable cryoprotectants are important factors for successful semen cryopreservation (Hammerstedt *et al*., 1990; Curry *et al*., 1994). The cryoprotectant agent is essential for the viability of spermatozoa after thawing, as these agents minimize intracellular ice formation and restrict the solution effect (Medeiros *et al*., 2002). Addition of intracellular cryoprotectant compounds, cause increased extender osmotic pressure, and potentially damage spermatozoa cells. Water will leaves of the cell so that spermatozoa shrink, then swell when cryoprotectant and water reenter the cell to maintain chemical equilibrium. At the time of cryoprotectant removal, the cell initially will swell due to the influx of water and then slowly return to the isoosmotic state when cryoprotectant and water leave the cell. Repeated changes in osmotic pressure can result in significant loss of spermatozoa functional integrity, such as motility, or even cell death without loss of plasma membrane integrity (Gao *et al*., 1997).

Since the discovery of glycerol (Polge *et al*., 1949), it has been used extensively for the cryopreservation of many types of cells, including mammalian spermatozoa (McGonagle *et al*., 2002). However, glycerol has osmotic and toxic effects on the plasma membrane and metabolism of cryopreserved cells (Hammerstedt *et al*., 1990). It is responsible for the disorganization of spermatozoa plasma membrane (Hammerstedt and Graham, 1992; Buhr *et al*., 2001) and reducing motility and fertilizing ability (Jeyendran *et al*., 1985). Higher concentrations of glycerol lead to cell death (Wündrich *et al*., 2006).

MATERIALS AND METHODS

Preparation of extenders

The basic ingredients of lactose extender was 9.3% w/v lactose (Merck, Darmstadt, Germany) and 1.24% w/v fructose (Merck, Darmstadt, Germany) in bidestilled water. Meanwhile, 100 ml pure sugar palm juice was heated up to 100°C and then filtered using a filter paper. Later, those basic ingredients were mixed with egg yolk and glycerol, as it is explained in following table (1). Moreover, 1,000 µg/ml Penicillin and 1,000 IU/ml streptomycin sulphate were added into each group of extenders in order to avoid the contaminations.

Semen collection and processing

Semen from a single buffalo bull was collected at the Artificial Insemination Center of South Kalimantan and processed at the Laboratory of Reproduction and Breeding, Department of Animal Science, University of Lambung Mangkurat (ULM), Banjarbaru, South Kalimantan.

Semen was collected weekly during six weeks in a row. After collection, fresh semen was evaluated immediately for following parameters, including volume, degree of acidity (pH), spermatozoa concentration, mass movement, progressive motility, viability, abnormality, and membrane integrity. Spermatozoa concentration

was counted using Neubauer chamber. While, progressive motility, viability, abnormality and membrane integrity were observed using 40x objective magnifications microscope.

The qualified fresh semen in this research had >2+ mass movement, ≥70% progressive motility, ≥5 x 10^8 spermatozoa/ml concentration, <10% abnormality. In each replication, the qualified fresh semen was divided in equal volume into four tubes. They have already contained four different extenders in each tube. Final concentration in each tube was 1 x 10^8 spermatozoa/ml. Later on, diluted semen from each tube was packaged into 0.25 ml plastic straw (IMV France), and equilibrated at 5°C for 4 h.

Freezing process was started by placing the straws 10 cm above surface of liquid nitrogen vapours in styrofoam for 15 minutes, and then straws were plunged into liquid nitrogen (-196°C). Straws were inserted into a goblet and stored in a liquid nitrogen container. After storage for 4 days, random straw samples from each group were thawed at 37°C for 30 seconds to evaluate the sperm quality.

Sperm quality following cryopreservation process

Three parameters including progressive motility, viability and membrane integrity were used to evaluate the spermatozoa quality in each group after dilution, equilibration and thawed process. A drop of semen sample was placed on object glass and covered by a thin cover glass to observe the progressive motility. The progressive motility was evaluated randomly in ten different locations under 40x objective magnifications microscope (Rasul *et al*., 2001).

Spermatozoa viability was evaluated using eosin nigrosin staining (Felipe-Perez *et al*., 2008). The percentage viability was the number of live cells divided by the total number of the cells in several sites of observation. The dead cells would reserve the staining and the spermatozoa head would become red/dark, while it would not happen in the live cells. At least 200 cells were counted from the different 10 sites of observation under 40x objective magnifications light microscope.

Spermatozoa membrane integrity was evaluated using osmotic resistance test (ORT) with hypo-osmotic swelling (HOS) solution (Revell and Mrode, 1994). About 20 µl of spermatozoa sample was diluted in 200 µl of HOS solution, and incubated at 37°C for 45 minutes. Hypo-osmotic swelling solution consists of 0.9 g fructose and 0.49 g natrium citrate in 100 ml bidestilled water. Intact plasma membrane would be swollen on the tail site, while damage plasma membrane would have the linear tail. At least 200 cells were counted from the different 10 sites of observation under 40x objective magnifications light microscope.

Statistical analysis

Obtained-data of six repetitions were analyzed using analysis of variance (ANOVA) by the linear model using SAS statistical software (SAS 9.1, 2001). The comparative analysis of mean value was analyzed using least significant difference (LSD) test with significant level of 0.05. The results were presented as the means ± standard error mean (SEM) on the table.

RESULTS AND DISCUSSION

Characteristics of fresh semen

In general, the mean fresh buffalo semen that was used in this research had a good quality. The important parameter such as the percentage of

progressive motility, abnormality, and membrane integrity were 74.17%, 5.17%, and 87.67%, respectively (Table 2). As standard quality said that fresh semen should have the percentage of progressive motility more than 70% and percentage of normal spermatozoa more than 80% (Swellum *et al*., 2011), and the membrane integrity more than 60% (Revell and Mrode, 1994).

Quality of spermatozoa after semen processing

The results of this research showed that after dilution and equilibration, there was no significant difference (P>0.05) among control and treatments on the percentages of progressive motility. Meanwhile after thawing, the percentage of progressive motility of buffalo spermatozoa in control group (45.83%) was significantly (P<0.05) higher than SPG6 (18.33%), SPG7 (30.83%), and SPG8 (18.33%) (Table 3). Nevertheless among the treatments group, the percentage of progressive motility, viability, and membrane integrity after thawing of SPG7 was significantly (P<0.05) higher than SPG6 and SPG8.

Our data also showed that the frozen-thawed spermatozoa of control and SPG7 were suitable to be used in AI program, with progressive motility more than 30%. Due to Indonesian national standard value (SNI), the minimum percentage of progressive motility for buffalo frozen-thawed semen should be more than 30% (SNI 4869.2-2008).

Equal to that, the percentage of spermatozoa viability after dilution and equilibration was also similar (P>0.05) among the groups. While after thawing, the percentage of viability was significantly (P<0.05) higher in control (59.37%) group (Table 4). As the previous parameter, the percentage of viability in SPG7 (47.67%) was higher (P<0.05) than in SPG6 (38.83%) and SPG8 (39.17%) groups.

In addition, there was no significant different on the percentage of membrane integrity after dilution and equilibration among the groups. Similar to other two parameters, the percentage of membrane integrity was higher (P<0.05) in control (57.83%) than other groups (Table 5). The percentage of membrane integrity is an important variable that strongly related to the motility and fertility of the spermatozoa. Furthermore, the percentage of membrane intergrity in SPG7 (48.83%) was higher than SPG6 (40.33%) and SPG8 (39.33%) groups.

Swellum *et al*. (2011) reported that percentage of progressive motility, viability, and membrane integrity of buffalo frozen-thawed spermatozoa in Tris extender containing 7% glycerol were 50.25%, 61.75%, and 68.50%, respectively. El-Sisy *et al*. (2016) reported that the best spermatozoa motility, viability, normal morphology, cell membrane and DNA integrities of buffalo frozen-thawed spermatozoa occured in Tris extender containing 7.3% glycerol. Abbas and Andrabi (2002) studied the effects of different concentrations of glycerol (2%, 3%, 4%, 5%, 6%, 7%, 8%, 10% or 12%) on post-thawed spermatozoa quality of Nili-Ravi buffalo bull. They reported that the spermatozoa frozen in 7% were significantly better to those in other concentrations of glycerol as judged by post-thawed motility, survivability, and plasma membrane integrity. While Barati *et al*. (2009) used 7% glycerol in cryopreservation of buffalo epididymal spermatozoa. Paralel to those previous results, SPG7 extender in our study that also contains 7% of glycerol showed a better results in three parameters compare to SPG6 and SPG8. Therefore, we assume that the glycerol concentration in the extender is the important key of high success rate of buffalo

Table 1. Extender compositions.

Extender group	Basic lactose (%)	Sugar palm juice (%)	Egg yolk (%)	Glycerol (%)
Control	73	-	20	7
SPG 6	-	74	20	6
SPG 7	-	73	20	7
SPG 8	-	72	20	8

Table 2. The mean characterics of fresh swamp buffalo semen.

Variable	Mean±SD
Volume (ml)	1.83±0.41
Degree of acidity (pH)	6.90±0.09
Mass movement (1-3)	2.00±0.00
Spermatozoa concentration (million/ml)	1.295±143.07
Progressive motility (%)	74.17±2.04
Live spermatozoa (%)	88.33±1.50
Abnormality (%)	5.17±0.75
Membrane integrity (%)	87.67±0.82

Table 3. Mean percentage of progressive motility after diluting, equilibrating, and thawing.

Treatment	Stage of semen processing		
	After diluting	After equilibrating	After thawing
Lactose	74.17±2.04	68.33±2.58	45.83±4.91[a]
SPG6	74.17±2.04	65.83±3.76	18.33±2.58[c]
SPG7	74.17±2.04	68.33±2.58	30.83±2.04[b]
SPG8	74.17±2.04	65.83±3.76	18.33±2.58[c]

[a,b,c] superscript in the same column showed that significantly different ($P<0.05$).

Table 4. Mean percentage of live spermatozoa after diluting, equilibrating, and thawing.

Treatment	Stage of semen processing		
	After diluting	After equilibrating	After thawing
Lactose	88.33±1.50	82.50±1.05	59.37±3.97[a]
SPG6	88.33±1.50	80.67±1.63	38.83±2.56[c]
SPG7	88.33±1.50	80.83±1.83	47.67±3.39[b]
SPG8	88.33±1.50	80.33±1.86	39.17±1.17[c]

[a,b,c] superscript in the same column showed that significantly different (P<0.05).

Tabel 5. Mean percentage of membrane integrity after diluting, equilibrating, and thawing.

Treatment	Stage of semen processing		
	After diluting	After equilibrating	After thawing
Lactose	87.67±0.82	82.67±1.21	57.83±2.17[a]
SPG6	87.67±0.82	80.50±1.38	40.33±5.28[c]
SPG7	87.67±0.82	81.00±1.26	48.83±2.32[b]
SPG8	87.67±0.82	81.67±1.94	39.33±2.06[c]

[a,b,c] superscript in the same column showed that significantly different (P<0.05).

semen cryopreservation. We can see that about 7% of glycerol in the basic extender is the best concentration that can maintain sperm quality during cryopreservation process. Spermatozoa are very sensitive to alterations in the osmolality of the surrounding solution. If the osmolality difference becomes intolerable, spermatozoa lose their motility, and it is an irreversible effect (Willoughby *et al*., 1996; Gilmore *et al*., 1998).

In conclusion, sugar palm juice might not as ideal as control group in maintaining frozen-thawed swamp buffalo semen quality. But this inform us that in a very remote area where chemical or commercial extenders are not easy to find, sugar palm juice can be an alternative due to the fact that it did not affect the pregnancy rate of AI program. Moreover, the cryoprotectant (glycerol) concentration play important role in order to maintain the post thawed quality. Further study to evaluate the consisting materials in sugar palm juice is needed in order to obtain stable results in future.

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