

FREEZING CAPABILITY OF PASUNDAN BULL SPERM USING TRIS-EGG YOLK, TRIS-SOY, AND ANDROMED® DILUENTS

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ABSTRACT

The aims of this study were to investigate the freezing capability of Pasundan bull spermatozoa in Tris-egg yolk (TEY), Tris-soy (TS), and AndroMed® as diluents. Semen were collected twice a week from four Pasundan bulls aged 3-5 years old using an artificial vagina and evaluated macro- and microscopically. Semen had $\geq 70\%$ sperm motility, $\geq 800 \times 10^6/\text{mL}$ sperm concentration, and less than 20% sperm abnormalities were divided into three parts and each of them diluted with TEY, TS, or AndroMed®. After an equilibration step at 5° C for four hours, diluted semen were packaged in 0.25 mL straw, frozen in liquid nitrogen for ten minutes and kept in liquid nitrogen container until examination. Motility test on fresh, diluted, equilibrated, and after-thawed semen was done using Androvision®. The results showed that after thawing motility of sperm diluted in AndroMed® (58.64±0.72%) was higher than in TEY (49.45±1.22%) and TS (39.34±6.33%). Sperm motility of Pasundan bulls diluted in these three diluents reduced around 33.27±2.45% during freezing process.

Key words: andromed, freezing capability, Pasundan bull, tris-egg yolk, tris-soy

ABSTRAK

Penelitian ini bertujuan mengetahui kemampuan bertahan spermatozoa sapi pasundan dalam proses pembekuan menggunakan pengencer tris kuning telur (TKT), tris soya (TS), dan AndroMed®. Empat ekor pejantan sapi pasundan dengan umur sekitar 3-5 tahun dikoleksi semennya menggunakan vagina buatan dua kali dalam seminggu. Semen hasil koleksi segera dievaluasi secara makroskopis dan mikroskopis. Semen dengan motilitas $\geq 70\%$, konsentrasi $\geq 800 \times 10^6/\text{mL}$, dan abnormalitas spermatozoa $\leq 20\%$ dibagi tiga bagian, masing-masing diencerkan menjadi $100 \times 10^6/\text{mL}$, menggunakan pengencer TKT, TS, atau AndroMed®, diekuilibrasi pada suhu 5° C selama empat jam, dan dikemas dalam straw 0,25 mL. Semen yang telah dikemas dibekukan dalam uap nitrogen cair selama sepuluh menit dan disimpan dalam kontainer untuk pengujian lebih lanjut. Pengujian motilitas dilakukan menggunakan Androvision® pada semen segar, setelah pengenceran, setelah ekuilibrasi, dan post thawing. Hasil penelitian menunjukkan motilitas spermatozoa post thawing dalam pengencer AndroMed® (58,64±0,72%) lebih tinggi dibandingkan pengencer TS (39,34±6,33%), namun tidak berbeda dengan TKT (49,45±1,22%). Pengencer TKT tidak memperlihatkan perbedaan ($P > 0,05$) dengan TS. Penurunan motilitas selama proses pembekuan pada sapi pasundan adalah sebesar 33,27±2,45%.

Kata kunci: andromed, freezing capability, sapi pasundan, tris kuning telur, tris soya

INTRODUCTION

Indonesia has a number of natural and genetically resources that need to be maintained their existence such as local beef cattle. These cattle have shown maximum productivity and economical efficiency limited breeding conditions. One of local beef cattle is Pasundan cattle that genetically have characteristic genes of Bali cattle, Javanese cattle, Ongole cattle and Madura cattle. This cattle have been stated as one of natural biodiversity of Indonesian beef cattle according to the Minister of Agriculture of the Republic of Indonesia declaration No. 1051/KPTS/SR.120/10/2014. Pasundan cattle are readily adapted to environment, easy to breed, have good quality carcass, and relatively resistant to tropical diseases.

Populations of Pasundan cattle are about 52,540 cattle and scattered in several regencies such as Ciamis (535 cattle), Pangandaran (5,130 cattle), Tasikmalaya (7,231 cattle), Cianjur (10,346 cattle), Sukabumi (12,897 cattle), Garut (1,842 cattle), Purwakarta (2,788 cattle), Kuningan (7,218 cattle), and Majalengka (4,553 cattle) (West Java Livestock Institution, 2014). The Province of West Java has planned to improve genetic quality of these cattle through artificial insemination (AI) program using frozen semen of selected bull in Regional Artificial Insemination Centre (AIC) of Ciamis-West Java).

For AI purposes, the semen must be collected from selected bulls, using breeding soundness evaluation (BSE) including semen quality evaluation. The capability sperm to survive during freezing process (freezing capability) is important aspect to select a bull in AI centre. During freezing process (cryopreservation) will reduce the viability of sperm caused by hyper-osmotic diluents and temperature changing during freezing. Freezing capability is also influence by individual aspect of animals (Arifiantini *et al.*, 2014; Nalley *et al.*, 2015), freezing technique, type and concentration of cryoprotectants (Aboagla and Terada, 2004) and type of diluents (Arifiantini and Yusuf, 2010). Sperm motility decreases about 40% during freezing. To achieve of good quality of frozen semen production, requires a diluents which able to maintain the quality of sperm during cooling and freezing processes as well as in the thawing steps (Aboagla and Terada, 2004).

Diluents commonly used for bull semen dilution are skim egg yolk, Tris-egg yolk (TEY) (Arifiantini *et al.*, 2005) and soya lecithin-based commercial diluents (Beran *et al.*, 2012) such as Biochiphos plus® and Bioxcell® (IMV, France) and AndroMed® (Minitub, Germany). Until this time, freezing process of the Pasundan bull semen only uses commercial diluents. This diluent not always available, which can potentially

disrupt the frozen semen production. Arifiantini and Yusuf (2010) have developed modified Tris-soy (TS) diluents for freezing bull semen and demonstrated comparable conception rates with commercial diluent.

The modified diluents contain Tris (hydroxymethyl) aminomethane, universal buffer commonly used for frozen semen from several animals such as buck (Tambing *et al.*, 2004), ram (Rizal *et al.*, 2003), deer (Saenz, 2007), dogs (Yildiz *et al.*, 2000), and buffalo (Asr *et al.*, 2011). So far, Tris combine with egg yolk is believed as a diluent which has better capability to maintain plasma membrane stability than TS. The property related to the high protein content in the egg-yolk can minimize deleterious effect of freezing process on sperm. Tris-soy diluent, on the other hand, may provide promising alternative soy lecithin-based diluents for semen. Considering a quality of Pasundan bull semen is not comprehensively studied, and diluents currently used for Pasundan bull semen is Andromed[®], this study was done to investigate the characteristic of semen and freezing capability of Pasundan bull sperm during freezing process using TEY and TS as diluents compare to that using Andromed[®].

MATERIALS AND METHODS

Four selected Pasundan bulls weighed 300-350 kg were individually caged which equipped with ration and drink containers. All bulls were fed twice a day with 10% grass and 1% concentrate of total BW and water was given *ad libitum*.

Preparation of Diluents

This study used modified Tris diluents namely TEY, TS, and commercial diluents Andromed[®]. The TEY and TS diluents were prepared using Tris A buffer consist of 3.03 g Tris hydroxymethyl aminomethane,

1.78 g citric acid and 1.25 g fructose added with aquabidest until 100 ml (TEY) and buffer B consist of 3.03 g Tris hydroxymethyl aminomethane, 1.78 g citric acid and 1.75 g fructose added with aquabidest until 100 mL (TS). Andromed[®] was prepared according to the brochure. The TEY contained 20 ml of egg yolk and TS diluents contained 2.5 g soy milk. Tris with and Tris with soy were initially centrifuged at 1,500 rpm for 10 minutes. Supernatants were collected, mixed each of them with 6 mL glycerol, penicillin 1000 IU/mL, and streptomycin 1 mg mL⁻¹ and used as diluents.

Semen Collection and Evaluation

Semen was collected twice a week in the morning using artificial vagina. Immediately after collection, semen was evaluated macros and microscopically. Macroscopic evaluation included volume, color, viscosity and pH using special indicator paper. Microscopic evaluation including mass movement conducted under microscope with 200 X magnifications. The percentage of progressive motility conducted using computer assisted sperm analyzed (CASA) instrument Androvision[®] (Minitub-Germany). Sperm concentration was measured using a photometer (SDM 6; Minitub-Germany) whereas their sperm viability and morphology percentages were determined using eosin negrosin staining. Intact sperm plasma membrane was measured by mixing 10 µL semen with 1 mL hypo-osmotic swelling (HOS-Test) using a solution prepared from 7.35 g sodium-citrate and 13.52 g fructose in 1000 mL distilled water.

Semen Freezing Process

Fresh semen had sperm motility higher than 70% and sperm concentrations more than 800x10⁶ mL⁻¹ were divided into three tubes. The semen was then

Table 1. Sperm motility of Pasundan bull fresh semen

Parameter	Bulls				Average±SE
	R5	R4	R3	R2	
Total motility	90.53±2.74 ^a	87.61±2.34 ^a	89.44±2.35 ^a	89.91±1.80 ^a	89.37±2.32 ^a
Progressive motility	84.08±3.61 ^a	80.55±3.03 ^a	83.67±2.92 ^a	81.35±2.30 ^a	82.41±2.97 ^a
Rapid motility	77.24±4.08 ^a	73.01±3.58 ^a	73.93±3.23 ^a	71.08±5.02 ^a	73.82±3.98 ^a
Slow motility	6.79±1.55 ^a	7.16±1.37 ^a	9.03±1.42 ^a	10.04±3.16 ^a	8.26±1.88 ^a
Circle motility	0.05±0.23 ^a	0.39±0.22 ^a	0.72±0.63 ^a	0.22±0.13 ^a	0.35±0.30 ^a
Local motility	6.44±1.93 ^a	7.09±0.98 ^a	5.45±1.19 ^a	8.58±3.09 ^a	6.89±1.80 ^a
Immotile	9.27±2.27 ^a	12.39±2.34 ^a	10.56±2.35 ^a	10.09±1.83 ^a	10.58±2.20 ^a

R5, R4, R3, R2 (bull identity). The same letters following numbers in the same row showed not significant different (P>0.05).

Table 2. Sperm motility of Pasundan bull after frozen and thawed in three different diluents

Parameters	Type of diluents (Average±SE)		
	TEY (%)	TS (%)	Andromed [®] (%)
Total motility	62.59±2.12 ^a	52.79±6.50 ^a	71.64±0.78 ^a
Progressive motility	49.45±1.22 ^{ab}	39.34±6.33 ^b	58.64±0.72 ^a
Rapid motility	40.65±1.35 ^{ab}	32.18±5.91 ^b	51.05±1.53 ^a
Slow motility	8.42±0.22 ^a	9.02±1.42 ^a	5.96±0.58 ^a
Circle motility	0.02±0.01 ^a	0.01±0.004 ^a	0.03±0.01 ^a
Local motility	11.25±2.04 ^a	11.02±1.02 ^a	7.94±0.93 ^a
Immotile	37.62±2.18 ^{ab}	48.16±6.58 ^b	28.36±0.78 ^a

^{a, ab, b}Different superscripts in the same row showed significant different (P<0.05); TEY= Tris-egg yolk; TS= Tris-soy

diluted in TEY, TS, or AndroMed® (final concentration was 100×10^6 sperm mL^{-1}). Semen were equilibrated in cool top (5°C) for four hours and then packed using an automatic filling and sealing machine. Freezing process was carried out using liquid nitrogen in $60 \times 40 \times 30 \text{ cm}^3$ Styrofoam box for ten minutes. Frozen semen was then stored in liquid nitrogen container (-196°C) for further examination.

Semen Evaluation

Evaluation of semen quality was done on fresh, diluted, equilibrated, and after thawed semen. Thawing was done in warm water (37°C) for 30 seconds after 24 hours of storage.

Data Analysis

This study used a complete randomized design with repeated measurement comprise of three diluents as treatment with three replications each. Data obtained was analysis by analysis of variance (ANOVA). The Duncan test was used to compare treatment means (Steel and Torrie, 1994).

RESULTS AND DISCUSSION

Quality of Pasundan Bull Fresh Semen

Evaluation on the quality of fresh semen was done to find out qualified semen for freezing processes. The results demonstrated macroscopically Pasundan bulls semen showed average volume of $3.80 \pm 0.58 \text{ mL}$, white to creamy in color, medium to thick consistency, and acidic grade (pH) of 6.43 ± 0.08 . Microscopic examination identified mass motility ranged from ++ up to +++ and progressive sperm motility of $82.41 \pm 2.97\%$. Sperm concentrations were $1355.85 \pm 6.06 \times 10^6 \text{ mL}^{-1}$ with sperm viability (the number of live sperm) of $84.37 \pm 1.05\%$. The numbers of sperm with intact membrane was $84.89 \pm 1.00\%$ and $11.13 \pm 0.39\%$ of sperm abnormality.

According to the results, the quality Pasundan bull fresh semen was normal. Semen volume of Pasundan bull was $3.80 \pm 0.58 \text{ mL}$. According to Arifiantini (2012), the normal volume of bull semen range from 2 to 15 mL with the average of 4-8 mL. Considering Pasundan cattle is a local breed, the semen volume was almost similar to that collected from other local breed such as Bali, Madura, Java, and Ongole crossbreed cattle, that range from 3.00 to 6.30 mL.

The semen color was range from milky white to creamy. This is in agreement with Garner and Hafez (2000) that observed the color of normal ejaculate of cattle semen is creamy to milky white, while semen with low sperm concentration will have muddy color. Consistency or viscosity levels of Pasundan bull semen were watery to medium with average pH of 6.43 ± 0.01 . Semen from normal bull has consistency ranged from medium to thick and pH range from 6.4 to 7.8 (Garner and Hafez, 2000).

According to microscopic examination, the mass movement of Pasundan bull semen ranged from ++ to +++, this is belonged to normal mass movement of bull sperm. The different in the motility of semen collected from the four Pasundan bulls was not significant (Table 1). Motility values observed still belonged to normal semen motility reported by Garner and Hafez (2000), 70% to 80%. Sperm concentration of Pasundan bull was $1355.85 \pm 6.06 \times 10^6 \text{ mL}^{-1}$. This value belonged to normal range of sperm concentration of semen of adult cattle i.e. 800 to $1200 \times 10^6 \text{ mL}^{-1}$. Sperm motility and concentration recorded from Pasundan bull were not different from those of Bali cattle reported by Arifiantini *et al.* (2006), $71.04 \pm 3.69\%$ and $1.340 \pm 447.85 \times 10^6 \text{ mL}^{-1}$, respectively. Viability and abnormality of Pasundan bull sperm were $84.37 \pm 1.05\%$ and $11.13 \pm 0.39\%$, respectively, that were not differ from those recorded from Bali bull sperm that were $84.60 \pm 2.04\%$ and $10.00 \pm 1.22\%$ (Matahine *et al.*, 2014).

According to Gordon (2003) the color, numbers, volume, concentration, consistency, mass movement,

Table 3. Decreasing of Pasundan bull sperm motility during freezing process

Process	Diluents			Mean $\pm$ SE
	TEY	TS	AndroMed®	
Fresh semen to after dilution	0.33 ± 2.05	1.7 ± 2.27	2.33 ± 1.80	1.45 ± 0.60
After dilution to after equilibration	8.17 ± 0.87	12.91 ± 1.27	1.98 ± 0.78	7.69 ± 3.16
After equilibration to after thawing	24.46 ± 0.57	19.1 ± 4.16	24.12 ± 1.21	22.56 ± 1.73
Fresh semen to after thawing	32.96 ± 1.75	43.07 ± 3.35	23.77 ± 2.25	33.27 ± 5.57

TEY= Tris-egg yolk; TS= Tris-soy

Table 4. Motility of Pasundan bull sperm in the fresh and after thawing

Parameter	Processing step	Diluents (Average $\pm$ SE)			RR (%)
		TEY (%)	TS (%)	AndroMed® (%)	
Progressive motility	FS		$82.41 \pm 2.97\%$		-
	AT	49.45 ± 1.22^{ab}	39.34 ± 6.33^b	58.64 ± 0.72^a	59.62 ± 5.57
Total motility	AT	49.45 ± 1.22^{ab}	39.34 ± 6.33^b	58.64 ± 0.72^a	-
Rapid motility	AT	40.65 ± 1.35^{ab}	32.18 ± 5.91^b	51.05 ± 1.53^a	-
Slow motility	AT	8.42 ± 0.22^a	9.02 ± 1.42^a	5.96 ± 0.58^a	-
Circle motility	AT	0.02 ± 0.01^a	0.01 ± 0.004^a	0.03 ± 0.01^a	-
Local motility	AT	11.25 ± 2.04^a	11.02 ± 1.02^a	7.94 ± 0.93^a	-
Immotile	AT	37.62 ± 2.18^{ab}	48.16 ± 6.58^b	28.36 ± 0.78^a	-

^{a, ab, b} Different superscripts in the same row showed significant difference ($P < 0.05$); TEY= Tris-egg yolk; TS= Tris-soy; RR= Recovery rate; FS= Fresh semen; AT= After thawing

pH, and motility of fresh sperm collected from a bull are vary. These are influenced by many factors such as individual condition, quality of reproductive organs, age, condition of breeding management, feeding, and breed.

The use of CASA in the examination of sperm motility was intended to overcome subjectivity in scoring. This method based on development of digital image technology to obtain quick and accurate results of analysis, is able to improve and standardize evaluation of parameters of sperm motility relevant for fertility evolutions. Study using CASA displayed eight parameters of sperm motility movements, namely total motility, progressive motility, rapid motility, slow motility, circle motility, hyperactive, local motility, and immotile.

Result of analysis showed that no difference ($P>0.05$) was found among four bulls (R5, R4, R3, and R2) in progressive motility movement (Table 1). Average of progressive motility value of Pasundan bull sperm was $82.41\pm2.97\%$, a prerequisite to determine the level of female fertility and as a consideration for the production of frozen semen. According to Garner and Hafez (2000), minimal motility percentage of fresh semen is 80% with mass motility of ++ to +++.

The Quality of Pasundan Bull Frozen Semen

In this study, after thawing motility of Pasundan bulls frozen semen in TEY, TS, and AndroMed[®] were $49.45\pm1.22\%$, $39.34\pm6.33\%$, and $58.64\pm0.72\%$, respectively (Table 2). No differences between TEY and AndroMed[®]. This results were also not differ from those obtained from Bali bull sperm in ultracentrifugation-egg yolk was able to enhance progressive motility up to $51.5\pm10.1\%$. These results were also comparable compared to that obtained from experiment using commercial diluent AndroMed[®], 46.4 ± 11.0 - $47.4\pm2.11\%$ (Mohamad *et al.*, 2009; Beran *et al.*, 2012). The use of AndroMed[®] and TEY diluents in this study were able to protect sperm membrane in the freezing process.

Diliyani *et al.* (2014) reported that the use of AndroMed[®] and egg yolk as diluents were able to protect integrity of sperm membrane. The integrity of sperm membrane was influenced by the content of phospholipids that play a role in maintaining membrane integrity and in forming a dynamic inter cell surface that in turn provide protective mechanism against the stress from environment. Disruption of membrane integrity caused by phospholipids detachment will influence sperm viability.

Decreasing of Pasundan Bull Semen Quality During Freezing

In order to evaluate the success and to find out sperm destruction in each step, evaluations on the percentage of progressive motility were done on fresh, after dilution, after equilibration, and thawing (Table 3). Without considering the type of diluents, reduced percentage of progressive motility of $1.45\pm0.60\%$ observed from fresh to after dilution semen.

Progressive motility reductions from after dilution to after equilibration were $7.69\pm3.16\%$, and those from after equilibration to after thawing were $22.56\pm1.73\%$. Therefore, total decline of progressive motility percentage of sperm from fresh to after thawing semen were $33.27\pm5.57\%$.

Reduced progressive motility percentage ($33.27\pm5.57\%$) indicated that a drastically reduction has been occurred starting from dilution step to after thawing. Semen motility decline in other animals ranged 10% to 40%, even reached 50% (Sorenson, 1979). Reduced progressive motility in freezing process of sheep and goat semen were 27.42% (Herdis, 2005) and 33.05% (Tambing *et al.*, 2004), respectively, which lower than those observed in this study. This probably related to cold shock caused by extreme temperature changes experienced by sperm during the treatment. Cold shock occurred due to immediate temperature reduction from body temperature to very low temperature (lower than 0°C) resulted in reduced cell viability (Rehman *et al.*, 2013). According to Medeiros *et al.* (2002) cell destruction was caused by the changes in membrane structure organization and the change in the function of cell metabolic.

Main effects of cold shock on sperm cells are able to reduce motility and viability, changes in permeability and lipid components in the membrane. The numbers of motile undergo reduction accompanied by enzyme release, ion movement through membrane, and changes in lipid contents such as phospholipids and cholesterol that play significant roles in maintaining the integrity of plasma membrane, as well as reduced capability of sperm cells in controlling Ca^{2+} movement (Ogbuewu *et al.*, 2010).

Recovery rate is an ability of sperm to recover after thawing compared to fresh sperm (Hafez, 2000). Regardless of the type of diluents, this study observed a recovery rate of sperm was $59.62\pm5.57\%$ (Table 4). This means that $59.62\pm5.57\%$ sperm of Pasundan bull was able to recover after freezing process.

The use of TEY and AndroMed[®] both are able to provide nutritional source for sperm to move progressively to survive during storage process. According to Aboagla and Terada (2004), the use of TEY and AndroMed[®] is basically to protect sperm during temperature change from room temperature (28°C) to equilibration temperature (5°C). AndroMed[®] contains soy lecithin that has similar function with egg yolk lecithin. The main function of lecithin (phosphatidylcholine) is as membrane coating to maintain normal configuration of phospholipids bilayer, the main organization structure of sperm membrane.

Sperm diluted in TS did not showed different in sperm ($P>0.05$) from those diluted in TEY, but have lower motility than those diluted in AndroMed[®]. This finding supported reports from Arifiantini and Yusuf (2010) that stated FH semen preserved using modified Tris-soy diluents have lower after thawing sperm motility than those prepared using AndroMed[®]. The in vivo conception rate of the first, however, was higher than the other. Although different from AndroMed[®],

TS can be used as semen diluents in the future because it also contains soy lecithin. Lecithin extracted from soybeans is appropriate choice for lecithin of semen diluents in the future (Aires *et al.*, 2003) and able to suppress oxidative stress (Ogbuewu *et al.*, 2010), but further research is required to obtain buffer composition with similar quality to AndroMed®.

The high sperm motility of frozen semen prepared using AndroMed®, because the diluent not only contain high lecithin (6.76 g/100 mL) but also several compounds important for sperm such as protein, carbohydrate (fructose, glucose, mannose, and maltotriose), minerals (sodium, calcium, potassium, magnesium, chloride, phosphor, and mangan), citric acid, glycerol, fat, and glycerilphosphoril choline (GPC) Insignificant different of quality of frozen semen prepared using diluents contain soy lecithin (AndroMed®) and those prepared using diluents of animal lecithin (egg yolk /Tris-egg yolk) is related to the property of egg yolk itself as buffer for osmotic pressure so that sperm are more tolerant to the hypotonic or hypertonic environments (Khalifa and El Saidy, 2006). Moreover, egg yolk contains phospholipids consisted of 77% lecithin (phosphatidylcholin), 18% phosphatidylethanolamine, and 3% sphingomyelin.

Results of the study showed that the quality of frozen semen in TEY and AndroMed® diluents has fulfilled the Indonesian National Standards (SNI) for frozen semen, SNI Number 4869.1:2008, therefore suitable for insemination purposes. This study also proved that TEY can be used as alternative diluents for Pasundan bulls frozen semen when AndroMed® is unavailable.

CONCLUSION

It can be concluded that freezing capability of Pasundan bull sperm using the AndroMed® and Tris-egg yolk diluents were similar.

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