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THE QUALITY OF BOAR FROZEN SEMEN DILUTED IN BTS® AND MII® WITH DIFFERENT CRYOPROTECTANT SUPPLEMENTED WITH SODIUM DODECYL SULPHATE

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ABSTRACT

This research was aimed to study the effect of administration of glycerol and dimetilacetamida (DMA) in BTS® and MIII®extender supplemented with sodium dodecyl sulphate (SDS) on boar frozen semen. A number of four boars were used in this study for semen collection (n=20). The collected semen was evaluated both macroscopically and microscopically. In this study, only the semen that demonstrated>70% sperm motility, >200.10^6/mL sperm concentration, and<20% sperm abnormalities were used and divided into eight tubes. A number of 4 tubes were diluted with 5 mL of BTS, and the rest with 5 mL MIII. The sampel was stored at 20-22° C for 2 hours, followed by centrifugation for 15 minutes (at 2000 rpm), and taken of pellet with 1 ml supernatant. The pellet that was resulted from centrifugation using BTS, then re-diluted with BTS-glycerol 5% (BTS_G), BTS DMA 5% (BTS_D), BTS-glycerol 5% and SDS (BTS_{G-S}), BTS-DMA 5% and SDS (BTS_{D-S}). Four other pellets that were centrifuged with MIII also re-diluted with MIII-glycerol 5% ($MIII_G$), MIII-DMA 5% ($MIII_D$), MIII-glycerol 5% and SDS ($MIII_{G-S}$), MIII-DMA 5% and SDS ($MIII_{D-S}$). Next, all of diluted semen were inserted into 0.5 mL straw and equilibrated for 2 hours (4° C), then frozen and stored in liquid nitrogen. The evaluation of frozen semen quality was conducted at 24 hours after frozen. The result of this study showed that post-thawing motility of spermatozoa in BTS_{D-S} (40.17±0.2%) was found higher (P<0.05) compared to seven other dilution processes. Therefore, it is concluded that the concentration of 5% DMA that supplemented with SDS in BTS dilution much better for maintaining boars frozen semen quality.

Key words: boar, cryopreservation, dimethylacetamide, glycerol, SDS

ABSTRAK

Penelitian ini bertujuan menguji penggunaan gliserol dan dimetilasetamid (DMA) dalam pengencer BTS® dan MIII® dengan sodium dedocyl sulphate (SDS) untuk pembekuan semen babi. Semen dikoleksi dari empat pejantan (n=20) dan dievaluasi secara makroskopis dan mikroskopis. Semen dengan motilitas >70%, konsentrasi >200 juta/ml, dan abnormalitas <20% dibagi dalam delapan tabung. Empat tabung diencerkan dengan 5 ml BTS, sedangkan yang lainnya diencerkan dengan 5 ml MIII. Semen didiamkan selama 2 jam (20-22° C), kemudian disentrifuga selama 15 menit (2000 rpm) dan diambil pelet bersama 1 ml supernatan. Pelet hasil sentrifuga dengan BTS masing-masing ditambahkan BTS-gliserol 5% (BTS_G), BTS-DMA 5% (BTS_D), BTS-gliserol 5% dengan SDS (BTS_{G-S}), BTS-DMA 5% dan SDS (BTS_{D-S}). Pelet hasil sentrifus MIII masing-masing diencerkan dengan MIII-gliserol 5% ($MIII_G$), MIII-DMA 5% ($MIII_D$), MIII-gliserol 5% dan SDS ($MIII_{G-S}$), MIII-DMA 5% dan SDS ($MIII_{D-S}$). Semen hasil pengenceran dikemas dalam straw 0,5 ml dan diekuilibrasi selama 2 jam (4° C), dibekukan di atas uap nitrogen cair dan disimpan dalam nitrogen cair. Pengujian kualitas semen beku dilakukan 24 jam setelah pembekuan. Hasil penelitian menunjukkan motilitas spermatozoa setelah thawing dalam pengencer BTS_{D-S} (40,17±0,2%), lebih tinggi (P<0,05) dibandingkan tujuh pengencer lainnya. Dari hasil penelitian disimpulkan bahwa konsentrasi DMA 5% yang disuplementasi SDS dalam pengencer BTS lebih baik dalam mempertahankan kualitas semen beku babi.

Kata kunci: babi, kriopreservasi, dimetilasetamid, gliserol, SDS

INTRODUCTION

Pig farming in Indonesia plays a very important role in providing meat for national market as well as for export to other countries. Therefore, this commodity has a good potency for further development. An effort to increase their productivity and improvement of genetic quality are through the application of artificial insemination (AI). In Indonesia, most of AI application in pigs were using liquid semen. On the other hand, the use of frozen semen is very limited. The reason is due to frozen semen has to be imported and, in turn, make it costly.

Research on boar semen cryopreservationin Europe showed more than 40% post thawing mortality (PTM)followingEquex STM paste administration . This is a commercial product with active substance of sodium dodecyl sulphate (SDS), which also available in Indonesia. A study of using SDS for cryopreservation of dogs semen has been reported could maintain motility, acrosome integrity, and high fertility (Ponglowhapan and Chatdarong, 2008).

In processing of frozen semen, normally the temperature will be decreased to -196° C. It will resulted in 20 to 30% spermatozoa mortality. In this process, the osmotic pressure is also changed extremely that compromise composition of lipid plasma membrane and reduced the sperm motility. Since the plasma membrane is the main target to be destroyed during frozen process, then needs to protect its integrity. Therefore, to prevent this problem during cryopreservation, they need appropriate type and concentration of cryoprotectant in semen extender.

The commercial extender for boar liquid frozen semen is available in the market, but only beltsville thawing solution (BTS®) and MIII®that mostly used in Indonesia. The BTS® is an extender that could be kept in short-time (1-3 days) while MIII®is an extender that could be kept in a longer time (5-7 days) (Gadea, 2003). The liquid boar semen is only possible to keep

at the temperature of 16-18° C. However, during cryopreservation, there is an equlibration time at the temperature of 4-5° C. Therefore, lecithine and phospholipid is needed to reduce cold shock. The addition of egg yolk in the extender resulted in mixingproble, therefore its needed to add SDS in the solution. The addition of SDS could mix and increase molecule dispersion from yolk. This could then increase a contact between phospholipid of yolk and plasma membrane of spermatozoa.

Furthermore, another substance that common used as cryprotectan for frozen semen is glycerol. However, it was reported the result is not as expected. Buranaamnuay *et al.* (2008) has suggested that the used of 9% glycerol in Modena extender with 1.5% of equex-STM paste could produces PTM of 34%. Then, Medeiros *et al.* (2002) suggested of using the group of amida (formamida; methyl- atau dimethyformamide, NF-DMF; acetamida atau dimethylacetamide, MA-DMA) as cryoprotectant. The amida has also been tested by Pinho *et al.* (2014) that produced PTM of 51.7% in BTS that combined with lactose and yolk.

So far, study on boar frozen semen received less attention in Indonesia. A study by Dapawole (2014) by using BTS with cryoprotectan glycerol 4% and added of 50 mM trehalose, only produced PTM less than 30%. It is important to study the technology of cryopreservation for boar frozen semen in Indonesia to support the development of good performance of this animal and economic value. Therefore, we have tested the effect of glycerol cryoprotectant and MDA with and without addition of SDS in BTS® and MIII® extenders.

MATERIALS AND METHODS

Fresh semen was obtained from four adult boars of breed of Duroc, Cross Breed and Yorkshire at ages of 1-3 years old. These boars were raised in individual cages that equiped with food and drinking facilities.Each of them was fed of concentrate at 3 kg/day and drink was given ad libitum.

Preparation of Basic Extender

The basic extender materials used in this study were commercial BTS® and MIII® (Minitub Germany). Each of 50 g BTS® and 60 g MIII®, was diluted with aquadest up to 1000 mL. Then, they were stored at temperature of 37° C.

Preparation of Frozen Semen Extender

As much as 80% basic extender material (either BTS or MIII) was added with 20% yolk (KT), then homogenized and centrifuge at 2000 rpm for 15 minutes. Next, the supernatant of BTS-KT and MIII-KT were taken to be processed for frozen semen. The extender for frozen semen was comprised of eight combinations (Table 1).

Semen Collection and Evaluation

Semen was collected two times every week, each of them for one ejaculate, using glove hand method and dummy sow. The collection tubes were equipped with screen cloth for filtering gelatin fraction. After collection, the semen was evaluated macroscopically for volume, color, and pH, then, for microscopic evaluation of motility, viability, concentration, and sperm morphology. The volume of fresh semen was measured using the scale at evaluation glass. The color was evaluated visually and the acidity was measured using pH indicator paper (Merck scale 6.4-8.0). The microscopic evaluation was conducted using binocular microscope (Olympus CX21) at 40x magnification. The percentage of spermatozoa motility was measured by compared the movement of progressive spermatozoa with the non-progressive one, such as reverse, circular, vibrator, and idle or death.

The concentration was evaluated by counting using Neubauer chamber that diluted at 1000 times (10 µL in 990 µL of formosaline). Then, the viability of spermatozoa was measured using eosin-nigrosin stain. The spermatozoa was counted at minimum of 200 cells of 10 observation field. The living/viable spermatozoa was indicated by not absorb the color (transparancy). On the other hand, the death spermatozoa will absorb the red color at the part of their head.

The morphology of spermatozoa was evaluated using carbofucshin stain, as conducted by Arifiantini *et al.* (2012). It was classified based on the abnormality of their head (primary) and neck as well as tail (secondary). Only the semen that showed motility >70% and concentration >200x10^6 cells/ml with abnormality <20% that were used in this experiment.

Table 1. The composition of BTS and MIII extenders for frozen semen processing

Extender	Materials				
	BTS (%)	MIII (%)	SDS (%)	G (%)	DMA (%)
BTS_G	95	-	-	5	-
BTS_D	95	-	-	-	5
BTS_{G-S}	95	-	0.05	5	-
BTS_{D-S}	95	-	0.05	-	5
$MIII_G$	-	95	-	5	-
$MIII_D$	-	95	-	-	5
$MIII_{G-S}$	-	95	0.05	5	-
$MIII_{D-S}$	-	95	0.05	-	5

BTS= Beltsville thawing solution; -= MII; BTS_G= BTS-glycerol; BTS_D= BTS-DMA; BTS_{G-S}= BTS-glycerol-SDS; BTS_{D-S}= BTS-DMA-SDS; $MIII_G$= MIII-glycerol; $MIII_D$= MIII-DMA; $MIII_{G-S}$= BTS-glycerol-SDS; $MIII_{D-S}$= MIII-DMA-SDS

Dilution and Processing Frozen Semen

The qualified semen qualified were divided into eight tubes. The first four tubes were added with BTS®and the other four tubes were added with MIII® with ratio of 8:5. The diluted semen was stored at the temperature of 20-22° C for two hours (holding time). Then, it was centrifuged with the speed of 2000 rpm for 15 minutes. The supernatant was discharged and 1 ml pellet was used. The pellet was re-diluted with frozen semen extender (Table 1).

The diluted semen was package in a straw of 0.5 ml, with a concentration of 200 million/0.5 mL. Then, they were placed in a frozen media and equilibrated at the temperature of 4-5° C for two hours. The processing of frozen semen was conducted above the air surface of liquid N_2 at the distance of 4 cm for 10 minutes. It was stored in a container of liquid N_2 (-196° C) for further analysis.

Quality Analysis of Frozen Semen

The evaluation of sement post frozen processing was conducted minimum at 24 hours after stored. The frozen semen was thawed in a waterbath at temperature of 37° C for 30 second, then spermatozoa motility and viability were evaluated.

Data Analysis

The data obtained in this study was analyzed using analysis of variance. When it was found a significant different between treatment groups, then was continued with Duncan test's (Steel and Torrie, 1995).

RESULTS AND DISCUSSION

The Quality of Fresh Semen

The aims of boar fresh semen study is to evaluate the quality and to determine the optimum level of dilution to be used. The results showed that the spermatozoa quality was very good and fulfill the standard for frozen semen (Table 2). The standards for processing of frozen semen are minimum 70% motility and $200x10^6$ cells/mL concentration, as well as maximum of 20% abnormal spermatozoa morphology (Johnson *et al*., 2000).

The macroscopic evaluation showed that the color of boar semen was muddy white and a washy consistency. Then, the pH was found at 7.40±0.07 and the semen volume at 176.00±4.65 mL. This results was in agreement with the study reported by Ax *et al.* (2000) that the semen volume of boar at the range of 100-450 mL. The results of microscopic evaluation showed that the motility of spermatozoa was 81.50±0.96%. This result was higher than observed by Dapawole (2014), which was 76.31±4.80%. The difference in motility was probably due to the different age of boar used in different study. In our study, we used a productive boar at the age of 1-3 years old. Then, the concentration of spermatozoa obtained in our study was $442.45±22.29x10^6$ cells/mL. Johnson *et al*. (2000) have suggested that several factors that could influence the motility, concentration, volume, and percentage of abnormality of spermatozoa are the amount of ejaculate, the internal of semen collection, the condition of boar, age, genetic, environment, and farm management.

The spermatozoa viability in this study was found 89.20±0.36% that was similar when compared to the study by Sumardani *et al*. (2008), which was found at 87.76±2.87%.The morphology of boar spermatozoa that observed in this study showed lower number of abnormality (6.79±0.37%). This result was lower than a study of Dapawole (2014) that showed an amount of 11.1±4.0%. It has been recommended by Johnson *et al*. (2000) that the percentage of spermatozoa for processing of frozen semen not more than 20%. A higher percentage of abnormality could affect the fertility of male animals, which could decrease the target of artificial insemination (Garner and Hafez, 2000).

Quality of Frozen Semen

The process of cryopreservation is comprised of several stages. At every stage needs to observe the motility and viability of spermatozoa to measure the success of this process. The observation is conducted after the addition of basic material of extender (BTS or MIII), two hours holding time, two hours centrifugation (the pellet was added with frozen semen), equilibration and thawing. The result showed that the motility and viability of spermatozoa were decrease 1.50% and 1.08%, after diluted in BTS, and 1.87% and 1.54% in MIII, respectively (Table 3). This reduction is assumed low because of the extender used was a patent extender and conducted immediately and timely.

Table 2. The characteristic of fresh semen of boars (mean±SEM)

Characteristic	Timberwolf brown	Heavy duty	Night train	Timberwolf black	Mean
Macroscopic					
Volume (mL)	206±9.80	170±10.00	188±11.83	138±10.00	176±4.65
Color	cloudy white	cloudy white	cloudy white	cloudy white	cloudy white
pH	7.28±0.05	7.42±0.02	7.34±0.06	7.58±0.05	7.40±0.07
Consistency	liquid	liquid	liquid	liquid	liquid
Microscopic					
Motility of Spermatozoa (%)	79±1.00	83.5±1.50	82±2.00	80±1.58	81.50±0.96
Velocity	3	3	3	3	3
Concentration of Spermatozoa(10^6sel/mL)	491±47.97	420±49.66	466±42.70	395±21.39	442.45±22.29
Viability of Spermatozoa (%)	88.46±0.43	89.34±0.34	88.84±0.55	88.59±0.44	89.20±0.36
Morphology abnormal (%)	7.28±0.74	6.14±1.73	6.19±1.15	7.57±1.5	6.79±0.37

Holding time is a time that needed by boar spermatozoa to adapt with prior frozen processing (Althouse and Casas, 2013). After holding time, the motility and viability of spermatozoa reduced to 2.50% and 0.23% in BTS, and to 3.38 and 0.31% in MIII, respectively (Table 3). The motility of spermatozoa after centrifugation and added with the extender of frozen semen was found not significant between treatment group (P>0.05). The quality of frozen semen after equilibration was decreased to 5.12-23.63% and 9.75-25.25% in BTS and MIII, subsequently (Table 4). This reduction was suggested quite significant, because the temperature was decreased from 20-22^{o} C to 4^{o} C for 2 hours in the refrigerator. Equlibration is the period of adaptation of spermatozoa in the extender and low temperature before the frozen processing. It is including the exchange of water with cryoprotectant in the spermatozoa to obtain a balance between intracellular and extracellular concentration. This equilibration is also aimed for the balance of osmotic of other type of active extender (Salamon and Maxwell 1995). After equilibration, the highest motility was found in the extender using BTS_{D-S} at 72.38±0.83% and the lowest in $MIII_D$ at 51.00±0.94%.

After frozen processing and thawing, the motility of spermatozoa in all extenders was dropped significantly (P<0.01), in an interval of 32.21% to 38.67%. The spermatozoa in BTS_{D-S} was showed a highest motility (40.17±0.20%) as compared to other extenders. The expected result was also obtained when using BTS_{G-S} with a motility of spermatozoa at 30.42±0.91%. The decrease of semen quality after frozen processing is caused by a sudden drop of temperature to below 0 ^{o}C. The effects of cold-shock to the spermatozoa are the decrease of motility and viability, folowed by the release of enzim and the transfer of ion through membran. The reduction of lipid, such as phospholipid and cholesterol, is important for maintaining the integrity of plasma membrane (Holt, 2000).

The viability of spermatozoa was led to a lower level at 4.95-16.71% in an extender of BTS and 6.68-21.41% in MIII. The highest viability of spermatozoa was showed in BTS_{D-S} (82.94±0.90%) and the lowest in MIII for only 65.94±1.39% (Table 5). After thawing, the viability of spermatozoa resulted in a significant reduction (P<0.01) in all extender for 29.98% to 38.28%. However, the viability of spermatozoa in BTS with DMA cryoprotectant that supplemented with SDS (BTS_{D-S}) was higher (52.96±0.96%) as compared to other treatment groups.

The sustainability of spermatozoa life is related to the strength of membrane. It is the result of the role of membrane for controlling the input and output of substrate and electrolite. The change of spermatozoa in

Table 3. The motility and viability of boars spermatozoa after dilution and holding time (mean±SEM)

Variable	Extender	
	BTS	MIII
After dilution		
Motility of spermatozoa (%)	80.00±0.96	79.63±0.77
Viability of spermatozoa (%)	88.12±0.19	87.66±0.34
After holding time		
Motility of spermatozoa (%)	77.50±1.08	76.25±0.41
Viability spermatozoa (%)	87.89±0.19	87.35±0.12

Table 4. The motility of spermatozoa after equilibration and after thawing in BTS and MIII using glycerol and MDA cryoprotectant with and without addition of SDS (mean±SEM)

Extender	After equilibration	After thawing
BTS_G	53.87±1.91^{de}	14.21±1.34^{e}
BTS_D	56.88±1.68^{d}	19.92±0.57^{d}
BTS_{G-S}	65.88±1.03^{b}	30.42±0.45^{b}
BTS_{D-S}	72.38±0.83^{a}	40.17±0.20^{a}
$MIII_G$	54.88±1.28^{de}	15.67±0.35^{e}
$MIII_D$	51.00±0.94^{e}	12.33±0.18^{f}
$MIII_{G-S}$	61.00±1.34^{c}	23.25±0.87^{c}
$MIII_{D-S}$	66.50±1.34^{b}	29.96±0.34^{b}

The same superscripts in the same column showed a non-signifcant at 5 %; BTS_G= BTS-glycerol; BTS_D= BTS-DMA; BTS_{G-S}= BTS-glycerol-SDS; BTS_{D-S}= BTS-DMA-SDS; $MIII_G$= MIII-glycerol; $MIII_D$= MIII-DMA; $MIII_{G-S}$= BTS-glycerol-SDS; $MIII_{D-S}$= MIII-DMA-SDS

Table 5. The viability of spermatozoa after equlibration and thawing in BTS and MIII using glycerol and MDA cryoprotectant with and without addition of SDS (mean±SEM)

Extender	After equilibration	After thawing
BTS_G	67.95±1.83^{cd}	29.09±1.43^{ef}
BTS_D	71.18±1.72^{c}	34.62±0.63^{d}
BTS_{G-S}	78.83±1.26^{ab}	44,84±0.14^{b}
BTS_{D-S}	82.94±0.90^{a}	52.96±0.96^{a}
$MIII_G$	69.46±1.88^{cd}	29.96±0.97^{e}
$MIII_D$	65.94±1.39^{d}	27.66±0.61^{f}
$MIII_{G-S}$	75.82±1.77^{b}	38.22±0.42^{c}
$MIII_{D-S}$	80.67±1.56^{ab}	44.63±0.31^{b}

The same superscripts in the same column showed a non-significant at 5%; BTS_G= BTS-glycerol; BTS_D= BTS-DMA; BTS_{G-S}= BTS-glycerol-SDS; BTS_{D-S}= BTS-DMA-SDS; $MIII_G$= MIII-glycerol; $MIII_D$= MIII-DMA; $MIII_{G-S}$= BTS-glycerol-SDS; $MIII_{D-S}$= MIII-DMA-SDS

frozen processing is caused as an effect of cold-shock. Therefore, the addition of yolk that contains phospholipid in the extender is needed to protect the spermatozoa from cold-shock during refrigeration and frozen process (Amirat *et al.,* 2007). The addition of SDS in the extender of frozen semen is aimed to break the bigger molecule to be a smaller one, so could easier engage with the membrane of spermatozoa.

In this study, it was proven that motility and viability of boar spermatozoa in the extender of BTS_D that added with SDS 0.05% was higher than those without SDS (Table 5). The presence of SDS in semen extender is could support the mixing and dispersion of yolk molecule, which resulted in an optimum contact between yolk and the membrane of spermatozoa as well as to increase the semen quality. SDS is a basic material of *equex* STM *paste*, which is available commercially in Indonesia and can be used for extender. The addition of *equex* STM *paste* in the extender has been reported effective to increase the membrane motility and integrity as well as to maintain the longevity of dog spermatozoa (Tsuitsui *et al.,* 2000). This substance has been used in semen extender of deer (Cheng *et al.,* 2004), goat (El-Kon *et al.,* 2010), and pig (Panida *et al.,* 2014).

The quality of frozen semen after frozen processing and thawing, and the motility and and viability of spermatozoa in the extender of BTS_{D-S} was better as compared to other extenders in this experiment. It is likely due to the molecular weight of DMA cryoprotectant smaller (87.12 g/mol) than glycerol (92.05 g/mol). As a result, the molecule of DMA is able to pass the plasma membrane faster than glycerol. The entering velocity of cryoprotectant into the cells as a substitute for water could prevent the presence of ice crystal. Medeiros *et al.* (2002) has suggested that the group of amida has a lower toxicity compared to glycerol, and it can be used for the semen that obtained from the male animals that sensitive to glycerol.

CONCLUSION

The best extender for boar frozen semen processing is BTS® that supplemented with SDS and using the DMA cryoprotectant of 5%.

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