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Cryopreservation of Aceh Swamp Buffalo (*Bubalus bubalis*) Semen with Combination of Glycerol and Lactose

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Abstract

Aceh swamp buffaloes *(Bubalus bubalis)* are decreasing their population and genetic quality. This study was conducted to determine the influence of lactose and glycerol cryoprotectants on spermatozoa of Aceh swamp buffaloes after thawing. This study used completely a factorial randomized design with nine treatments, and five replications. Fresh semen of the Aceh swamp buffalo were diluted by using a combination extender lactose cryoprotectants 0 mM (L_0), 60 mM (L_{60}), 120 mM (L_{120}) and glycerol 3% (G_3), 5% (G_5), 7% (G_7) with the equilibration of 4 hours. The results showed that the combination of cryoprotectants $L_{120}G_7$ influenced significantly ($P < 0.05$) on the quality of spermatozoa of the Aceh swamp buffalo *(B. bubalis)* after thawing. The percentage of sperm motility $L_{120}G_7$ (42.60 ± 1.14); viability $L_{120}G_7$ (55.00 ± 0.71); acrosome integrity $L_{120}G_7$ (52.00 ± 0.71); and plasma membrane integrity $L_{120}G_7$ (53.20 ± 1.48). The combination of lactose cryoprotectants 120 mM (L_{120}) and glycerol 7% (G_7) was the best combination to maintain the quality of spermatozoa of swamp buffalo. This finding could be used to define a policy for the spermatozoa storage of Aceh swamp buffalo to artificial insemination (AI).

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INTRODUCTION

Semen cryopreservation contributes to the expansion of reproductive techniques, such as artificial insemination (AI) and in vitro fertilization. The extensive use of AI has enabled an accelerated rate of genetic selection and improvement in animal production, especially in dairy cattle (Medeiros *et al.,* 2002); buct etawah (Ariantie *et al.,* 2013); goat (Bezerra *et al.,* 2011). Aplication of AI with frozen thawed semen is limited in buffalo due to poor freezability of buffalo bull spermatozoa compared to cattle bull spermatozoa (Kumaresan *et al.,* 2005). The main hindrance to utilize cryopreserved buffalo bull semen is the damage of spermatozoa plasma membrane during cryopreservation and thawing, which reduces post-thaw spermatozoa motility and viability (Holt, 2000). In addition, spermatozoa of swamp buffalo are easily defected during the cryopreservation process (Herdis *et al.,* 1999). The defect is caused by a cold shock applied to the frozen cells. To deal with that problem, cryoprotectant can be added to extenders to prevent the defect of cell membrane mechanically as the temperature drops (Tambing *et al.,* 2000).

The extenders must be able to protect spermatozoa during cooling, freezing and thawing process. The defect that occurs during freezing until thawing will affect the spermatozoa especially their cellular membrane (plasma and mitochondria) and nucleus. The defect of cellular membrane will lead to a negative impact on metabolism process. Therefore, a change in spermatozoa's integrity will influence the life sustainability and fertility of spermatozoa.

Cryopreservation is a non-physiological method using an adaptation of cells that being cryopreserved. Several studies on freezing buffalo spermatozoa have been done to find the optimum level of glycerol as a cryoprotectant (Mughal *et al.,* 2017). Since the result was not satisfying, more studies are being developed to reach the better outcomes.

There are two kinds of cryoprotectants; intra and extracellular cryoprotectants. Intracellular cryoprotectant such as glycerol is commonly used in spermatozoa cryopreservation. Glycerol can penetrate into spermatozoa cell and bind the water and so prevents the forming of ice crystals in the extenders during the freezing process (Azizah & Arifiantini 2009).

Andrabi (2009) studied the influence of varying glycerol concentration (2%, 3%, 4%, 5%, 6%, 7%, 8%, 10% or 12%) on the quality of spermatozoa after thawing process. The result showed that the quality of frozen spermatozoa with 7% glycerol addition was better than other concentrations. It can be seen from spermatozoa motility, sustainability and the integrity of plasma membrane. Adding glycerol step by step is suitable for buffalo spermatozoa motility. However, adding a larger amount of glycerol at once can be toxic to spermatozoa. Therefore, for a higher level of glycerol, mixing with semen is done gradually for more than one hour.

El-Harary *et al.,* (2011) have studied on how to improve glycerol ability as a cryoprotectant in extenders. Besides glycerol that can serve as an intracellular cryoprotectant, there are other forms of sugar such as lactose that can also be used as an extracellular cryoprotectant (Rizal & Herdis, 2005). Commonly, lactose as extracellular cryoprotectant can replace water molecules (Viswanath & Shannon, 2000). These qualities can help stabilize plasma membrane of spermatozoa cell during the transition, through critical temperature zone, and change mechanical characteristics of extenders through enhancement of viscosity (Labetubun & Siwa, 2011). The combination of intracellular and extracellular cryoprotectants is expected to give optimum protection to spermatozoa during semen processing, especially during freezing and thawing process. This study aimed to determine the right combination of glycerol and lactose as a cryoprotectants to be used in cryopreservation of Aceh swamp buffalo (*B. bubalis*) semen. The combination extenders of both glyserol and lactose to freeze semen hopefully could improve Aceh swamp buffalo (*B. bubalis*) genetically and expectancy.

METHODS

The Aceh swamp buffaloes used as the object of this study had been trained and well adapted to semen collecting condition. The buffaloes were trained by experts to be able to get on female buffalo and copulate in an unusual environment. Semen was collected from a 3.5-year-old male buffalo. Semen was collected using artificial vagina with the help of a 3.5-year-old female buffalo. The semen collection process was done in the morning at 08.00 AM.

Semen that had been collected was evaluated macro- and microscopically. Macroscopic evaluation included observation of semen color, pH, volume, smell and consistency, while the microscopic observation included mass movement, motility and viability spermatozoa, spermatozoa concentration, and abnormality.

Basic extenders were added with each

combination lactose [L_0(0 mM), L_{60} (60 mM), L_{120} (120 mM)] and glycerol [G_3 (3%), G_5 (5%), G_7 (7%)] resulted in combination L_0G_3, L_0G_5, L_0G_7, $L_{60}G_3$, $L_{60}G_5$, $L_{60}G_7$, $L_{120}G_3$, $L_{120}G_5$, and $L_{120}G_7$. The extenders were then placed in a tube and stored in an ice thermos and were taken to spermatozoa collection site. The extenders were added with fresh semen that met the standard (motility ≥ 70%, concentration ≥ 1000 million cells per ml, mass movement (++) or (+++) and abnormality < 15%).

Equilibration process was started as semen was diluted into extenders kept in an ice thermos. The diluted semen was packed into a mini straw (0.25 ml) with a concentration of 60 million motile spermatozoa and then equilibrated in a refrigerator at a temperature of around 5°C for 4 hours (Eriani *et al.,* 2017). Prefreezing was done by putting straw on a straw shelve in a styrofoam containing liquid nitrogen in a position of 2 cm above the liquid nitrogen. Then the styrofoam was closed and let stand for 15 minutes; then the straw was placed in liquid nitrogen container (temperature -196°).

The thawing process was done by placing the straw into a basin containing warm water at 37°C for 30 seconds. Spermatozoa were transferred from a straw onto object glass for evaluation using a microscope.

The parameters of this study were motility, percentage of spermatozoa viability, percentage of acrosome integrity, the percentage of the plasma membrane integrity during pre-equilibration, post equilibration and after thawing. Spermatozoa motility was determined by placing a drop of diluted spermatozoa on an object glass and covered by a thin glass cover. The progressive motility was observed subjectively using 400x objective magnifications light microscope in eight different fields. The number given was between 0% until 100% with a scale of 5% (Toelihere, 1993).

Viability of spermatozoa were determined using eosin coloring method (Toelihere, 1993). One drop of spermatozoa was added with eosin, mixed until homogenous and thinly spread on the object glass. Viability spermatozoa were recognized from their white head, while dead spermatozoa were marked by the red head. At least 200 spermatozoa were observed under a light microscope with 400x magnifications.

Acrosome integrity was evaluated using the method developed by Saacke and White (1972). A 25 µl semen was added into 100 µl NaCl physiologic solution which contained 1% formalin, slowly mixed until homogenous and let stand for 5 minutes. Semen was thinly spread on object glass and observed using a light microscope with 400x magnification to more than 200 spermatozoa. Spermatozoa with acrosome integrity were marked by pitch-black heads, while damaged spermatozoa did not appear pitch-black.

Plasma membrane integrity was evaluated using Osmotic Resistance Test (ORT) method (Revell & Mrode, 1994). A 25 µl of semen was added into 200 µl hypoosmotic solution and incubated at 37°C for 60 minutes. Semen was thinly spread on object glass and observed using a light microscope with 400x magnification to more than 200 spermatozoa. Spermatozoa with plasma membrane integrity were marked by the curved or swollen tail, while the damaged ones were recognized from the straight tail.

To determine if there were significant differences ($P < 0.05$) in treatments, five replications were employed in this study. Data were analyzed using ANOVA. The significant differences between treatments were further tested using Tukey (Hanafiah, 1997; Walpole, 1992).

RESULTS AND DISCUSSION

Fresh semen quality

The acquired semen must meet the standard for further processing from diluting until freezing. The analysis result of Aceh swamp buffalo fresh semen quality showed that it was fit to the required criteria (Table 1).

The acquired fresh semen had good quality and could be used for the cryopreservation. The evaluation of average sperm quality was mass movement (++), motility (81.8%), viability (82.2%), and sperm concentration (1267×10^6/ ml). This result showed that the sperm was fit for cryopreservation. It is in consistence with Vale (2010) who stated that for semen to be able to be frozen, they must show movement more than 50% and motility more than 70%.

Table 1. Average result of Aceh swamp buffalo fresh semen evaluation

Parameter	Result
Volume (ml)	1.16 ± 0.21
Color	Milk white
Consistency	Medium
Concentration (10^6/ ml)	1267 ± 26.60
Mass movement	(++)
Motility (%)	81.8 ± 2.49
Viability spermatozoa (%)	82.2 ± 5.18
Abnormality	10.2 ± 1.92

Semen quality after cryopreservation process

Cryopreservation process can cause damage to spermatozoa due to the cold shock. Therefore, several parameters must be evaluated after the cryopreservation process to see the quality of spermatozoa.

Percentage of sperm motility with different treatments showed different results after extenders were added. The result of observation on sperm motility can be seen in Table 2.

Based on data analysis, the highest motility percentage is shown by the combination L_{120} G_7 (Tabel 2). The amount of glycerol 7% is assumed to have penetrated cell membrane as intracellular cryoprotectant and protect spermatozoa. According to Gazali and Tambing (2002), as an intracellular cryoprotectant, glycerol can penetrate into spermatozoa by diffusion and therefore binds intracellular water and replace some free water and releases intracellular electrolytes. According to Kwon *et al.,* (2002), glycerol also prevents water from freezing and reduces the forming of ice crystals which can damage spermatozoa cell organelle and thus maintain the quality of spermatozoa. Cell damage can occur from dehydration, increase in electrolyte concentration and the forming of intracellular ice which influence wall permeability so that spermatozoa loses its motility.

Generally, the decreasing sperm quality at post equilibration was lower than post thawing. It is suggested that the equilibration time applied before freezing was four hours (Eriani *et al.,* 2017) so that cryoprotectant could well penetrate the spermatozoa without causing toxicity. This finding is aligned with Mughal *et al.,* (2017) stating that cryopreservation of buffalo bull semen should be kept at 5 °C for not less than 2 h and not more than 6 h before freezing. After post thawing, there was a decrease of semen due to cold shock of liquid nitrogen (-198 °C). However, the results for spermatozoa motility was above 40% which was still acceptable for AI.

Percentage of viability spermatozoa (Figure 1a) with different treatments showed different results after the addition of extenders. The results of observation on Aceh swamp buffalo spermatozoa in every treatment can be seen in Table 3.

Based on data analysis of viability percentage (Table 3) the combination of $L_{120}G_7$ had the highest viability percentage among other combinations of lactose and glycerol. Glycerol with a concentration of 3% (G_3) and 5% (G_5) were assumed to inadequately protect for Aceh swamp buffalo spermatozoa especially against freezing damage. It is different from 7% glycerol which could prevent spermatozoa from damage leading to death. Glycerol prevents spermatozoa from death due to damaged organelles that play a role in cell energy metabolism and therefore makes cell obtain energy. Lactose as disaccharide acts not only as a cryoprotectant and maintains extenders osmotic pressure and keeps plasma membrane integrity, but also supplies energy substrate for spermatozoa during storing process. If the energy cannot be metabolized, it will bring death to spermatozoa. Therefore, there must be an optimum energy in extenders to be used by spermatozoa to stay alive and move actively (Yildiz *et al.,* 2000; Salamon & Maxwell, 2000).

Table 2. Percentage of spermatozoa motility

Phase	Lactose (L) Treatment	Glycerol (G) Treatment			Average L
		G_3	G_5	G_7	
Pre equilibration	L_0	51.60 ±1.52	51.00 ± 1.00	51.60 ± 0.55	51.40 ± 1.02[a]
	L_{60}	46.40 ± 0.89	53.20 ± 1.30	53.60 ± 0.55	51.06 ± 0.92[a]
	L_{120}	54.00 ± 1.00	54.40 ± 0.89	60.60 ± 2.07	56.33 ± 1.53[b]
	Average G	50.66 ± 1.14[a]	52.87 ± 1.06[b]	55.27 ± 1.06[c]	
Post equilibration	L_0	42.20 ± 0.84	43.60 ± 2.51	47.40 ± 0.55	44.40 ± 1.29[a]
	L_{60}	46.40 ± 0.89	47.40 ± 0.55	45.00 ± 0.71	46.27 ± 0.72[b]
	L_{120}	45.00 ± 0.71	48.40 ± 0.55	50.80 ± 1.64	48.07 ± 0.95[c]
	Average G	44.53 ± 0.81[a]	46.47 ± 1.20[b]	47.73 ± 0.97[c]	
Post thawing	L_0	32.40 ± 1.82	32.60 ± 1.67	39.80 ± 0.45	34.93 ± 1.31[a]
	L_{60}	34.40 ± 0.89	37.60 ± 0.55	37.60 ± 0.55	36.53 ± 0.66[b]
	L_{120}	37.60 ± 0.55	41.40 ± 1.34	42.60 ± 1.14	40.53 ± 1.01[c]
	Average G	34.80 ± 1.09[a]	37.20 ± 1,19[b]	40,00 ± 0,71[c]	

Note: Superscripts with different letters in the same lines and column show a significant difference ($P<0.05$)

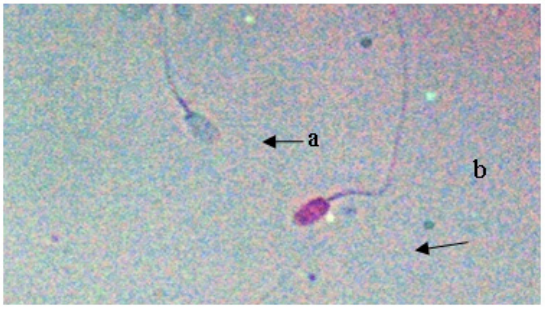

Figure 1. Spermatozoa colored with eosin; a) living spermatozoa b) dead spermatozoa

Percentage of spermatozoa acrosome integrity with different treatments showed different results after thawing. The results of the observation on Aceh swamp buffalo acrosome integrity with different treatments can be seen in Table 4.

Damaged acrosome cap is marked by a pitch-black head after being exposed to NaCl fisiologic-1% formaldehyde. According to Rizal (2005), formalin fixates the enzymes of acrosome vesicle at the tip of spermatozoa's head. Based on this study, the combination of $L_{120}G_7$ showed better protection for spermatozoa after thawing. The data analysis a 7% glycerol (Tabel 4) succeeded in protecting acrosome cap from damage due to freezing which is one of spermatozoa death causes. It is in accordance with Abbas and Andrabi (2002) who studied the effects of several glycerol concentrations (2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10 % and 12 %) with the result that the addition of 7% glycerol had the best percentage of viability sperm. According to Krishna and Rao (1987) acrosome can swell, disrupted, wrinkle and be torn after sperm was frozen. That is why cryoprotectant with the right concentration is required to protect spermatozoa during cryopreservation.

Lactose was assumed to be able to protect the sperm as it is a reducing compound that will neutralize hydrogen peroxide which is known for its ability to damage double bond of unsaturated fatty acid from spermatozoa plasma membrane fosfolipid bilayer. As a result, spermatozoa plasma membrane remained its stability and integrity, and at the same time protects acrosome cap from damage during cryopreservation process (Tambing *et al.,* 2003).

Percentage of spermatozoa plasma membran integrity with different treatments showed different results after thawing. The results of observation on Aceh swamp buffalo spermatozoa plasma membran integrity can be seen in Table 5.

Plasma membrane integrity in spermatozoa is marked by its curved or swollen tail after being incubated in hypo-osmotic solution (Figure 2a). According to Rizal (2005), it is related to the integrity of cell membrane. Water entering the cell will be halted for a while, so that spermatozoa swollen or curved. Based on this study, the addition of 120 mM lactose (L_{120}) combined with 7% glycerol (G_7) showed a relatively high plasma membrane integrity of Aceh swamp buffalo spermatozoa after thawing (Table 5) compared to the other cryoprotectant combinations. The difference is caused by the characteristics of spermatozoa semi permeable cell membrane and therefore glycerol must be in the right concentra-

Table 3. Percentage of viability spermatozoa

Phase	Lactose (L) Treatment	Glycerol (G) Treatment			Average L
		G_3	G_5	G_7	
Pre equilibration	L_0	72.00 ± 1.87	74.20 ± 0.84	76.60 ± 0.55	74.27 ± 1.09^a
	L_{60}	76.40 ± 0.89	77.40 ± 0.89	78.20 ± 0.45	77.33 ± 0.73^b
	L_{120}	80.40 ± 0.55	83.20 ± 0.84	84.80 ± 1.30	82.80 ± 0.89^c
	Average G	76.27 ± 1.10^a	78.27 ± 0.86^b	79.87 ± 0.77^c	
Post equilibration	L_0	49.40 ± 1.14	52.80 ± 2.78	72.40 ± 0.55	58.20 ± 1.67^a
	L_{60}	51.00 ± 0.71	53.60 ± 0.55	74.40 ± 0.55	59.66 ± 0.60^b
	L_{120}	54.60 ± 0.55	64.00 ± 0.71	74.80 ± 2.28	64.47 ± 1.18^c
	Average G	51.67 ± 0.79^a	56.80 ± 1.35^b	73.86 ± 1.13^c	
Post thawing	L_0	34.60 ± 1.14	34.60 ± 1.82	42.00 ± 0.71	37.07 ± 1.01^a
	L_{60}	38.60 ± 0.55	42.60 ± 0.55	52.40 ± 0.55	44.53 ± 0.55^b
	L_{120}	44.40 ± 0.55	48.00 ± 0.71	55.00 ± 0.71	49.13 ± 0.66^c
	Average G	39.00 ± 075^a	41.73 ± 0.81^b	49.8 ± 0.66^c	

Note: Superscripts with different letters in the same lines and column show a significant difference ($P<0.05$)

Table 4. Percentage of spermatozoa acrosome integrity

Phase	Lactose (L) Treatment	Glycerol Treatment (G)			Average L
		G3	G5	G7	
Preequilibration	L0	71.40 ± 0.89	73.80 ± 1.64	75.60 ± 0.55	73.60 ± 1.03[a]
	L60	74.40 ± 0.89	75.00 ± 0.71	81.20 ± 0.84	76.87 ± 0.81[a]
	L120	77.60 ± 0.55	86.00 ± 1.00	86.80 ± 1.30	83.47 ± 0.95[b]
	Average G	74.47 ± 0.78[a]	78.27 ± 1.12[b]	81.20 ± 0.89[c]	
Post equilibration	L0	49.00 ± 1.41	51.60 ± 1.82	53.20± 0.84	51.33 ± 1.36[a]
	L60	51.00 ± 0.71	59.60 ± 0.55	61.40± 0.55	57.30 ± 0.60[b]
	L120	63.80 ± 0.84[a]	65.00 ± 0.71[b]	76.20 ±1.48[c]	68.33 ± 1.01[c]
	Average G	54.60± 0.98[a]	58.73 ± 1.02[b]	63.60 ± 0.96[c]	
Post thawing	L0	40.60 ± 0.89	42.40 ± 1.95	45.40 ± 0.55	42.80 ± 1.13[a]
	L60	43.60 ± 0.55	44.60 ± 0.55	46.00 ± 0.71	44.73 ± 0.60[b]
	L120	45.40 ± 0.55	48.60 ± 0.89	52.00 ± 0.71	48.67 ± 0.72[c]
	Average G	43.20 ± 0.67[a]	45.20 ± 1.13[b]	47.80 ± 0.66[c]	

Note: Superscripts with different letters in the same lines and column show a significant difference (P<0.05)

Table 5. Percentage of spermatozoa plasma membrane integrity

Phase	Lactose (L) Treatment	Glycerol (G) Treatment			Average L
		G_3	G_5	G_7	
Pre equilibration	L_0	68.40 ± 0.89	70.20 ± 1.48	73.40 ± 0.55	70.67 ± 0.97[a]
	L_{60}	71.40 ± 0.89	71.40 ± 0.55	73.60 ± 0.55	72.13 ± 0.67[b]
	L_{120}	74.60 ± 0.55	80.80 ± 0.87	83.80 ± 0.84	79.73 ± 0.74[c]
	Average G	71.47± 0.77[a]	74.13± 0.77[b]	76.93 ± 0.65[c]	
Post equilibration	L_0	45.80 ± 0.84	51.40 ± 1.14	54.60 ± 0.55	50.60 ± 0.84[a]
	L_{60}	53.60 ± 0.55	54.40 ± 0.55	61.00 ± 1.00	56.33 ± 0.70[b]
	L_{120}	56.20 ± 0.45	60.80 ± 0.85	74.60 ± 2.07	63.87 ± 1.13[c]
	Average G	51.87 ± 0.61[a]	55.53 ± 0.84[b]	62.20 ± 1.22[c]	
Post thawing	L_0	40.80 ± 0.45	40.60 ± 0.55	42.80 ± 0.45	41..0 ± 0,48[a]
	L_{60}	41.00 ± 1.23	42.80 ± 1.64	44.40 ± 0.55	42.77 ± 1.13[b]
	L_{120}	42.60 ± 1.14	49.40 ± 1.14	53.20 ± 1.48	48.4 ± 1.25[a]
	Average G	41.47 ± 0.94[a]	44.27 ± 0.95[b]	46.80 ± 0.83[c]	

Note: Superscripts with different letters in the same lines and column show a significant difference (P<0.05)

tion. It is presumed that glycerol of 3% and 5% did not work well and thus cannot protect Aceh swamp buffalo spermatozoa plasma membrane. Water leaves cell because plasma membrane is damaged. According to Rizal (2005), not plasma membrane integrity will cause water release and no mechanical increase so that tail remains straight when exposed to hypo osmotic solution. In this study, sugar (lactose) functions as extracellular cryoprotectant that protects spermatozoa cells from damage due to the forming of ice crystals. According to Salamon and Maxwell (2000) frozen sugar takes form like glass but not sharp so that it does not mechanically damage spermatozoa cells.

The result of this study showed that lactose concentration of 0 mM dan 60 mM was not sufficient to protect spermatozoa plasma membrane from damage during preservation process in low temperature. The addition of 120 mM lactose (L_{120}) influenced spermatozoa motility. The concentration met the requirements of spermatozoa to maintain their quality. According to Singh *et al.,* (1995), the addition of 180 mM lactose in tris extenders can improve the quality of goat frozen semen.

In general, lactose as extracellular cryoprotectant compound has the qualities to replace water molecules (Viswanath & Shannon, 2000). The qualities help stabilized spermatozoa cell plasma membrane during the transition period through the critical zone and change extenders mechanical quality through enhancement in viscosity (Labetubun & Siwa, 2011). The decrease in viability spermatozoa percentage presumably happens due to a drastic change in temperature during freezing and thawing process. Darnel *et al.,* (1990) stated that when there is extracellular unusual temperature change, then damaged permeability of fosfolipid hydrophilic can disturb membrane fluidity that leads to spermatozoa death.

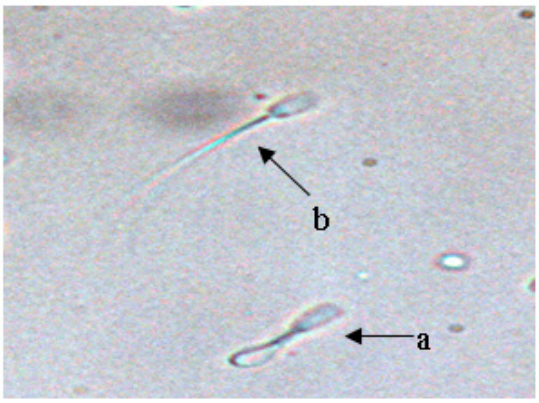

Figure 2. Microscopic image showing sperm membrane integrity colored with hypo-osmotic swelling test (HOST); (a) plasma membrane integrity, and (b) plasma membrane un-integrity

Based on data analysis on plasma membrane integrity percentage, 7% glycerol and 120 mM lactose was the optimum combination between intracellular and extracellular cryoprotectant in the cryopreservation process of Aceh swamp buffalo semen. One of the primary functions of these two cryoprotectants is to maintain spermatozoa cell plasma membrane from damage during semen processing, especially during freezing and thawing phases. An plasma membrane integrity will ensure metabolism process inside the cell so that ATP as energy will be produced and spermatozoa movement (motility) is ensured by the presence of ATP. An intact cell plasma membrane also has a real impact on acrosome cap integrity. It is because acrosome cap is located right under the plasma membrane as cell's outermost part.

Future research for cryopreserved semen should emphasize on freezing protocol upgrade for lower spermatozoa damage during cryopreservation. To achieve that goal then for the next research diluent media will be added antioxidant as extenders.

CONCLUSIONS

The combination of 120 mM lactose and 7% glycerol as cryoprotectants in tris-egg yolk extenders worked best in maintaining Aceh buffalo swamp spermatozoa until post thawing in this study. This finding could be used to define a policy for the spermatozoa storage of Aceh swamp buffalo with percentage motility, viability, acrosome integrity and plasma membrane integrity of spermatozoa were 42.60%, 55.00%, 52.00%, and 53.20%, respectively. However, further in vivo studies are needed to large of numbers to insemination of buffalo female with frozen sperm.

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