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The effect of equilibration time on semen freezing of local swamp buffalo (*Bubalus bubalis*) with combination extender of lactose and glycerol

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Abstract. *Eriani K, Sari N, Ihdina M, Rosnizar. 2017. The effect of equilibration time on semen freezing of local swamp buffalo* (Bubalus bubalis) *with combination extender of lactose and glycerol. Nusantara Bioscience 9: 77-82.* This study was to determine the different time of equilibration on semen of swamp buffalo (*Bubalus bubalis*) which was diluted by adding Tris-Egg Yolk extender of lactose and glycerol cryoprotectant combination. Fresh sperm of the swamp buffalo (*B. bubalis*) was diluted by using Tris-Egg Yolk extender of lactose cryoprotectants combination 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 2,5, 3, and 4 hours. The parameter used in this study was the percentage of sperm motility and live treatment. The results of the 2.5-hour equilibration with a combination of cryoprotectants $L_{60}G_7$ showed the best quality in all parameters. The results of the 3-hour equilibration with combination of cryoprotectants $L_{60}G_7$ showed the best quality in all parameters whereas the best quality in all parameters in 4-hour equilibration was the combination of cryoprotectants $L_{120}G_7$.

Keywords: Cryopreservation, equilibration, glycerol, lactose, swamp buffalo semen

INTRODUCTION

Swamp buffalo (*Bubalus bubalis*) is livestock commonly used by the Indonesian people. Buffalo livestock business as one of the Indonesian people's livelihood proves that Buffalo have benefits for the lives of the people of Indonesia. However, the availability of buffalo meat declines because the production is limited due to a decrease in buffalo population. Therefore, the efforts should be made to save the buffalo population and development. One of the efforts is the application of appropriate technology such as artificial insemination (AI). According to Rizal (2005), AI is one of the proper techniques to be used to maintain the level of population and livestock genetic material.

The quality of the sperm is one of the important factors in the success of AI. The buffalo sperm can be easily damaged in the process of freezing (Herdis et al. 1999). The damage is caused by the effect of cold shock on the semen. One of the efforts to overcome these problems is to adapt semen optimally at each stage of the freezing process. The determination of the appropriate equilibration time is one effort to prevent the occurrence of premature death on semen.

According to Toelihere (1985), equilibration time is the time required by the semen to adapt to the extender, so the excess of the semen deaths could be avoided in the freezing process. The equilibration time can be determined within a few hours at a low temperature and conducted after the semen is diluted. Toelihere (1985) argued that to obtain a high fertility rate; the semen should be preserved for some time. The optimum temperature in the storage process of semen is 5°C or lower. It depends on the cooling temperature. Temperatures above five ºC can inhibit the metabolic activity and motility of the sperm.

Extended sperm is placed at 5 °C before being placed the freezing stage (Pradiee et al. 2016). This is to reduce the metabolic activity of spermatozoa (Dong et al. 2008). Also, osmotic adaptation helps cryoprotectants entering the spermatozoa membrane (Gao and Zhou 2012). The equilibration time is required for the semen on matching themselves with an extender to prevent the death of spermatozoa on the freezing process (Tolihere 1993). The process is aided by the extender as spermatozoa safeguard. The appropriate addition of cryoprotectants also able to inhibit damage to the cell membrane mechanically at the time of temperature decrease (Tambing et al. 2000). The effort is also made with the addition of cryoprotectants in the extender. There are two types of cryoprotectants compounds, that is, intracellular and extracellular cryoprotectant. The intracellular cryoprotectants, such as glycerol, are used in the process of cryopreservation (freezing) of the semen. The extracellular cryoprotectants, such as lactose (carbohydrate), are used in the cryopreservation (freezing) process and preservation of semen at low temperature (generally 3-5°C). The preservation process, as in the equilibration stage, provides an opportunity for lactose to protect the semen from early death due to cold shock.

The combination of the two types of glycerol and lactose cryoprotectant with the optimal equilibration time is expected to provide the best protection to keep the quality

of the semen which will be frozen. Andrabi (2009) had examined the effect of various concentrations of glycerol (2%, 3%,4%, 5%, 6%, 7%, 8%, 10% or 12%) on the quality of semen after thawing and concluded that glycerol 7% provided the best results. The lactose as extracellular cryoprotectants (Rizal and Herdis 2005) can support the work of the glycerol as intracellular cryoprotectants. The lactose has the capability of replacing the water molecule (Viswanath and Shannon 2000). The properties of the cell plasma membrane help stabilize the semen during the transition through the critical temperature zone, as well as changing the mechanical properties of the extender through viscosity escalation (Labetubun and Siwa 2011). This study aims to determine the equilibration time with the appropriate combination cryoprotectant of glycerol and lactose to freeze the semen of Acehnese local buffalo (*Bubalus bubalis*).

MATERIALS AND METHODS

Procedures

Evaluation of fresh semen of buffalo

The collected semen is evaluated macroscopically and microscopically. The macroscopic evaluation includes observation of color, pH, volume, smell, and consistency of semen. The microscopic evaluation includes mass movement, motility percentage, the percentage of live semen and semen concentration. The qualified semen will go to the dilution process.

Extender and addition of cryoprotectants

Basic extender is added to each combination of cryoprotectants, namely, lactose L_0 (0 mM), L_{60} (60mm), L_{120} (120mm) and glycerol which G_3 (3%), G_5 (5%), G_7 (7%) in order to obtain a combination of L_0G_3, L_0G_5, L_0G_7, $L_{60}G_3$, $L_{60}G_5$, $L_{60}G_7$, $L_{120}G_3$, $L_{120}G_5$ dan $L_{120}G_7$. The extender is then inserted into the tube and stored in the ice bucket to be brought into the semen storage site. Next, the extender is added with fresh semen derived from a reservoir that has been met the good quality (motility ≥ 70%, concentration ≥ 2000 million cells per ml, the mass movement (++) or (+++).

Equilibration and semen observation (*pre- and post-equilibration*)

The equilibration process is carried out for three times, i.e., W1 (2.4 hours), W2 (3 hours) and W3 (4 hours) for each combination. At any time of W1, W2, and W3 the parameters before (pre) equilibration and after (post) equilibration are observed. The equilibration is carried out in the refrigerator at a temperature of about 5°C.

Data analysis

The treatment was done in three cycles. Data about semen quality was analyzed by ANOVA. The differences between treatment were tested with Tukey Significant Difference test.

RESULTS AND DISCUSSION

Results

The percentage of dead spermatozoa

The percentage of motile is observed before and after treatment of equilibration time. Each equilibration treatment results can be seen in Tables 1-3.

The percentage of live spermatozoa

The percentage of spermatozoa life were observed before and after treatment of equilibration time. Each equilibration treatment can be seen in Tables 4-6.

Discussion

The percentage of dead spermatozoa

Based on the results, it can be seen that there was significant difference between treatments of $L_{60}G_7$ and $L_{120}G_7$ at the equilibration time between 2.5 hours and 3 hours with an equilibration time of 4 hours. The combination of Lactose 60 mM with glycerol 7% ($L_{60}G_7$) has been able to work on time equilibration of 2.5 hours and 3 hours. It happened because the sperm cell membrane is semipermeable so glycerol and lactose must have the optimum time to protect sperm from damage. Glycerol allegedly has not been able to work optimally at a concentration of 3% and 5%, so it does not provide protection on spermatozoa plasma membrane of Buffalo. If the sperm membrane is not well protected, the membrane will be damaged. According to Rizal (2005), the plasma membrane which is not intact will undergo water discharge and lead to no mechanical improvement, so the tail remains straight when it is dissolved into hypo-osmotic solution.

As intracellular cryoprotectants, the glycerol enters the sperm cells diffusion by binding water in the cell and replacing partial free water and pulling outside of the intracellular electrolyte. This can reduce the possibility of spermatozoa damage. During freezing, glycerol will modify the water in the cells and form small and blunt ice crystals to reduce the effects of damage to sperm cells mechanically. Glycerol is also an antifreeze which can prevent water to freeze in sperm cells (Gazali and Tambing 2002). In addition to the important role of glycerol as cryoprotectants, egg yolk is added to the extender to provide protection and nutrients needed by spermatozoa during the process of freezing. Egg yolk on dilution allegedly affects equilibration time difference. The equilibration time of 4 hours as the longest time shows the highest percentage of motility. Anzara et al. (2011) stated that in the long equilibration time, lipoproteins in egg yolk interact with the plasma membrane of spermatozoa to be prepared for low temperature.

The equilibration time is the time of spermatozoa to adapt to the extender to avoid excessive damage during the freezing process. The difference of the equilibration time causes the difference in sperm motility after freezing. The optimum equilibration will provide the best opportunity for glycerol to protect the spermatozoa from the effects of *cold shock*. Glycerol will penetrate into sperm cells to form balanced intracellular and extracellular concentrations. In addition to the balanced equilibrium concentration of glycerol at this stage, there are also other active osmotic extenders components (Salamon and Maxwell 2000).

Table 1. The percentage of dead Spermatozoa of equilibration time of 2.5 hours

Stages	Treatment of lactose (L)	Treatment of Glycerol (G)			Mean L
		G_3	G_5	G_7	
Pre equilibration	L_0	47.50 ± 1.52	50.00 ± 1.00	51.60 ± 0.55	49.70 ± 1.02[a]
	L_{60}	49.00 ± 0.89	55.20 ± 1.30	56.50 ± 0.55	53.57 ± 0.91[b]
	L_{120}	51.00 ± 1.00	54.40 ± 0.89	53.60 ± 2.07	53.00 ± 1.32[b]
	Mean G	49.17 ± 1.14[a]	53.20 ± 1.06[b]	53.87 ± 1.06[b]	
Post equilibration	L_0	43.20 ± 0.84	44.40 ± 0.55	43.60 ± 2.51	44.73 ± 1.30[a]
	L_{60}	45.40 ± 0.89	50.00 ± 0.71	47.40 ± 0.55	47.67 ± 0.72[b]
	L_{120}	45.00 ± 0.71	48.80 ± 1.64	46.40 ± 0.55	46.77 ± 0.95[b]
	Mean G	44.53 ± 0.79[a]	45.73 ± 0.97[b]	45.80 ± 1.20[b]	

Note: superscript with different letters in the same row and column shows significant differences (P <0.05)

Table 2. The percentage of dead Spermatozoa of equilibration time of 3 hours

Stages	Treatment of lactose (L)	Treatment of Glycerol (G)			Mean L
		G_3	G_5	G_7	
Pre equilibration	L_0	45.20 ± 1.00	51.00 ± 1.04	51.20 ± 0.46	49.10 ± 0.83[a]
	L_{60}	46.00 ± 0.55	55.20 ± 1.21	60.60 ± 0.50	55.06 ± 0.92[b]
	L_{120}	50.00 ± 1.00	52.40 ± 0.75	53.00 ± 2.00	53.93 ± 1.25b
	Mean G	47.06 ± 0.85[a]	52.87 ± 1.00[b]	54.93 ± 0.97[b]	
Post equilibration	L_0	42.20 ± 0.84	43.60 ± 2.51	47.40 ± 0.55	44.40 ± 1.30[a]
	L_{60}	44.40 ± 0.89	47.40 ± 0.55	51.00 ± 0.71	47.60 ± 0.72[b]
	L_{120}	46.00 ± 0.71	46.40 ± 0.55	50.80 ± 1.64	47.73 ± 0.95[b]
	Mean G	44.20 ± 0.81[a]	45.27 ± 1.20[b]	49.73 ± 0.97[b]	

Note: superscript with different letters in the same row and column shows significant differences (P <0.05)

Table 3. The percentage of dead Spermatozoa of equilibration time of 4 hours

Stages	Treatment of lactose (L)	Treatment of Glycerol (G)			Mean L
		G_3	G_5	G_7	
Pre equilibration	L_0	43.50 ± 1,52	52.00 ± 1.01	54.60 ± 0.56	49.70 ± 1.03[a]
	L_{60}	48.00 ± 0,89	52.20 ± 1.30	53.40 ± 0.55	51.53 ± 0.91[b]
	L_{120}	50.00 ± 1,00	53.33 ± 0.84	60.58 ± 2.05	54.97 ± 1.30[b]
	Mean G	45.17 ± 1,14[a]	45.87 ± 1.05[b]	47.27 ± 1.06[b]	
Post equilibration	L_0	41.04 ± 0,82	41.40 ± 2.51	45.40 ± 0.08	42.73 ± 1.29[a]
	L_{60}	43.10 ± 0,91	46.30 ± 0.55	46.00 ± 0.71	45.13 ± 0.72[b]
	L_{120}	45.00 ± 0,60	49.40 ± 0.51	50.80 ± 1.00	48.40 ± 0.70[c]
	Mean G	43.04 ± 0,78[a]	45.70 ± 1.41[b]	47.40 ± 0,60[c]	

Note: superscript with different letters in the same row and column shows significant differences (P <0.05)

Table 4. The percentage of live Spermatozoa of equilibration time of 2.5 hours

Stages	Treatment of lactose (L)	Treatment of Glycerol (G)			Mean L
		G_3	G_5	G_7	
Pre equilibration	L_0	71.00 ± 1.87	74.20 ± 0.84	75.20 ± 0.15	73.46 ± 0.95[a]
	L_{60}	76.40 ± 0.89	81.60 ± 1.00	84.20 ± 0.10	81.53 ± 0.66[b]
	L_{120}	80.40 ± 0.55	80.20 ± 0.50	81.30 ± 0.00	80.63 ± 0.35[b]
	Mean G	75.93 ± 1.10[a]	78.60 ± 0.78[b]	79.90 ± 0.05[b]	
Post equilibration	L_0	49.40 ± 1.14	52.80 ± 2.78	66.00 ± 0.10	56.07 ± 1.34[a]
	L_{60}	51.00 ± 0.71	62.00 ± 0.55	71.40 ± 0.55	61.47 ± 0.60[b]
	L_{120}	54.60 ± 0.55	61.00 ± 0.71	70.80 ± 2.20	60.47 ± 1.15[b]
	Mean G	51.47 ± 0.8[a]	58.60 ± 1.35[b]	69.40 ± 0.95[b]	

Note: superscript with different letters in the same row and column shows significant differences (P <0.05)

Table 5. The percentage of live Spermatozoa of equilibration time of 3 hours

Stages	Treatment of lactose (L)	Treatment of Glycerol (G)			Mean L
		G_3	G_5	G_7	
Pre equilibration	L_0	72.00 ± 1.70	73.00 ± 0.84	76.60 ± 0.55	73.87 ± 1.03[a]
	L_{60}	80.20 ± 0.90	83.00 ± 0.89	78.20 ± 0.45	80.47 ± 0.75[b]
	L_{120}	80.20 ± 1.00	82.20 ± 0.84	83.80 ± 1.30	80.80 ± 1.05[b]
	Mean G	77.46 ± 1.20[a]	79.40 ± 0.86[b]	79.53 ± 0.77[c]	
Post equilibration	L_0	48.00 ± 0.00	50.00 ± 1.75	72.40 ± 0.10	56.80 ± 0.62[a]
	L_{60}	53.00 ± 1.15	62.60 ± 0.50	74.00 ± 0.20	64.87 ± 0.62[b]
	L_{120}	55.80 ± 0.50	66.00 ± 0.64	72.80 ± 0.00	64.47 ± 0.37[b]
	Mean G	52.27 ± 0.81[a]	59.53 ± 0.96[b]	73.06 ± 0.10[b]	

Note: superscript with different letters in the same row and column shows significant differences (P <0.05)

Table 6. The percentage of live Spermatozoa of equilibration time of 4 hours

Stages	Treatment of lactose (L)	Treatment of Glycerol (G)			Mean L
		G_3	G_5	G_7	
Pre equilibration	L_0	73.00 ± 1.87	74.20 ± 0.68	75.80 ± 0.40	74.33 ± 0.98[a]
	L_{60}	76.40 ± 0.89	78.40 ± 1.00	81.15 ± 0.00	78.65 ± 0.63[b]
	L_{120}	81.40 ± 0.55	83.45 ± 0.00	85.10 ± 1.10	83.31 ± 0.89[c]
	Mean G	76.93 ± 1.10[a]	78.68 ± 0.56[b]	80.68 ± 0.50[c]	
Post equilibration	L_0	49.40 ± 1.14	52.80 ± 2.78	72.40 ± 0.55	58.20 ± 1.49[a]
	L_{60}	51.00 ± 0.71	53.60 ± 0.55	74.80 ± 0.35	59.80 ± 0.53[b]
	L_{120}	54.60 ± 0.55	64.00 ± 0.71	76.62 ± 2.28	66.41 ± 1.18[c]
	Mean G	51.67 ± 0.79[a]	56.80 ± 1.35[b]	75.94 ± 1.06[c]	

Note: superscript with different letters in the same row and column shows significant differences (P <0.05)

Lactose as extracellular cryoprotectant can protect sperm from damage caused by ice crystals formed. This study shows that the concentration of 60 mM lactose has allegedly entered the cell membranes of spermatozoa during equilibration time of 2.5 hours and 3 hours. The concentration is adequate to protect the cell plasma membrane of semen from damage during the preservation process at low temperatures. However, the addition of 120 mM lactose (L_{120}) gives the best effect to motility of semen at the 4-hour equilibration time. This concentration is alleged to have met the needs of the semen in maintaining its quality. Singh et al. (1995) stated that the lactose concentration of 180 mM on Tris extenders could improve the quality of frozen goat semen. The equilibration time of wild species typically takes a long equilibration (Santiago-Moreno et al. 2009; Watson and Holt 2001) and the buffalo used in this study was wild buffalo. This is in line with the findings that the equilibration time of 4 hours, the longest time, is the best preservant for sperm quality. Lactose as the extracellular cryoprotectant compound has the capability to change normal water molecules (Viswanath and Shannon 2000). The characteristics help to stabilize the plasma membrane of sperm cells during the moving process through critical temperature zone and also change the mechanical characteristics of extenders by increasing the viscosity (Labetubun and Siwa 2011).

The percentage of live semen

Based on the results, the concentration of 7% glycerol makes no difference without the combination of time equilibration. The differences in quality have been seen at the time of the equilibration time between 2.5 and 3 hours to 4 hours. This is in line with the statement of Leite et al. (2010) that the equilibration time is more important than the concentration of glycerol in the process of adaptation to sperm membranes at low temperatures. The survival of spermatozoa during the freezing process is highly dependent on the degree of cooling (Rizal 2005). Therefore, the temperature of the freezing process is one of the important factors that needs special attention to keep spermatozoa alive. The differences of equilibration time will cause the differences in the percentage of spermatozoa after freezing. The study shows that equilibration time affects the percentage of survival of spermatozoa. However, it also can not be separated by the influence of a combination of cryoprotectants. A decline in the percentage of live sperm takes place after the treatment of equilibration. It occurs due to changes in temperature drastically. In keeping the state of sperm cells, glycerol takes considerable time to get into the cell membrane and keep the cell organ from damage due to freezing treatment. At the equilibration of 4 hours, glycerol in the extenders can work optimally to inhibit the formation of ice crystals,

thereby inhibiting the death of spermatozoa. Glycerol which binds to water molecules is free to form small and delicate ice crystals , so they are not harmful to sperm cells. The damage to the membrane and organelles can be avoided so the function of each organelle runs normally.

Carbohydrate can also serve as a substrate of energy source for spermatozoa during the process of preservation so that it can prolong its life. Lactose and maltose are carbohydrates of disaccharide class consisting of two units of monosaccharides, i.e. one unit of glucose and galactose for lactose, or two units of glucose to maltose, which all can be metabolized by the spermatozoa to produce energy in the form of ATP. Next, spermatozoa utilizes ATP as a source of energy in the process of movement so that it can remain motile and will maintain its vitality (Labetubun and Siwa 2011). The addition of lactose (Rizal 2006) and maltose (Herdis 2005) into Tris extenders can improve the quality of liquid semen of Garut sheep which is stored at 3-5°C.

Based on the data analysis which was obtained from percentages life, it showed that the combination of L120G7 with the equilibration time of 4 hours is the highest life percentage among other combinations. These results are similar to the results of research conducted by Tuli et al. (1981) and Herdis et al. (1999) which stated that the equilibration time of 4 hours is the best time. Graham et al. (1957) stated in the results of his study with an equilibration time of 4, 8, and 12 hours that the equilibration time of 4 hours showed the best semen quality. The 4-hour equilibration helps spermatozoa adapt to the low temperatures.7-percent glycerol has been able to protect the spermatozoa from plasma membrane damage and from organelles damage which leads to the success in cell energy metabolism. Harpahmi (2012) stated if plasma membrane is damaged, spermatozoa metabolism will be disturbed so that spermatozoa loses motility and leads to death. Graham (1994) added that semen plasma has an important role because it contains a wide variety of compounds that support the life of spermatozoa. Cell plasma membrane repair will have a positive impact on motility and the life of sperm. This is due to the motility of spermatozoa is very dependent on adenosine triphosphate (ATP) which is resulted in the metabolism process. Metabolism can work well if the plasma membrane of the cell is in state of intact. It makes the traffic to and from the cells goes well.

The combination of glycerol with lactose $L_{120}G_7$ is considered to be able to maintain the wholeness of the plasma membrane at the equilibration time of 4 hours which is alleged as optimal time. However, the equilibration time of 2.5 and 3 hours showed difference indicating that lactose L60 has been able to protect spermatozoa. The difference of the equilibration time causes the difference in the percentage of spermatozoa. At the 4-hour equilibration, glycerol in the extenders can work optimally to inhibit the formation of ice crystals, thereby inhibiting the occurrence of the death of spermatozoa,

Based on these results, it can be concluded that the best equilibration time is 4 hours with the combination of cryoprotectant $L_{120}G_7$. The equilibration time with appropriate cryoprotectants protects spermatozoa to obtain a good semen quality.

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