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Effect of Extenders with TCG and DMSO on the Viability of Rabbit Sperm

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ABSTRACT The purpose of this study was to evaluate the effect of addition of ethylene glycol, glycerol and sucrose to TCG (Tris, Citric Acid, Glucose, Egg Yolk) and DMSO Frozen. The extender containing Egg yolk concentration (10%, 20%) affects viability and acrosome morphology of rabbit sperm. Sperm viability was then assessed for the freezing extenders TCGD (Tris + Citricacid + Glucose + DMSO), TCGED (Tris + Citricacid + Glucose + Egg yolk + DMSO), TCGGD (Tris + Citricacid + Glucose + Glycerol + DMSO) and TCGSD Tris + Citricacid + Glucose + Sucrose + DMSO) during thawing at 38°C. for 20 seconds, respectively. TCG + 10% egg yolk (viability: 77.0 ± 0.8, NAI: 73.3 ± 0.9) was significantly (sperm viability and normal acrosome interaction (NAI)) higher than TCG + 20% egg yolk (70.7 ± 1.1, 70.0 ± 0.9) in the sperm normalcy analysis according to the yolk concentration. TCGGD (53.4 ± 0.1, 62.3 ± 0.4), TCGSD (61.3 ± 0.0, 67.1 ± 0.1) sperm viability and normal acrosome interaction (NAI) in frozen spermatozoa are TCGD (46.4 ± 2.8 and 56.3 ± 1. 4) and TCGED (23.0 ± 1.1 and 54.6 ± 1.4) extenders was thawed at 38°C for 20 seconds. According to the results from each frozen bulking agent, sperm membrane integrity by hypotonic swelling test (HOST) analysis in TCGGD (59.8 ± 0.7), TCGSD (59.3 ± 0.5) was significantly high compared to other experimental groups ($p < 0.05$). In conclusion, these results suggested that TCGGD and TCGSD extenders enhance survivability of rabbit sperm after frozen-thawing.

Keywords: extender, HOST, rabbit, sperm, TCG

INTRODUCTION

The market for rabbit meat industry is known to be an important source for future food resources. In particular, United Nations Food and Agriculture Organization (FAO) focuses on rabbit meat as a solution to the world food problem (Foote and Carney, 2000). However, studies on the improvement and mass production of rabbits had not been completed, and research on the application of frozen semen had also been lacking. The sperm of rabbits was suggested to the differential morphological and physiological on the sperms of the other economic animals. The extender causes cold shock on sperm acrosome or survival rate during frozen (Watson, 2000). Semen freezing with egg yolk and Tris, Citric acid, Glucose (TCG) was known as a commonly used extenders for freezing preser-

vation of rabbit sperm. There were many studies on freezing method by adding glycerol, dimethylsulfoxide (DMSO) and sucrose to the second extender with freezing extender. However, studies on the effect of buffer concentration and composition on sperm viability are insufficient (Cortell and Viudes, 2008). In particular, the frozen of sperm using glycerol is highly effective in preventing frostbite from frozen in many animal sperm (Polge et al., 1949). Especially, rabbit sperm have been difficult to freeze successfully (Hanada and Nagase, 1980; Chen et al., 1989a, 1989b; Bamba, 1990; Chen and Foote, 1994). The incorporation of detergents in seminal extenders has improved the quality of cryopreserved sperm from boars (Pursel et al., 1978), rams (El-Alamy and Foote, 2000), bulls (Arriola and Foote, 1987), dogs (Rota et al., 1997), and mice (Dewit et al., 2000). No similar studies have been reported for rabbit sperm. However, the concentration of glycerol in some animals (bovine and pigs etc.) is controlled in the toxicity about sperm. In animal species including chickens, sheep, pigs and horse's glycerol had been reported to reduce fertilization rate and capacitation (Swanda et al., 1964; Hammerstedt et al., 1992). Therefore, this study analyzed the viability of spermatozoa according to the freezing extender of rabbit sperm.

MATERIALS AND METHODS

Semen collection

The rabbit semen collection was performed by using a self-made sperm sampler at intervals of one week. The semen sample was quantitated using a micropipette, and 10 μL of diluted semen was collected from the sampler into a chamber and then diluted with a primary agent to obtain a sperm concentration of 4×10^5 sperm/mL. The extender was stored in a thermostat warmed at 37°C and was either used for artificial insemination within 2 hours or transferred to a laboratory for freezing.

Preparation of extender

The first extender prepared with Tris 250 mM (T-6791, Sigma, USA), Citric acid 88 mM (C-2404, Sigma, USA), and Glucose 47 mM (G-7021, Sigma, USA).

The osmotic pressure of the first extender was adjusted to Osmol and the pH was adjusted to 7.0. Ten and 20% of the egg yolk was added to the first extender and it was disclosed to the experiment comparing the viability of the sperm with the long term preservation (Table 1).

The second extender composed of 10% of egg yolk, 3.5 M of DMSO (D-2650, SIGMA, USA), 3% of ethylene glycol, 3.5 M of DMSO, 3% of glycerol and 3.5 M, DMSO, 0.1 M sucrose and 3.5 M DMSO in primary extender (Table 2).

Low-temperature storage and examination of survival rate of sperm

One of treatment groups was in a double boiler at 16 to 37°C and another of those was from 4 to 37°C. Semen dilution cooled down at 4°C and 16°C for 2 hour and kept five days. Survival rate was measured at 12 hour intervals at 37°C with a 100 × magnification microscope.

Semen freezing and examination of survival rate of sperm

The semen transferred to the laboratory was dispensed by experimental treatments. The hot water at 37°C was placed in a beaker and the semen injected into the beaker was stored in a refrigerator at 4°C for 2 hour. The equilibrated semen was preliminarily frozen in 0.5 mL straw from 10cm above the surface of liquid nitrogen, frozen by immersion in liquid nitrogen stored until the survival rate

Table 1. Composition of first extenders for semen dilution

First extender	Composition in extender 100 mL (g)	
	TCG10	TCG20
Tris	3.028	3.028
Citric acid	1.690	1.690
Glucose	0.847	0.847
OSM	303	303
pH	6.9	6.9
Egg yolk	10 mL	20 mL

*TCG10 (Tris, Citric, Glucose and 10% egg yolk), TCG20 (Tris, Citric, Glucose and 20% egg yolk), OSM (osmoles mater).

Table 2. Composition of second extenders for semen dilution

Second extender	Composition in extender (g/mL)			
	TCGD	TCGED	TCGGD	TCGSD
Ethylene glycerol		1.5		
DMSO	13.7	13.7	13.7	13.7
Glycerol			1.5	
Sucrose				1.7

*TCGD (Tris, Citric, Glucose and DMSO), TCGED (Tris, Citric, Glucose, Ethylene glycerol and DMSO), TCGGD (Tris, Citric, Glucose, Glycerol and DMSO), TCGGD (Tris, Citric, Glucose, Sucrose and DMSO).

was checked. The frozen semen was thawed at 38°C for 20 seconds.

Survival rate and acrosomal normaliztion

De Leeuw (1991) was improved and frozen semen was melted at 38°C for 20 seconds. Then, 100 μL of sperm dilution and 600 μL of 0.3% BSA were mixed and centrifuged at 1500 rpm for 5 minutes. After centrifugation, the supernatant was removed, Hoechst 33258 was added well at a concentration of 100 μg/mL and stored in the dark for 1 minute 30 seconds, then mixed with 10 μL of 4% paraformaldehyde. On the slides, 50 μL of the treated semen was covered with cover grass, and then 200 of sperm were observed at × 400 magnification. Two hundred sperm were counted and dyed sperm were counted as dead sperm. For analysis of acrosomal integrity of sperm, semen was stained with Coomassie Brilliant Blue (R-266, TNT, Republic of Korea) as described previously (Larson and Miller, 1999). Frozen-semen thawed at 38°C for 20 seconds, and then 100 μL of frozen-thawed semen was centrifuged at 1500 rpm for 5 minutes. The supernatant was removed. 50 μL of 1 × PBS was added to 10 μL of sperm pellet and centrifugation was performed twice at 1500 rpm for 5 minutes.

The semen was diluted in 500 μL of 1 × PBS and 50 μL of the solution was placed on a slide and dried at 37°C. The cells were stained by 0.25% Coomassie Briliant Blue (R-266) solution for 5 minutes and washed twice with 1 × PBS. Acrosomal normality was determined by observing the presence or absence of blue dyeing and the degree of staining of the sperm's head in a microscope at × 400 magnification. 300 sperm were counted and converted into percentages. Sperm dyed in blue were classified as normal sperm.

Plasma membrane function test

Plasma membrane function test of sperm was performed as described previously (Jeyendran RS, 1984). The frozen semen thawed in water at 38°C for 20 seconds and then 100 μL of frozen-thawed semen was centrifuged at 1500 rpm for 5 minutes. After removal of the supernatant, added 1 mL of hypoosmotic solution and stored at 37°C for 30 minutes. 50 μL of treated semen dilution was placed on a slide and more than number of 300 sperm were counted to analyzed the degree of fin and buoyancy of the tail with × 400 magnification.

Statistical analysis

Percentage of motility, viability and survivability of spermatozoa of among treatment groups were compared by Duncan's multiple range test in application of ANOVA and GLM using SAS package (version 9.1).

RESULTS

Low temperature preservation of rabbit semen

The collected rabbit semen was stored at 16°C and 4°C for 72 hours according to the low temperature preserva-

Table 3. Addition effect of Tris in freezing extender on frozen-thawed sperm motility and viability of rabbit spermatozoa (Mean ± SD)

Type of semen extenders	Temperature (°C)	Preservation periods (h)						
		0	12	24	36	48	60	72
TCG	4	90%	78.0 ± 0.7	51.1 ± 1.6	23.4 ± 0.6	16.5 ± 0.4	0.0	0.0
	16	85%	78.3 ± 0.8	55.6 ± 0.9	23.0 ± 0.1	19.5 ± 1.6	16.5 ± 0.2	0.0
TCGE	4	90%	78.5 ± 0.4	72.7 ± 0.6	64.4 ± 1.5	53.5 ± 1.2	26.8 ± 1.5	17.8 ± 1.5
	16	90%	85.8 ± 1.2	81.7 ± 1.1	76.1 ± 1.2	60.8 ± 1.2	39.2 ± 0.1	19.3 ± 1
TCGP	4	90%	85.6 ± 1.7	81.3 ± 1.8	62.9 ± 1.5	47.0 ± 1.4	30 ± 1.3	13.7 ± 1.3
	16	85%	85.7 ± 0	78.8 ± 1.6	72.2 ± 1.1	61.3 ± 1.9	34.7 ± 1.0	14.6 ± 1.6
TCGEC	4	90%	78.75 ± 1.8	70 ± 1.8	60 ± 0.3	53.7 ± 3.5	30.5 ± 1.8	10.5 ± 0.3
	16	90%	85 ± 1.5	78.75 ± 1.8	66.2 ± 1.8	60 ± 0.3	30 ± 1.5	10 ± 0.3
TCGM	4	90%	78.0 ± 0.7	51.1 ± 1.6	23.4 ± 0.6	16.5 ± 0.4	0.0	0.0
	16	95%	78.3 ± 0.8	55.6 ± 0.9	23.0 ± 0.1	19.5 ± 1.6	16.5 ± 0.2	0.0
TCGPEM	4	85%	62.3 ± 1.1	63.5 ± 1.6	51.2 ± 1.8	41 ± 1.4	32.7 ± 1.0	0.0
	16	85%	65.4 ± 1.0	67.5 ± 1.2	62.5 ± 0	50 ± 0	30.8 ± 1.2	10 ± 1.4

*TCG (Tris, Citric and Glucose), TCGE (Tris, Citric, Glucose and Ethylene glycerol), TCGP (TCG + PVP), TCGec (TCG + Ethyleneclycol), TCGM (TCG +Mercaptoethanol), TCGPEM (TCG + PVP + Egg yolk+Mer captoethanol).

tion extender prepared by the research team. The survival rate and the change of vitality were measured.

Table 3 shown the survival rates of low-temperature preserved agent by TCG (Tris + Citricacid + Glucose) dilution, TCGE (TCG + Egg yolk), TCGP (TCG + PVP), TCGec (TCG + Ethyleneclycol), TCGM (TCG + Mercaptoethanol), and TCGPEM (TCG + PVP + Eggyolk + Mercaptoethanol).

As a result of evaluating the cold storage property according to each frozen agent by temperature, the survival rate was higher at 16°C than at 4°C in all the low temperature frozen agent. In the extenders analysis, survival rate of the samples was 0.0% at 72 hours on 16°C. The survival rate was 14.6% ± 1.6% in TCGP, 10 ± 0.3% in TCGec, 0.0% in TCGM, 10 ± 1.4% in TCGPEM, and the survival rate of TCGM on 19.3 ± 1% was higher than other frozen agents (Table 3).

Survival and vitality of rabbit sperm according to the egg yolk addition concentration

In order to investigate the effect of egg yolk addition on the vitality of sperm during low - temperature storage of rabbit sperm, the addition of egg yolk was added to a concentration of 10%, 20% according to result preserving in TCGE cold storage agent at 16°C. The results are shown in Table 4. From 12 to 84 hours, survival rate of TCGE10 with 10% egg yolk was 85.8 ± 1.1% at 12 hours, 77 ± 0.8% at 48 hours, 67.5 ± 1.9% at 36 hours, and 48.2 ± 1.2% at 60 hours and 36.0 ± 0.9% at 72 hours. The survival rate of TCGE20 was 80.8 ± 1.2% at 12 hours, 76.7 ± 1.1% at 24 hours, 70.1 ± 1.2% at 30 hours, 60.8 ± 1.2% at 48 hours and 39.2 ± 0.1% at 60 hours and 19.3 ± 1% after 72 hours. the low temperature frozen agent of TCGE10 was significantly ($p < 0.05$) higher than the low temperature frozen agent of TCGE20. In addition, in the plasma membrane test of Table 5, TCGE10 showed 73 ± 1.4% of plasma membrane function normal. TCGE20 was 70 ± 1.4% of plasma membrane function normal and the low temperature frozen agent of TCGE10 was significantly higher than TCGE20 ($p < 0.05$).

Ability rate of sperm according to frozen extender

To analyze the effect of extender on viability and acrosome test after frozen thawing, added 3.5 M of DMSO, 3% ethylene glycol, 3.5 M DMSO in dilution (below TCGED), 3% glycerol, supplement 3.5 M DMSO (below TCGGD), 0.1 M sucrose, 3.5 M DMSO (below TCGSD) were used for the experiment in the semen of rabbits diluted with cold extenders. The survivability and acrosome stability of the rabbit semen after freezing and thawing according to each frozen extenders were shown in Fig. 1 and Table 6. The results of acrosome normality and the survival rate of rabbit sperm and was analyzed by Hoechst fluorescence staining in classifying fluorescently stained sperm is dead sperm and unstained sperm is living sperm. High survival rates were identified in TCGGD and TCGSD semen frozen agent. Based on the results of fluorescence staining analysis, the survival and acrosome stability of rabbit sperm in frozen agent were evaluated using computer assisted sperm analysis system. In TCGGD, 53.4 ± 0.1% and 62.3 ± 0.4%, 53.4 ± 0.1% and 62.3 ± 0.4% in TCGGD, 61.3 ± 0% and 67.1 ± 0.1% in TCGSD. In TCGD 46.4 ± 2.8% and 56.3 ± 1.4% was significantly higher than 23.0 ± 1.1% and 54.6 ± 1.4% in TCGED, respectively ($p < 0.05$).

Table 5. Plasma membrane function of sperm in rabbit (Mean ± SD)

Type of extender	Tail changes (%)
TCGD	73 ± 1.4*
TCGED	70 ± 1.4

*Different letters within same column represent a significant difference ($p < 0.05$).

*TCGD (Tris, Citric, Glucose and DMSO), TCGED (Tris, Citric, Glucose, Ethylene glycerol and DMSO).

Table 4. Effect of 10 and 20% of egg yolk concentrations in semen extender on survival ability

Type of semen extenders	Preservation periods (h)							
	0	12	24	36	48	60	72	86
TCG10	86.4 ± 0.5	77.0 ± 0.8	67.5 ± 1.9	65.8 ± 1.1	48.2 ± 1.2	36.0 ± 0.9	11.1 ± 1.7	0
TCG20	85.9 ± 1.2	76.7 ± 1.1	70.1 ± 1.2	60.8 ± 1.2	39.2 ± 0.1	19.3 ± 1.0	15.5 ± 0.9	0

*TCG10 (Tris, Citric, Glucose and 10% egg yolk), TCG20 (Tris, Citric, Glucose and 20% egg yolk).

*Different letters within same column represent a significant difference ($p < 0.05$).

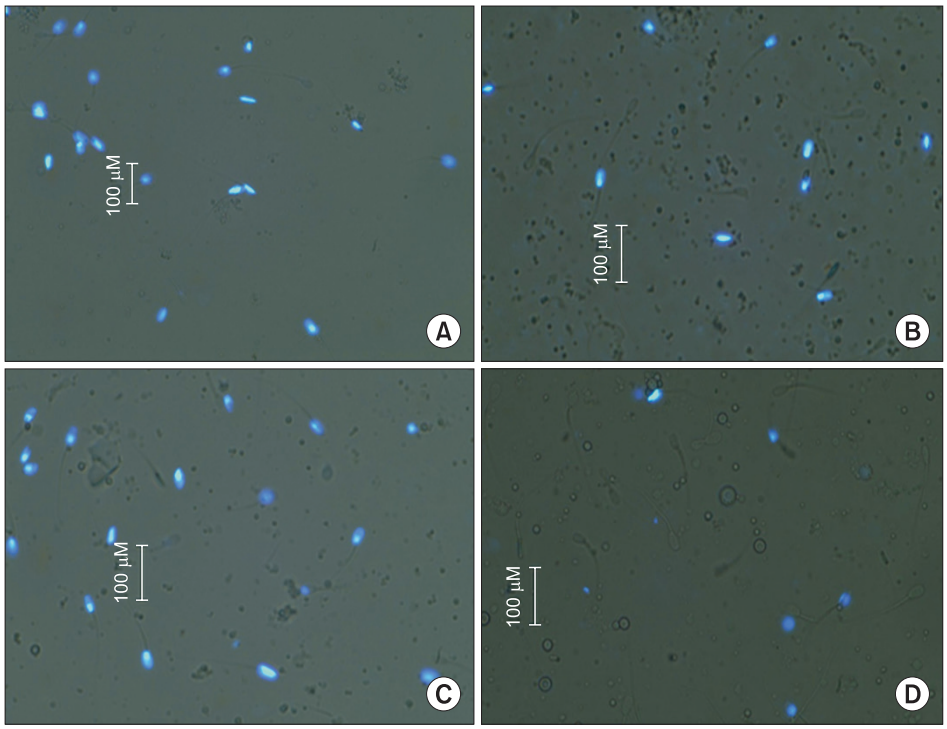

Fig. 1. Effect of different frozen extender for spermatozoa Nucleic acid normalization on the rabbit sperm. (A) effect of survival ability to the TCGD on frozen sperm, (B) effect of survival ability to the TCGED on frozen sperm, (C) effect of survival ability to the TCGGD on frozen sperm, (D) effect of survival ability to the TCGSD on frozen sperm.

Table 6. Effect of freezing extender and thawing method on the survival ability of frozen Rabbit spermatozoa

Type of extender	Viability (%)	Acrosome (%)		
		F	B	AR
TCGD	46.4 ± 2.8	56.3 ± 1.4	24.8 ± 0.3*	18.9 ± 1.2*
TCGED	23.0 ± 1.1	54.6 ± 1.4	33.6 ± 0.3**	11.8 ± 1.0
TCGGD	53.4 ± 0.1*	62.3 ± 0.4*	20.3 ± 0.7	17.4 ± 0.4*
TCGSD	61.3 ± 0**	67.1 ± 0.1**	21.1 ± 0.7	11.8 ± 0.8

F: Normal Acrosome Intact , B: Capacitated , AR: Reactive.
*TCGD (Tris, Citric, Glucose and DMSO), TCGED (Tris, Citric, Glucose, Ethylene glycerol and DMSO), TCGGD (Tris, Citric, Glucose, Glycerol and DMSO), TCGGD (Tris, Citric, Glucose, Sucrose and DMSO).
*,**: Different letters within same column represent a significant difference (p <0.05).

DISCUSSION

This study analyzed the effects of acrosome normality and viability of sperm to TCG (Tris, Citric acid, Glucose, Egg yolk) agent to ethylene glycerol, glycerol, sucrose, regulation of DMSO concentration is compared to the many research. According to a study by Gotzeand Paufler (1976), changes of egg yolk concentration in TCG suggested that rabbit sperm could have a great effect on frozen sperm, but the difference was not known. In the present study, 10% egg yolk supplementation resulted in a survival rate of 77 ± 0.8, acrosome steadiness of 73.3 ± 0.9%, and plasma membrane function of 73 ± 14%, the survival rate of long term sperm could be maintained during storage at low temperature rather than 20% egg yolk supplementation. In addition, low survival rate was recorded by cold shock in free egg yolk TCG. It was considered the survival rate would be lowered in the plasma membrane damage due to exposure to low temperatures in rabbit sperm head (Cortell and Viudes de Castro, 2008). Based on the present study, the second frozen agent by mixing the solution related to freezing preservation after the first low temperature preservation in each frozen agent using 20% egg yolk was resulted in differences in the survival rate and acrosome normality. According to Arriola (1982), glycerol on frozen of rabbit sperm wastoxic compared to other species, it could affect the survival rate (Arriola, 1982). It was recommended to use methyl, amide and DMSO as alternative materials for freeze protection but that was known to have highly no effect at living rate of sperm and changes of acrosome (Hanada and Nagase, 1980). However, the addition of DMSO and egg yolk couldhave a positive effect on the normality and survival rate of rabbit sperm. In a result study of improving IVF correction rate, the final 1.5% glycerol or 0.1 M sucrose and 14%

DMSO to the first frozen agent supplement 10% egg yolk showed higher survival rates than a result of the existing 10% DMSO and 4.8% glycerol added frozen agent. It was compared to the 4.5% DMSO and 1% glycerol frozenagent of Polgar et al. (2004), high acrosome normality and survival rate were shown during sperm freezing and thawing. Compared to the methods presented in many other studies, high concentrations of DMSO and low concentrations of glycerol additions could be considered effective in protecting the plasma membrane of rabbit sperm head (Foote et al., 2000; Cortell et al., 2008). These findings are considered to have a significant effect on sperm conservation in rabbits according to the concentration control of the frozen agent.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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