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Effect of Supplementation of Trehalose, Glycerol on Conventional Freezing and Vitrification of Boar Sperm

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ABSTRACT

The boar sperm has more lipid droplets and specialty of seminal plasma compared with other species, causing difficulties of freezing sperm and decreases for the utilization of frozen semen into the artificial insemination. However, several studies reported significant results for the recovery of sperm motility and reproductive by addition of cryoprotectants and seminal plasma after thawing. This study was designed to investigate the effects of supplementation of trehalose or glycerol in the LEY (lactose and egg yolk in BTS) solution for the conventional freezing and vitrification process. Two boars aged 16 months were used to collect semen for 2 times in a week. The samples were allotted to 3 freezing solutions (LEY + glycerol 10.5% + OEP 1.5%, LEY + trehalose 1M + OEP 1.5%, and sucrose 1.5M + trehalose 1 M + OEP 1.5%) after centrifugation at 800 g for 10 minutes. Semen was equilibrated in freezing solutions for 10 minutes and injected into plastic straws with 2～3 air bubbles to minimize freezing damages. Vitrification was performed to locate sperm in 5 cm above LN_2 for 5 minutes, and the conventional freezing was conducted with an automatic freezer. Motility and survival rates were measured by CASA (Computer assisted sperm an alyzing system) and FITC (Fluorescein isothiocyanate), respectively after thawing semen at 50℃ for 12 seconds. The results were analyzed by ANOVA with STATVIEW statistical program. The vitrificatioin solution (LEY + 10.5% glycerol + 1.5% OEP) presented higher motility (20.9%) than other solutions while the solution (LEY + 1M trehalose + 1.5% OEP) showed the lowest (motility : 5.2%). However, survival rates of vitrified sperms detected by FITC showed 1~4% live sperms in almost of dead sperms at all vitrification solutions' groups, but survival rate of freezing solution of LEY + 1M trehalose + 1.5% OEP LEY and LEY + 10.5% glycerol + 1.5% OEP were showed 49%, and 79%, respectively. There were differences (P<0.05) survival rate of conventional freezing in LEY + 10.5% glycerol + 1.5% OEP and LEY + 1M trehalose + 1.5% OEP and the remaining showed no differences. The results suggested that vitrified boar semen was not enough to be utilized for the artificial insemination, but it showed possibility to utilize for ICSI and conventional freezing with glycerol would be useful method for artificial insemination in pig while we choose the outstanding semen against tolerance to freezing damages.

(Key words : sucrose, trehalose, glycerol, boar semen, vitrification)

INTRODUCTION

Boar sperm cryopreservation is an important basic element to improve the artificial insemination and additionally develop the animal genetic improvement (Bailey *et al.*, 2008). Frozen semen offers long term storage of livestock and wild rare animals. However cryopreservation methods of boar semen are still unstable about the diversity of freezing methods. The boar sperm has more lipid droplets and specialty of seminal plasma as with fractions of the ejaculated semen compared with other species, causing difficulties of freezing sperm and decreases. However, several studies reported significant results for the recovery of sperm motility and reproductivity by addition of cryoprotectants (Mazur *et al.*, 2008), such as glutathione and ascorbic acid and seminal plasma (Alkmin *et al.*, 2014; Gonzalez-Cadavid *et al.*, 2014) after thawing. Freezing extenders of boar semen is based on egg yolk (Westendorf *et al.*, 1975) with other additives (Andrabi *et al.*, 2008; Bathgare *et al.*, 2006) and cryoprotectants. The most used cryoprotectant is glycerol that used low concentration owing to the toxicity (Murdoch and Jones, 1978; Jones *et al.*, 1992; Bhur *et al.*, 2001; Gutierrez-Perez *et al.*, 2009; Malo *et al.*, 2010) in bull and so on. The

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other one is trehalose which acts in various organisms in response to dehydration (Leslie *et al.*, 1995), temperature (Ramlov and Westh, 1992) and osmotic damage (Hounsa *et al.*, 1998; Gutierrez-Perez *et al.*, 2009). And trehalose probably protects biomolecular structures the replacement of water in hydrogen bonds (Chen *et al.*, 2000; Hu *et al.*, 2009) and essential hydration water molecules (Patisr and Zoerb, 2005) and maintain the viscosity of biomolecular structure (Sampedro and Uribe, 2004). Some of the reports suggested that trehalose has significant effects on sperm freezing in mouse, bull, ram and goat and indicated the mitochondria membrane potential frozen-thawed with trehalose in boar spermatozoa (Athurupana and Funahashi, 2014). The other cryoprotectant frequently used in boar semen freezing with dry ice, is Equex STM paste (OEP) which extracts more lipid or lipoproteins from egg yolk to the freezing extender (Axner *et al.*, 2004; Wu *et al.*, 2012). These cryoprotectants were avoided intracellular formation of ice crystal during freezing of spermatozoa and penetrated to cells for taking place intercellular water. To inhibition of crystallization, intercellular water is to convert the vitrified form with non-permeable agent, large molecules such as sucrose, polyvinyl-pyrrolidone (PVP). Currently, methods of vitrification are also developed, Nawroth *et al.* (2002) and Isachenko *et al.* (2003) reported the vitrification of human spermatozoa without cryoprotectants which was effective and could be recommended for human IVF. But vitrification of animal sperm is not established yet to use for commercial fields and has no trial. In this regards, the objective of this study was to investigate the motility and survival rate on cryoprotective effects of trehalose, glycerol and Equex STM paste and adaptation of vitrification in boar spermatozoa.

MATERIALS AND METHODS

1. Experimental Animals and Semen Sample Treatment

Two boars aged 16 months were used to collect semen for 2 times in a week. Semen was diluted to 1:1 with BTS (Bestville Thawing solution) and measured the survival rate and concentration of raw semen. Sperm morphology was detected by hyposolution contained 3% salt at 37℃. One aliquot of was mounted on glass slide and counted 200 sperms and evaluated the percentage of sperm with survivals, damaged membrane or straight or coiled tail, respectively.

2. Freezing of Boar Sperm

The samples were diluted with lactose egg-yolk (LEY) solution contained with 20% fresh egg yolk separated from whole egg. Experimental groups were allotted to 3 freezing solutions (LEY + glycerol 10.5% + OEP 1.5%, LEY + trehalose 1 M + OEP 1.5%, and sucrose 1.5 M + trehalose 1 M + OEP 1.5%) after centrifugation at 800 g for 10 minutes. Semen was equilibrated in freezing solutions for 10 minutes and injected into 0.5 ml plastic straws with 2～3 air bubbles to minimize freezing damages. Vitrification was performed to locate straws contained equilibrated sperm with glycerol in 5 cm above LN_2 for 5 minutes, and the conventional freezing was conducted with an automatic freezer. Frozen semen was thawed with 50℃ for 12 sec and then measured the survival rates.

3. Detection of Motility and Survival

Motility and survival rates were measured by CASA (Computer assisted sperm analyzing system) and FITC (Fluorescein isothiocyanate) staining methods, respectively.

4. Statistical Analysis

The results of vitrified sperm were analyzed by ANOVA with STATVIEW statistical program.

RESULTS

1. Survival Rates after Freezing

Semen sample used in this study was normal morphology and 70% or more living sperms at raw status. And we had been found the phenomenon about the ability to tolerate the freezing damages in some of boars. So boar selection was occurred among boars breeding in NIAS (National Institute of Animal Science). The results of conventional freezing and vitrification in boar sperm were represented in Table 1 and Table 2. And the results of survival rates on conventional freezing and vitrification were showed in Table 3 and Table 4.

The conventional freezing in solution (LEY + 10.5% glycerol + 1.5% OEP) presented higher motility (20.9%) than other solutions while the solution (LEY + 1 M trehalose + 1.5% OEP) showed the lowest (motility 6.9%). Almost of vitrified sperm showed lower motility as 5.2～9.9% than conventional freezing. And the movements measured by CASA were also weak, it seems to be unavoidable to induce AI injection to sows.

However, survival rates of vitrified sperms detected by FITC showed 1～4% live sperms of almost of vitrified sperms in all

Table 1. Effects of cryoprotectants on conventional freezing in boar sperm

Freezing with various cryoprotectants	Result of CASA				
	Motility	VCL	VSL	VAP	LIN
LEY + glycerol + Equex	20.98	41.98	18.92	25.3	52.07
LEY + trehalose + Equex	6.92	45.03	12.41	21.18	43.45
Sucrose + trehalose + Equex	8.32	41.34	14.13	20.46	56.87

※ VCL: Curvilinear line velocity, VSL: Straight line velocity, VAP: Average path velocity, Lin: Linearity.

Table 2. Effects of cryoprotectants on vitrification in boar sperm

Vitirfication with various cryoprotectants	Result of CASA				
	Motility	VCL	VSL	VAP	LIN
LEY + glycerol + Equex	8.38	19.47	13.08	18.34	78.92
LEY + trehalose + Equex	5.22	18.99	13.37	18.45	80.1
Sucrose + trehalose + Equex	9.99	17.46	13.38	16.48	82.82

※ VCL: Curvilinear line velocity, VSL: Straight line velocity, VAP: Average path velocity, Lin: Linearity.

Table 3. Results of survival rates of frozen-thawed sperm by FITC staining in boar sperm

Freezing with various cryoprotectants	No. of sperms		
	Live	Dead	Survival rates (%)
LEY + glycerol + Equex	49	151	24.3±4.7[a]
LEY + trehalose + Equex	79	121	39.7±3.1[b]
Sucrose + trehalose + Equex	0	200	0±0

[a,b] within same column means significantly different (p<0.05).

vitrification solutions' groups, but survival rate of freezing solution of LEY + 1M trehalose + 1.5% OEP LEY and LEY + 10.5% glycerol + 1.5% OEP were showed 49%, and 79%, respectively. There were differences (P<0.05) survival rate of

Table 4. Results of survival rates of vitrified-thawed sperm by FITC staining in boar sperm

Freezing with various cryoprotectants	No. of sperms		
	Live	Dead	Survival rates (%)
LEY + glycerol + Equex	4	196	2.0±0.9
LEY + trehalose + Equex	1	199	0.3±0.6
Sucrose + trehalose + Equex	0	200	0±0

conventional freezing in LEY + 10.5% glycerol + 1.5% OEP and LEY + 1M trehalose + 1.5% OEP and the remaining showed no differences. In conventional freezing and vitrification, the groups of LEY plus cryoprotectants were showed live sperm after freezing while treatment of sucrose + trehalose + OEP was showed all of dead sperm. It seems that sucrose could not be taken place with LEY.

DISCUSSION

This results agreed the effects of the trehalose-containing freezing extender used for boar semen is possible to preserve of membrane structure. And acrosomal integrity was increased by detecting live sperm with FITC when trehalose was added freezing extender (Gutierrez-Perez *et al.*, 2009). In report of Athurupana and Funahashi (2014), trehalose without glycerol attributes effect of mitochondria membrane potential in boar sperm, that seems trehalose maintains the activity of mitochondria during freezing damage. In our results, survival rate of conventional freezing with trehalose was better than glycerol group. And trehalose protects osmotic effect and specific interaction with membrane phospholipids. It appears connection of replacing water at the membrane-solution interface. Although glycerol had been used for cryoprotectans in some species, but it is not profitable to satisfy protecting freezing tolerance (Hu *et al.*, 2009; Malo *et al.*, 2010). When trehalose was added into freezing media, Hu *et al.* (2009) reported the synergic effects of trehalose and glycerol on boar sperm integrity. Because extraction of intercellular water from cells is induced through stable membrane in the freezing media. Trehalose gives the specific cryoprotecting ability on the lipidic bilayer (Patist and Zoerb, 2005; Hu *et al.*, 2009). And Hu *et al.*(2009)

suggested the optimal concentration of trehalose is 100 mM and sperm membrane integrity significantly decreased when more adding of 100 mM of trehalose. But we used 10 times trehalose than theirs, survival rates of frozen-thawed boar sperm was showed lower for this reason.

The useful compound of Equex STM paste is sodium dodecyl sulfate (SDS). It suggested SDS probably acts modifying the structure of egg yolk lipoproteins in the extracellular medium. The mechanism of cryoprotective effect of Equex STM paste on sperm is not completely known. Axner *et al.* (2004) reported Equex did not affect the intact sperm membrane or motility immediately after thawing and motility and membrane integrity decreased at a faster rate in Equex treated cat sperms. Compared with dog sperms, addition of Equex to freezing extender significantly improves sperm membrane integrity, motility and longevity (Rota *et al.*, 1997). The cryoprotective effect of Equex seems to be exerted by modification of egg yolk lipoproteins and SDS is known to be toxic effect on sperm membrane. If Equex would have the useful effects if frozen-thawed spermatozoa would be use for artificial insemination. In this regarding, our results were showed lower survival rates than the results of Wu *et al.* (2013). They reported the optimal concentration of boar sperm freezing extender is 0.5% Equex and frozen-thawed sperms were detrimental in 2% Equex. Even if our results were lower than that of theirs, it is not easy to estimate the harmful inducer in our freezing extender because of mixing high concentration of trehalose and Equex. To confirm this difference, more research may be needed to change the concentration and/or cryoprotective agents (Buranaamnuay *et al.*, 2009). Vitirification of boar sperm was not found in any articles but in the case of boar, these type was required the freezing of boar semen, because it is not established yet and we need another trial for boar semen cryopreservation. Isachenko *et al.* (2002, 2003) tried to vitrify human spermatozoa without permeable cryoprotectants (Isachenko *et al.*, 2012) and reported success to extend the reproduction methods with IVF, ICSI and so on. And they applied to vitrify sperm of animals such as sperm of fish, dog and tissues of human organisms. They vitrified the human sperm with 0.5% sucrose in HTF (human tubal fluid) + 1% HSA (human serum albumin) of vitrification solution and produce two babies. We think that it is nice trial for the breeds which is difficult to cryopreserve. They indicated the cryopreservation is normally achieved tertiary combination of cells, permeable cryoprotectants and a low temperature environment and cryoprotectants has negative effects on spermatozoa including damaging the cytoplasm, functional destabilization and mutagenesis. As mentioned above, numerous researchers reported the low concentration of cryoprotectants and maintenance of the membrane status for stream of intercellular water. And number of frozen-thawed sperm is promised on artificial insemination in animals, sperms for fertilizing in oviduct need few thousands sperms and few millions in uterus. Even though AI technique is developed as manipulating injection of sperms, it is essential to prepare live sperms. In conclusion, our results are not enough to use AI for reproducing pigs, but trial of vitrification can be used in assisted reproduction. And numerous reports have been indicated the diversity of cryopreservation on breeds (Nicolas *et al.*, 2012), further studies are needed to investigate the possible optimal cryopreservation of boar semen.

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