韓國家畜繁殖學會誌 25(4) : 409~419 (2001)
Korean J. Animal Reprod.

Enhancement of Fertilizing Ability of Frozen-Thawed Bovine and Human Spermatozoa Treated with Fertilizing Promoting Peptide or Pentoxifylline

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ABSTRACT

This study was to examine whether the *in vitro* viability, motility and intact acrosome of frozen -thawed bovine and human sperm can be improved by adding Pentoxifylline (PF) or Fertilization Promoting Peptide (FPP). Human semen was frozen ultra-rapidly using Test yolk-buffer (TYB) freezing medium. Additive (PF, FPP) effects in frozen-thawed bovine and human sperm were analyzed by microscopic count for sperm motility and coomassie brilliant blue staining method for sperm acrosome intact. The *in vitro* motility of frozen-thawed bovine sperm with 5 mM PF treatment group (50.0%) was significantly higher than that of control (34.0%) ($P<0.05$). In the frozen-thawed bovine sperm was examined, the intact acrosome rate of 50 nM FPP treatment (49.0%) was significantly higher than those of control (30.0%) and 25 nM FPP (38.0%) treatment groups ($P<0.01$). In human semen, when *in vitro* motility of sperm with PF addition prior to freezing was examined, the result of 5 mM treatment group (51.0%) was significantly higher than those of control and 2.5 mM treatment group (39.0, 40.0%) ($P<0.01$). In addition, 50 nM (75.5%) FPP adding in all treatment procedures for human semen freezing (before freezing, freezing and after thawing) was significant effect on maintenance of the sperm intact acrosome percentage (control: 45.0; 25 nM: 53.0; 100 nM: 68.0%) ($P<0.01$). Also, the intact acrosome rate of human sperm with FPP (65.0%) was significantly higher than that with PF (43.0%) ($P<0.05$), although sperm motility was slightly higher in PF treatment group. These results suggest that improved sperm motility and intact acrosome of frozen thawed bovine and human sperm can be obtained by addition of PF or FPP, and that the enhanced *in vitro* viability, motility and intact acrosome can be obtained by addition of FPP in all semen freezing procedures.

(Key words: Bovine and Human sperm, Cryopreservation, Viability, Motility, Intact acrosome)

Ⅰ. INTRODUCTION

Cryopreservation of mammalian semen is a routine technique for storing donor or superior semen prior to artificial insemination. Sperm may be frozen before cancer therapy, vasectomy or as a method that makes the required delay possible to ensure human immune deficiency virus-free sperm specimens prior to insemination of recipient. Improvements in

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cryopreservation of human spermatozoa have been attempted in the past by use of different cryoprotectants and extenders and in particular, by altering the cooling rate, usually a linear reduction in temperature with time (Gilmore et al., 1997). However, sperm cryopreservation results is adverse changes in sperm motility (Critser et al., 1987), viability (Alvarez and Storey, 1993), the ability of spermatozoa to penetrate into cervical mucus, zona-free hamster egg, and acrosome reaction (McLaughlin et al., 1992). The fertilizing ability of human spermatozoa decrease after cryopreservation and can be partially explained by the reduced percentage of spermatozoa with normal, intact acrosomes and diminished acrosin activity (Mack and Zaneveid, 1987). Therefore, efforts to improve the fertilizing ability of cryopreserved spermatozoa are clinically useful.

Capacitation encompass the many steps that transform a non-fertilizing (uncapacitated) sperm into a potentially fertilizing (capacitated) cell, i.e., one able to bind to the zona, undergo the acrosome reaction, and fertilize an oocyte. Although experimental evidence indicates that sperm undergo numerous changes that include membrane modification, biochemical and change in the pattern of motility during capacitation, the mechanism that modulate capacitation are still poorly understood. Recent investigators, phosphodiesterase inhibitor such as pentoxifylline (PF) has been used to stimulate sperm motility (Sikka and Hellstrom, 1991; Tesarik et al., 1992; Sharma et al., 1996). The treatment of highly selected motile sperm populations by incubation with PF stimulates *in vitro* fertilization rates (Yovich, 1993). PF exerts its effects on sperm motility probably by inhibiting cyclic adenosine monophosphate (cAMP) phosphodiesterase, thus increasing the intracellular cAMP concentration (Garbers et al., 1971). Treatments that increase intracellular cAMP concentration often cause an increase in the agonist -induced acrosome reaction (Tesarik et al., 1992) and fertilzation rate (Yovich, 1993). Therefore, PF is one of the most effective compounds for enhancing sperm motility and inducing capacitation and acrosome reactions in human spermatozoa.

Fertilizing promoting peptide (FPP) is tripeptide (pyroglu-tamylproineamide; pGlu-Gln-ProNH2) related to thyrotrophin releasing hormone (TRH; pGlu -His-ProNH2) that has been identified in several tissues, including the reproductive tract and pituitary gland (Ashworth, 1994). It was initially characterized in the rabbit prostate complex (Cockle et al., 1989) and has subsequently been found in the prostate and/or semen of several mammalian species including humans, rabbits, and rodents (Cockle, 1995). The relative abundance of the tripeptide in human seminal plasma (Cockle et al., 1994) suggested a possible biological role relating to spermatozoa which would come into contact with the peptide at ejaculation. Recent study have demonstrated that nanomolar concentrations of prostatic tripeptide FPP stimulate capacitation and fertilizing ability in mouse (Green et al., 1994) and human sperm (Green et al., 1996a), along with the demonstrable effects of FPP on sperm function. In addition, recent evidence suggests that prostate-derived FPP may play an important role *in vivo* to optimize the fertilizing potential of the few sperm cell that reach the site of fertilization (Fraser, 1998). Indeed, FPP has been shown to elicit a biphasic response in mouse sperm, first stimulating capacitation and then inhibiting spontaneous acrosome loss.

Thus this study was to examine whether the *in vitro* viability, motility and intact acrosome of frozen-thawed bovine and human sperm can be improved by adding PF or FPP.

Ⅱ. MATERIALS AND METHODS

1. Sperm Preparation

Frozen bull semen was obtained from Livestock

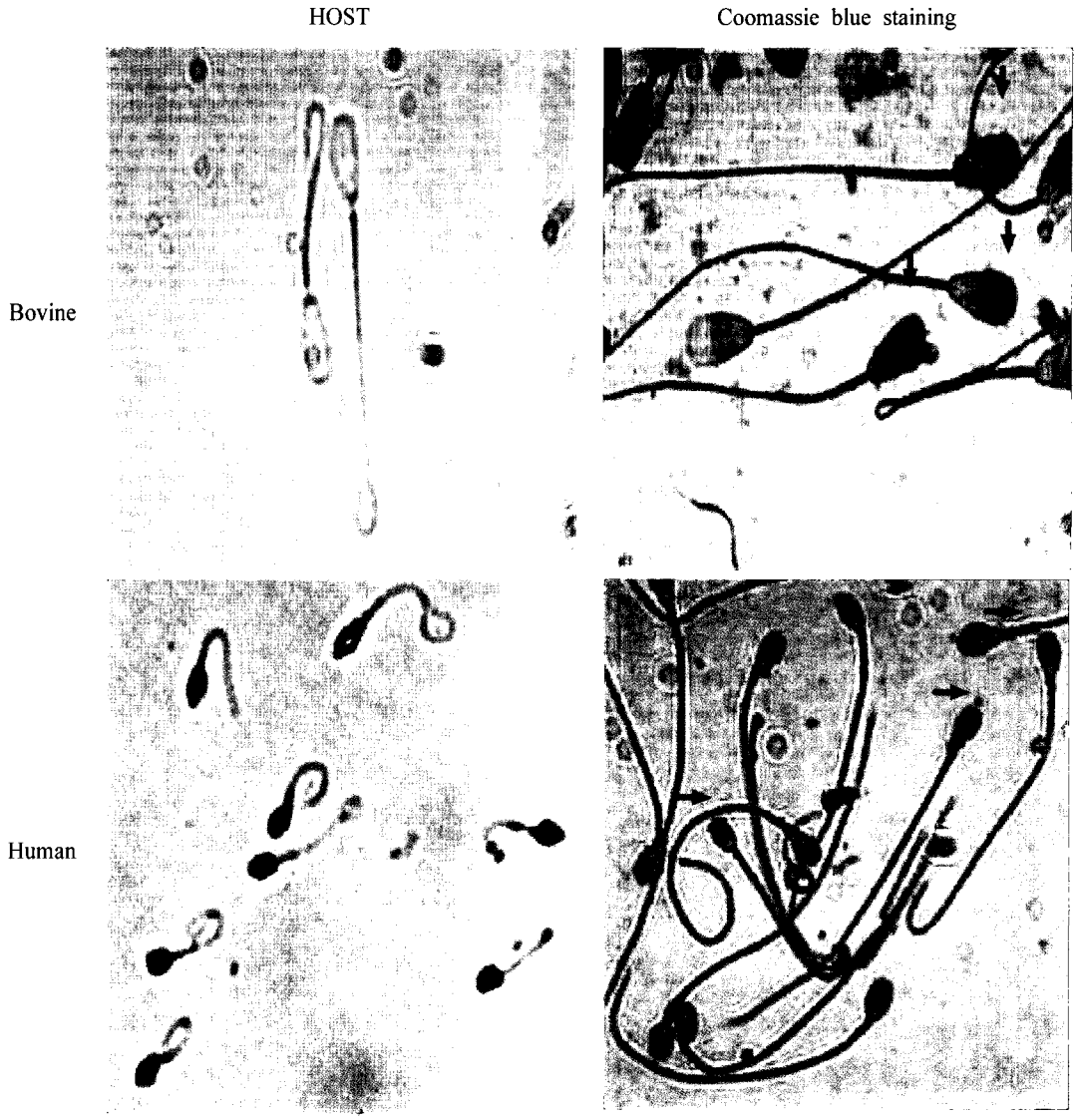

Fig. 1. Hypo-osmotic swelling test for observation of sperm viability and Coomassie blue staining for observation of sperm intact acrosome (arrows indicate acrosome intact sperm).

Improvement Main Center, NLCF. At thawing, the straw was removed from the liquid nitrogen and placed at room temperature for 10 sec. and at 37℃ for 30 sec.. Before experiment, recovered semen was estimated sperm concentration, motility, viability by hypo-osmotic swelling (HOS) test and intact acrosome percentage by coomassie brilliant blue staining method. TALP solution was used for the bovine sperm preparation.

Ejaculated human sperm was allowed to liquefy for 30 min. at 37℃. Raw semen was taken to evaluate sperm concentration (by makler chamber), percentage of motile spermatozoa and normal morphology by strict criteria (Meukveld and Kruger, 1995). And then semen was loaded onto 1 ml aliquots of 80% isotonic percoll (Pharmacia, Uppsals,

Table 1. Effect of pentoxifylline on the motility, viability and intact acrosome of bovine sperm

Pentoxifylline concentraion	0h (%)				3h (%)		
	Sperm count	V	M	A	V	M	A
Control	14×10^6	63.0	55.0	46.0	50.0	34.0^a	28.0
2.5 mM	14×10^6	63.0	55.0	46.0	52.0	41.0^{ab}	31.0
5.0 mM	14×10^6	63.0	55.0	46.0	56.0	50.0^b	40.0
10.0 mM	14×10^6	63.0	55.0	46.0	55.0	44.0^{ab}	35.0

[a,b] Means in the same column without common superscripts are significantly different ($P<0.05$).
V : Viability rate, M: Motility rate, A: Intact acrsome.

Sweden) solution, centrifuged for 20 min. at 3,000 rpm and the sperm pellet was collected re-suspended in 1 ml of Ham's F-10 medium supplemented with 0.3% BSA. After centrifugation at 3,000 rpm for 3 min., the pellet was resuspended in 1 ml of Ham's F-10 medium with 0.3% BSA.

2. Cryopreservation of Human Semen

For human sperm freezing, TYB freezing medium (contained glycerol at a concentration 15% egg yolk, citrate, glycine, glucose and antibiotic) was purchased from Irvine Scientific (Santa Ana, CA, USA). Human sperm was carefully diluted by the addition of droplets in equal volume to the freezing medium TYB. After dilution, the mixed sperm with cryoprotectant was loaded in 0.5 ml french straws (IMV, Paris, France). Briefly, a 2.5 cm length was

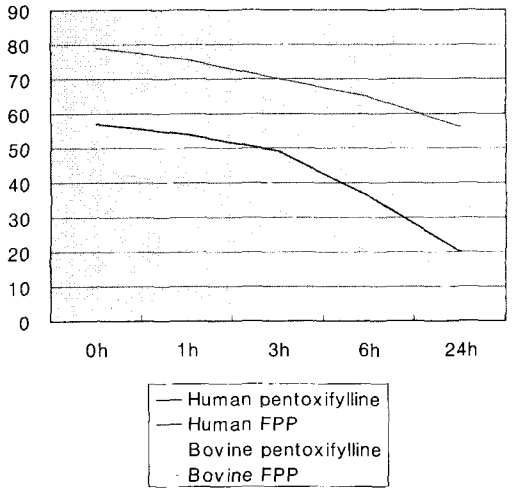

Fig. 2. Changes in motility and intact acrosome of human and bovine sperm suspension incubated for 1, 3, 6 and 24 h in the pentoxifylline and FPP.

Table 2. Effect of FPP on the motility, viability and intact acrosome reaction of bovine sperm after freezing-thawing

FPP concentration	0 h (%)				3 h (%)		
	Sperm count	V	M	A	V	M	A
Control	10×10^6	68.0	57.0	54.0	48.3	40.0	30.0^a
25 nM	10×10^6	68.0	57.0	54.0	46.0	42.5	38.0^{ab}
50 nM	10×10^6	68.0	57.0	54.0	61.5	53.6	49.0^c
100 nM	10×10^6	68.0	57.0	54.0	51.0	49.0	42.0^{bc}

[a,b,c] Means in the same column without common superscripts are significantly different ($P<0.01$).
V: Viability rate, M: Motility rate, A : Intact acrsome

filled with Ham' F-10 followed by a 0.8 cm air bubble, 5.5 cm mixed sperm with cryoprotectant, 0.8 cm air bubble and the remaining part of straw was filled with Ham's F-10. After filling of semen, the 0.5 ml french straws were then rapidly frozen in liquid nitrogen vapor by placing them horizontally 5cm above the liquid nitrogen, nitrogen vapors for 5 min., 2 cm above the liquid nitrogen, nitrogen vapors for 3 min. and finally submersion in liquid nitrogen at −196℃ for long term storage. At thawing, straw was removed from the liquid nitrogen and placed at room temperature for 3 min. thawed at room temperature. Straw from each experiment treatment was estimated sperm concentration, motility, HOS testing and acrosome intact test.

3. Reagents

The semen sample aliquot was treated by adding 2.5 mM, 5.0 mM and 10.0 mM pentoxifylline (Sigma Chemical Company, St Louis, MO, USA, P-1784) for 30 min. at 37℃. One mg FPP (Sigma P-2053) was dissolved in distilled water. Then 10 μl aliquots, containg 10 mM each, were placed in microcentrifuge tubes and stored at −20℃. Also, the semen sample aliquot was treated by adding 25 nM, 50 nM, 100 nM FPP (Sigma P-2053) for 3 h at 37℃.

4. Semen Analysis and Sperm Function Testing

Sperm concentration and motility were assessed according to the methods described by the WHO (1992). Hypoosmotic swelling technique consisted of 0.1 μl of liquefied semen mixed with 1.0 μl of hypo-osmotic solution (7.35 g sodium citrate + 13.51 g fructose in 1000 ml 150 mosm/kg) incubated at 37℃ for 30 min., sperm was then examined for typical tail abnormalities indicative of the occurrence of hypo-osmotic swelling under the phase contrast microscope. Spermatozoa were scored for the presence or absence of swelling in the tail region.

Acrosome function was reaction count using the Coomassiblue staining method. Sperm was fixed with 4% paraformaldehyde solution for 10 min. at 24℃ and centrifuged and washed twice using 1.5 ml of 100 mM ammonium acetate (pH 9.0). The final sperm pellet was resuspended in 1 ml of 100 mM ammonium acetate and 50 μl of the sperm suspension was smeared on slid glass and air dry. Coomassie stain (0.22% Coomassie Blue G-250 + 50% methanol + 10% glacial acetic acid + 40% water) for 2 min. The slides were washed throughly using distilled water to removed excess stain, and slides were air dry and coverslips were place on slides and sealed using mounting solution. The stained sperm was examined under bright filed microscopy. At least 200 spermatozoa per slides were evaluated. Acrosome-intact sperm stained darkly near the apical portion of the sperm head, the location of the sperm acrosome, acrosome-reacted sperm exhibited

Table 3. *In vitro* survival of frozen-thawed human sperm according to equilibration time

Treatment	Pre-freezing (%)			Post-thawing (%)		
	Sperm count	V	M	Sperm count	V*	M
30 min/4℃	12×10^6	64.2	50.0	7×10^6	58.0	48.7
Direct	12×10^6	64.2	50.0	5×10^6	41.6	38.1
10 min/RT	12×10^6	64.2	50.0	6×10^6	50.0	45.2

* No significant difference.

V: Viability rate, M: Motility rate

Table 4. Effect of pentoxifylline on the motility, viability and intact acrosome of human sperm

Pentoxifylline concetration	Pre-freezing (%)				Post-thawing (%) 0 h				Post-thawing (%) 3 h		
	Sperm count	V	M	A	Sperm count	V	M	A	V	M	A
Control	30×10^6	65.0	57.0	78.0	15×10^6	50.0	40.0	58.0	40.5	39.0^a	50.0
2.5 mM	30×10^6	65.0	57.0	78.0	14×10^6	46.6	45.0	55.0	42.0	40.0^a	51.0
5.0 mM	30×10^6	65.0	57.0	78.0	18×10^6	60.0	54.0	53.0	54.0	51.0^b	40.0
10.0 mM	30×10^6	65.0	57.0	78.0	16×10^6	53.0	49.0	50.0	50.0	46.0^{ab}	46.0

[a,b] Means in the same column without common superscripts are significantly different ($P<0.01$).

V: Viability rate, M: Motility rate, A : Intact acrsome

Table 5. Effect of FPP treatment to the motility, viability and intact acrosome of human sperm after frozen-thawed

FPP concetration	Post-thawing (%) 0 h				Post-thawing (%) 3 h		
	Sperm count	V	M	A	V	M	A*
Control	15×10^6	54.0	46.0	61.0	40.4	31.0	39.0
25 nM	15×10^6	54.0	46.0	61.0	41.6	36.0	43.0
50 nM	15×10^6	54.0	46.0	61.0	46.5	40.0	48.0
100 nM	15×10^6	54.0	46.0	61.0	45.0	39.0	45.0

* No significant difference.

V: Viability rate, M: Motility rate, A : Intact acrsome

Table 6. Effect of FPP treatment in all semen freezing procedures to the motility, viability and intact acrosome of human sperm before freezing

FPP concetration	Pre-freezing (%)				Post-thawing (%) 0 h				Post-thawing (%) 3 h		
	Sperm count	V	M	A	Sperm count	V	M	A	V	M	A
Control	24×10^6	72.4	60.0	88.5	11×10^6	46.0	42.0	62.0	37.4	33.2	45.0^a
25 nM	24×10^6	72.4	60.0	88.5	13×10^6	54.0	47.0	64.0	39.0	35.0	53.0^a
50 nM	24×10^6	72.4	60.0	88.5	16×10^6	67.0	54.0	79.0	54.5	50.0	75.5^c
100 nM	24×10^6	72.4	60.0	88.5	15×10^6	63.5	51.0	70.0	43.0	40.0	68.0^b

[a,b,c] Means in the same column without common superscripts are significantly different ($P<0.01$).

V: Viability rate, M: Motility rate, A: Intact acrsome

very faint or no staining in the region of the acro- some.

Table 7. Effect of Pentoxifylline and FPP treatment to the motility, viability and intact acrosome reaction of human sperm after frozen-thawed

FPP concetration	Pre-freezing (%)				Post-thawing (%) 0 h				Post-thawing (%) 3 h		
	Sperm count	V	M	A	Sperm count	V	M	A	V	M	A
Control	32×10^6	66.0	59.0	80.0	15×10^6	46.6	45.0	60.0	39.0	38.0[a]	50.0[a]
Pentoxifylline	32×10^6	66.0	59.0	80.0	17×10^6	53.0	51.0	59.0	47.0	46.0[b]	43.0[a]
FPP	32×10^6	66.0	59.0	80.0	19×10^6	59.0	50.0	71.0	55.0	42.0[b]	65.0[b]

[a,b] Means in the same column without common superscripts are significantly different ($P<0.05$).
V: Viability rate, M: Motility rate, A: Intact acrsome

5. Statistics

Statistical analyses were performed using GLM (General Linear Model) procedure of SAS, Categorical data was analyzed using Duncan's Multiple Range Test to determine statistically significant differences between means.

Ⅲ. RESULTS

When the *in vitro* motility and intact acrosome of frozen-thawed bovine and human sperm according to the culture time after PF and FPP treatment were examined, the motility and intact acrosome rates in all treatment groups were tend to decrease fastly from 3 h. Thus, the assessment of PF and FPP treatment on bovine and human sperm was taken at 3 h after incubation. When the *in vitro* motility of frozen-thawed bovine sperm by PF addition of various concentration was examined, the result of 5 mM treatment group (50.0%) was significantly higher than that of control (34.0%), although there are not different among the PF treatment groups ($P<0.05$). Also, when the effect of FPP treatment on the frozen-thawed bovine sperm was examined, the intact acrosome rate of 50 nM treatment group (49.0%) was significantly higher than those of control (30.0%) and 25 nM (38.0%) treatment group ($P<0.01$). When the effect of pre-freezing equilibration time on the *in vitro* survival of frozen-thawed human sperm was examined, the viability and motility of 30 min./4℃ group (58.0, 48.7%) was higher than those of the other treatment groups (Direct: 41.6, 38.1%; 10 min/RT: 50.0, 45.2 %), although there are not significantly different. In human sperm, when *in vitro* motility according to PF addition prior to freezing was examined, the result of 5 mM treatment group (51.0%) was significantly higher than those of control and 25 mM treatment group (39.0, 40.0%) ($P<0.01$). In addition, although the significant effect of FPP addition in frozen-thawed human sperm was not found among the treatment groups including control group, 50 nM (75.5%) FPP adding in all treatment procedures for human semen freezing (before freezing, freezing and after thawing) was significant effect on maintenance of the sperm intact acrosome percentage (control: 45.0; 25 nM: 53.0; 100 nM: 68.0%) ($P<0.01$). Based on these data, when the additive effects of PF and FPP on sperm motility and intact acrosome of cryopreserved human sperm were compared simultaneously, intact acrosome rate in FPP treatment group (65.0%) was significantly higher than that in PF treatment group (43.0%) ($P<0.05$), although sperm motility was slightly higher in PF treatment group.

Ⅳ. DISCUSSION

The results from the study demonstrated that the more improved sperm motility and intact acrosome of frozen-thawed bovine and human sperm can be obtained by addition of 5 mM PF or 50 nM FPP. Especially, we found that intact acrosome of human sperm is highly maintained by addition of FPP in all semen freezing procedures (pre-freezing, freezing, post-thawing).

Generally, it has been known that sperm cryopreservation results is adverse changes in sperm. Thus, to maximize the chances of achieving successful fertilization, the few sperm to reach the oviduct should be capacitated but remain acrosome intact until they encounter an oocyte. Sperm that undergo spontaneous acrosome loss at some distance from an oocyte will no longer be fertile, even if they retain motility (Yanagimachi, 1994). A lot of efforts to improve the fertilizing ability of cryopreserved spermatozoa are clinically useful in human. However, despite the availability of sophisticated assisted reproductive techniques, the cryosurvival rates, and pregnancy rates with frozen semen are lower than those with fresh semen (Polanski and Lamb, 1988). In this study, to investigate *in vitro* motility of frozen-thawed human sperm, when PF of various concentration prior to freezing was examined, the result of 5 mM treatment group (51.0 %) was significantly higher than those of control and 2.5 mM treatment groups (39.0, 40.0%) ($P< 0.01$). This result was similar to that of PF addition in frozen-thawed bovine sperm. In addition, prostate -derived FPP has been proposed to play an important role in regulating mammalian sperm fertilizing ability *in vivo* (Fraser, 1998). Current evidence indicated that sperm acquire inhibitory surface associated molecules (decapacitation factors) of an either epididymal or seminal plasma origin which are then lost during capacitation, resulting in the acquisition of fertilizing ability. Like decapacitation factors, FPP may well bind to the sperm cells when they contact seminal plasma and remain bound following ejaculation. Funahashi et al., (2000) have demonstrated that boar sperm do respond to FPP ($\geq$12.5 mM) *in vitro*. In caffeine-free medium, exogeneous FPP stimulated capacitation and inhibited spontaneous acrosome loss. This apparent conservation of the FPP receptor, despite the low endogenous levels of ligand, suggests that it is part of a functionally important pathway in mammalian sperm.

In human, FPP (25～100 nM) induced a significant increase in the capacitated sperm, and a significant decrease in the proportion of uncapacitated ones without significant stimulation of acrosomal exocytosis (Kang et al., 2000). Given that the stimulation of capacitation occurred at a range of concentrations similar to the concentration of FPP detected in human seminal plasma (～50 nM; Cockle et al., 1994), In our study, when the frozen -thawed bovine sperm was examined according to treatment FPP concentration, the intact acrosome rates of 50 nM treatment group (49.0%) were significantly higher than that of control group (30.0%) ($P<0.01$). Especially, 50 nM (75.5%) FPP adding in all treatment procedures for human semen freezing (before freezing, freezing and after thawing) was significant effect on maintain the sperm intact acrosome percentage (control: 45.0; 25 nM: 53.0; 100 nM: 68.0%) ($P<0.01$). Also, when the additive effects of PF and FPP on sperm motility and intact acrosome were compared simultaneously, intact acrosome rate in FPP treatment group (65.0 %) was significantly higher than that in PF treatment group (43.0%) ($p<0.05$), although sperm motility was slightly higher in PF treatment group. However, the effects of PF or FPP added in sperm freezing procedures on fertilizing ability *in vivo* in clinics may be needed the more complicated further

study.

In conclusion, improved sperm motility and intact acrosome maintenance of cryopreserved bovine and human sperm can be obtained by addition of chemical agents PF or FPP. Also, in human sperm, the significantly high *in vitro* viability, motility and intact acrosome can be obtained by addition of FPP in all semen freezing procedures (freezing, thawing and after thawing).

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요 약

Fertilizing Promoting Peptide와 Pentoxifylline으로 처리된 소와 사람 동결 정액의 수정능 향상

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본 실험은 PF과 FPP가 소 와 사람 동결 정자에 첨가되었을 때 융해 후 정자의 체외 생존성, 운동성 그리고 intact acrosome 향상에 기여할 수 있는지의 여부를 조사하고자 실시하였다. 사람의 정액은 TYB 동결 배양액을 사용하여 초 급속 동결하였다. PF과 FPP 첨가 효과는 각각의 시약이 동결-융해된 소나 사람 정자의 현미경적 조사에 의한 운동성에 미치는 영향과 Coomassie brilliant blue 염색방법에 의한 intact acrosome의 비율에 미치는 영향으로 조사하였다. Bovine 동결 융해 정자에 PF을 첨가하여 운동성을 조사하였던 바, 5 mM 처리군 (50.0%)이 대조군 (34.0%) 보다 유의하게 높은 운동성을 보여주었다 ($P<0.05$). 동결 융해된 소 정자에 FPP를 농도에 따라 처리하여 intact acrosome을 조사하였던 결과, 50 nM 처리군 (49%)이 대조군과 25 nM 처리군 (30.0, 38.0%) 보다 유의하게 많은 intact한 acrosome을 유지하였다 ($P<0.01$). 사람 정자에서 동결에 앞서 PF을 농도에 따라 첨가하여 동결 융해 후 운동성을 조사한 결과, 5.0 mM 처리군 (51.0%)이 대조군과 2.5 mM (39.0, 40.0%) 처리군의 운동성보다 높았다 ($P<0.01$). 사람 정액의 모든 동결 처리과정 (동결전, 동결, 융해후)에서 50 nM (75.5%) FPP 첨가가 intact acrosome percentage 유지하는데 유의한 효과가 있었다 (대조군: 45.0; 25 nM: 53.0; 100 nM: 68.0%) ($P<0.01$). PF와 FPP 첨가하여 사람 정자의 동결융해 후 운동성과 intact acrosome에 미치는 영향을 동시에 비교해본 결과, 운동성에서는 PF 처리군이 약간 높지만 intact acrosome rate는 FPP 처리군의 결과 (65.0%)가 PF 처리군 (43.0%)보다 유의하게 높았다 ($P<0.05$). 따라서 본 실험은 동결-융해된 소 정자에 PF이나 FPP 첨가는 정자의 운동성이나 intact acrosome 비율을 좀더 개선시킬 수 있고, 특히 사람 정자는 동결 전 과정에 FPP를 첨가하는 것이 정자의 체외 생존성, 운동성 그리고 intact acrosome을 유의하게 향상시킬 수 있다는 것을 시사한다.

(접수일자 : 2001. 10. 25. / 채택일자 : 2001. 11. 20.)