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Effect of Fertilization Promoting Peptide (FPP) on the Acrosome Status of Cryopreserved Human Sperm

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Fertilization Promoting Peptide (FPP)가 동결 보존 사람정자의 첨체 상태에 미치는 영향

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연구목적: 정자의 첨체상태는 수정능과 상관관계가 있다. 본 연구는 사람 정자의 동결보존 시 Fertilization promoting peptide (FPP) 처리가 첨체 유지에 효과가 있는지를 알아보고자 실시하였다.

연구재료 및 방법: 사람 정자는 정액검사를 의뢰한 시료를 사용하였으며, 적정농도를 조사하기 위하여 25, 50, 100 nM FPP를 신선정자에 처리한 뒤 시간별로 첨체의 변화를 조사하였다. 또한 적정화된 50 nM FPP를 정자의 동결-융해 시에 처리한 뒤 첨체 변화를 조사하였다. 첨체 변화는 FITC - pisum sativum lectin (PSA) 염색방법을 이용하여 조사하였다.

결 과: FPP 농도 변화와 처리시간에 따른 사람 정자의 첨체 변화를 조사하였던 바, 50 nM FPP 처리군에서 대조군보다 높은 온전한 첨체비율을 얻을 수 있었다. 정자의 동결-융해 시, 동결액과 융해액에 50 nM FPP 첨가가 온전한 첨체를 유지하는 비율을 조사하였던 바, 신선 정자의 결과보다는 유의하게 낮지만 무 처리군보다 유의적으로 높은 온전한 첨체를 얻을 수 있는 것을 알 수 있었다. 또한 동결액에만 또는 융해액에만 50 nM FPP 처리를 하더라도 무 처리군보다 유의하게 높은 온전한 첨체 비율을 획득할 수 있음을 알 수 있었다 ($p<0.001$).

결 론: 사람 정자의 동결보존 시 50 nM FPP 첨가는 자발적으로 발생하는 첨체반응을 억제하고, 온전한 첨체를 유지할 수 있어 수정능 보유에 기여할 수 있을 것으로 사료된다.

Key Words: Human sperm, Fertilizing promoting peptide (FPP), Acrosome, FITC-PSA

Cryopreservation of human sperm is widely used in assisted conception units to preserve male gamete and provide the opportunity for future fertility. However, it has been known that current procedures of cryopreservation have detrimental effects on the metabolism and motility of spermatozoa, as well as on the status of sperm membrane domains[1,2] and activity of acrosome protease and acrosin.[3~5] The structural damage is principally in the peripheral sperm membrane and quite often there is considerable damage to the acrosome with the leakage of the acrosome membrane and contents.[4,5] The fertilizing ability of human spermatozoa is more reduced after cryopreservation, which can be

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explained partially by a reduction in the percentage of normal intact acrosomes and in total acrosin activity.[3] Therefore, lower pregnancy rates have been reported both in intrauterine insemination and in vitro fertilization/ intracytoplasmic spermatozoa injection programmes using cryopreserved spermatozoa compared with fresh semen.[6] Recently, it was demonstrated that progesterone, platelet activating factor, fertilization promoting peptide (FPP) and adenosine, are rapid and potent activator of human sperm fertilizing ability. Especially, FPP (pGlu-Glu-ProNH_2), structurally similar to thyrotrophin releasing (pGlu-His-ProNH_2), is produced by the prostate gland and secreted into seminal plasma.[7,8] The relative abundant of this tripeptide in human seminal plasma (49.5±10.3 nM) suggests possible biological activity relating to spermatozoa which would be exposed to the peptide at ejaculation.[9] *In vivo*, a mechanism that would promote capacitation but inhibit spontaneous acrosome loss and help to maximize the number of functionally competent spermatozoa capable of undergoing acrosomal exocytosis. FPP probably binds to sperm when they contact seminal plasma and then remains bound for some time, exerting a stimulatory effect on capacitation as spermatozoa move up through the female tract.[10]

This study was to investigate whether the FPP treatment on spermatozoa during freezing and thawing makes effect reducing spontaneous acrosome loss using FITC-PSA staining.

MATERIALS AND METHODS

1. Semen sample

Twenty-five seman samples were obtained by masturbation following at least 3 days of abstinence from the healthy men. After liquefaction, routine semen analysis was performed according to World Health Organization guidelines (WHO, 1999). Sperm morphology was determined using the strict criteria laid down by Kruger et al.[11] Only normal ranged sperm were used.

2. Medium and Reagents

The basic medium for sperm incubation was Ham's F-10 containing 0.3% BSA. FPP, FITC-PSA, calcium ionophore A23187, and bis-benzimide (Hoechst 33342) were supplied by Sigma chemical company and their stock solutions were prepared into 1 μM, 1 mg/ml, 5 mM and 1 mg/ml, respectively. TEST yolk-buffer freezing medium was purchased from Irvine Scientific (Santa Ana, CA, USA).

3. FITC-PSA staining and acrosome status assessment

Acrosome status was assessed in only viable sperm with FITC-PSA stain, in conjunction with bis-benzimide stain. For the viability check, sperm suspension was treated with 10 μg/mL Hoechst 33342 for 7 min. Sperm were then washed in PVP-40 solution by centrifugation at 600 g for 5 min. A 10 μl aliquots of this suspension was smeared on a slide glass and allowed to air-dry. Air-dried slides were immersed in ice-cold 95% ethanol for 5 min and allowed to air dry. The fixed smears were covered with a 100 μl drop of 100 μg/ml FITC-PSA and placed in a dark humidified chamber at 4℃ for 15 min. Slide was washed thoroughly using distilled water to remove excess stain. A drop of 0.22 M 1,4-diazabicyclo [2,2,2] octan (Sigma) was used to retard fading of fluorescence. Assessment was carried out under a Nikon epifluorescence microscope. To begin with, sperm were confirmed live and dead status using ultraviolet filter and then subsequently acrosome status of viable sperm was assessed using green filter. A minimum of 10 fields per slide (>100 sperm) were observed.

4. Experimental design

Experiment 1. Effect of 25, 50 and 100 nM FPP on fresh human sperm

To investigate the effect of FPP concentration and treatment time variation to the acrosome status, fresh and normal ranged motile sperm were obtained using 90% percoll column and centrifugation at 600 g for 5 min. For each treatment, at least 2×10^5/ml motile sperm suspension was prepared. Each sample was divided into five aliquots, for control as no treatment, Ca^{2+} ionophore treatment as negative control of acrosome reaction, 25 nM FPP, 50 nM FPP and 100 nM FPP treatment. All treatment groups were incubated for 1, 3, 6 and 24 h in 37℃, 5% CO_2 incubator and then their acrosome status was assessed. Calcium ionophore-induced AR was measured using control sperm at each time. For each ionophore challenge, sperm suspension was divided into two aliquots. One was challenged with 10 µM A23187 stock, and the other was treated with DMSO working solution to serve as a control. Both aliquots were then incubated for 30 min under the same conditions.

Experiment 2. Effect of FPP treatment on the cryopreserved human sperm

For freezing, liquefied semen was layered on top of the 90% percoll and centrifuged at 600 g for 20 min. The final sperm pellet was suspended in a suitable volume of Ham's F-10 (5×10^6 spermatozoa/ml). This suspension was divided into two aliquots. An aliquots from each sample was diluted (1:1) with TYB freezing medium which was slowly added dropwise. The one suspension was received 50 nM FPP (+/) and the other was remained 50 nM FPP-deficient (−/). Before an equilibration period at 4℃ for 30 min, the mixture was aspirated into 0.25 ml straw, which were powder sealed. The straws were frozen in liquid nitrogen vapour and then plunged into liquid nitrogen. For dilution, straws were removed from liquid nitrogen and left until thaw at room temperature. When samples were totally thawed, 3 ml of Ham's F-10 medium was added to cornical tube and the sperm were centrifuged at 600 g for 5 min to remove any traces of TYB freezing medium. The supernatant was removed and the pellet was resuspended in Ham's F-10. Two suspensions were subdivided into two groups, one receiving 50 nM FPP (+/+ and −/+) and the other remaining FPP-deficient (+/− and −/−); all treatment groups were incubated for 3 h in 37℃, 5% CO_2 incubator and then they were stained using bis-benzimide and FITC-PSA for assessment.

5. Statistical analysis

Statistical analyses of FPP treatments in four or five replicate experiments for treatment comparisons were carried out by Duncan's multiple range test. Data are expressed as mean ± SEM and $p<0.05$ was considered to be statistical significance.

RESULTS

Experiment 1. Effect of 25, 50 and 100 nM FPP on fresh human sperm

When modulation effect of FPP concentration (25, 50 or 100 nM) and treatment time (1, 3, 6 and 24 h) to the sperm acrosome status was examined, as shown in Table 1, there were significant differences among the treatment groups ($p<0.001$). Especially, 50 nM FPP treatment group has presented the most stable intact acrosome sperm (1 h; 87.25%, 3 h; 79.50%, 6 h; 73.00% and 24 h; 63.75%). It means 50 nM FPP help inhibition of spontaneous acrosome loss of sperm. As negative control, Ionophore A23187 treatment produced the lowest acrosome intact sperm by artificial induction of acrosome reaction.

Table 1. Effect of FPP concentration and treatment time on the acrosome status of fresh human sperm

Treatment time (h)	% of intact acrosome sperm in treatment group*				
	Control	Ionophore A23187	FPP conc.		
			25 nM	50 nM	100 nM
1	81.00[a]	53.00[b]	86.00[a]	87.25[a]	75.00[a]
3	79.75[a]	48.00[c]	67.50[b]	79.50[a]	65.00[b]
6	65.50[b]	36.50[c]	67.00[b]	73.00[a]	62.00[b]
24	64.50[a]	30.50[c]	61.50[a]	63.75[a]	56.00[a,b]

*Acrosome intact level was counted using FITC-PSA staining and dead sperm count was excluded by Hoechst staining.
a-d Values within the rows with different superscripts differ ($p<0.001$)

Table 2. Effect of 50 nM FPP on the acrosome status of cryopreserved human sperm

Control*	% of intact acrosome sperm in treatment group			
	FPP (freezing/thawing)**			
	−/−	−/+	+/−	+/+
88.30[a]	50.20[d]	61.00[c]	64.20[b,c]	71.94[b]

*Control means sperm before freezing.
**− or + means no treatment or treatment 50 nM FPP during freezing or/and thawing of sperm.
a-d Values within the row with different superscripts differ ($p<0.001$)

Experiment 2. Effect of FPP treatment on the cryopreserved human sperm

When the effect of 50 nM FPP treatment during freezing or/and thawing procedures was examined, as shown in Table 2. even it was added, intact acrosome percentages of cryopreserved sperm were significantly decreased than that of control ($p<0.001$). Among the treatment groups, the best result was obtained in freezing and thawing all treatment group (+/+, 71.94%). However, one-side treatment groups (−/+, 61.00%; +/−, 64.20%) between freezing and thawing procedure were also indicated significantly better effect to maintain intact acrosome than that of no treatment group (−/−, 50.20%) ($p<0.001$). However, dead sperm count was excluded from FITC-PSA count.

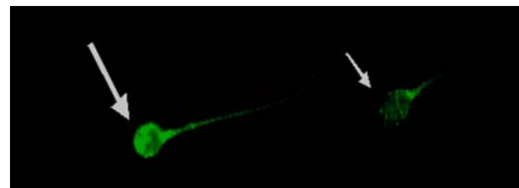

Figure 1. FITC-PSA stained acrosome intact sperm are discernible from acrosome reacted sperm. Large and small arrows indicated acrosome intact and reacted sperm, respectively (×1000).

DISCUSSION

This study demonstrated that the FPP treatment on sperm during freezing and thawing procedures function to reduce spontaneous acrosome loss through the pseudo-effect similar to seminal plasma. In fertilization, only spermatozoa with normal intact acrosomes can undergo acrosome reaction

(AR), which is essential for the spermatozoa to penetrate the zona pellucida (ZP) and to fuse with the oolemma. Also, the AR must occur at the correct time for the spermatozoa to be able to penetrate the ZP. The fertilizing ability of human spermatozoa is reduced after cryopreservation, which can be explained partially by a reduction in the percentage of normal intact acrosomes and in total acrosin activity. However, there were a number of different procedures utilizing different freezing protocols and cryoprotectants have been proposed, cryopreservation impairs sperm motility, viability, penetration into cervical mucus, penetration of zona-free hamster eggs, acrosome structure and activity of acrosome protease and acrosin.[3,12~14] For these reasons, conception rates with frozen-thawed spermatozoa are inferior to those obtained with freshly ejaculated specimens.[15] In this study also indicated that no treatment of FPP during freezing and thawing procedures makes significantly low acrosome intact percentage of sperm compared to treatment. It proved that FPP treatment function the reducing activity spontaneous acrosome reaction with preserving fertilization ability as shown in Figure 1.

Prostate-derived FPP has been proposed to play an important role in regulating mammalian sperm fertilizing ability *in vivo*.[16] FPP can stimulate capacitation and fertilizing ability in vitro in both epididymal mouse and ejaculated human spermatozoa.[8,15] These results suggest that FPP promotes the acquisition of fertilizing ability without pushing them into the acrosome reacted state where fertility would be lost. This apparent ability of FPP to stimulate early events in capacitation but to inhibit spontaneous acrosomal exocytosis. Current evidence indicates that spermatozoa 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.[17] Like decapacitation factors, FPP may well bind to the sperm cells when they contact seminal plasma and remain bound following ejaculation. In the female tract, decapacitation factors are lost, leaving FPP to exert a stimulatory effect on capacitation.

In this study, we investigated the effect of FPP on the functional status of ejaculated and frozen-thawed human spermatozoa *in vitro*. It has reported that FPP can significantly stimulate mouse sperm capacitation only at concentrations ≥25 nM.[8] However, in this study, we confirmed the significant response at 50 nM FPP treatment to inhibit of the spontaneous acrosome reaction, similar to the concentration of FPP detected in human seminal plasma (~50 nM).[9] Because of the relatively high concentrations of FPP found in seminal plasma, FPP may provide human spermatozoa with a stimulus that promotes capacitation following deposition in the female tract. In some men, impaired fertility might reflect insufficient FPP or a defective FPP-sperm interaction.

Therefore, this study indicated that a beneficial effect of FPP treatment on the acrosome status of post-thaw sperm, which is represented into the reducing activity spontaneous acrosome reaction with preserving fertilization ability.

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