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# The Effect of Seminal Plasma on Chilling and Freezing of Canine Spermatozoa

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**Abstract :** Seminal plasma (SP) is usually removed from semen that is to be cryopreserved. However, some reports indicate that SP has beneficial effects on spermatozoa during chilling and freezing. The purpose of this study was to determine the effect of SP on sperm survival by adding SP to the extender before cooling and freezing canine spermatozoa. In replicate experiments, ejaculates obtained from four healthy dogs (1-4 years old) of various breeds were pooled, centrifuged at 300×g for 10 min at 25°C, and the supernatant of seminal plasma was decanted. Spermatozoa were suspended in egg yolk-Tris (EYT) buffer. The study comprised two experiments: [Exp I] Sperm were suspended in EYT extender containing either 0, 20, 40, 80 or 100% SP and were slowly cooled to 4°C for 2 h or held at 25°C as controls. Sperm concentration was adjusted to $2\times10^8$/ml. [Exp II] Sperm samples, each of which contained $1\times10^8$/ml, were assigned to nine groups to be frozen. In the first four groups, sperm in EYT containing either 20, 40, 80 or 100% SP were cooled to 4°C, then diluted to contain final concentrations of EYT + 0.6 M glycerol and then were frozen. The final concentrations of SP were 10, 20, 40 or 50%. In the other four groups, sperm in EYT alone were first cooled slowly to 4°C, then diluted to contain final concentrations of EYT + 0.6 M glycerol plus 10, 20, 40 or 50% SP and then were frozen. Spermatozoa, which chilled in EYT alone and diluted to contain final concentrations of EYT + 0.6 M glycerol without seminal plasma, and then frozen, was regarded as control. Spermatozoa were frozen at 25°C/min of cooling rate in plastic straws that were suspended above liquid nitrogen and thawed in water at 38°C for 1 min. Sperm survival was assayed by determining progressive motility and integrity of plasma and acrosome membranes. Progressive motility was determined by microscopic examination at 200× magnification. Membrane integrity was assessed by use of a double fluorescent dye, and acrosome integrity by staining sperm with *Pisum sativum agglutinin*. The results of the first experiment showed that adding SP did not improve motility of spermatozoa compared to those incubated without SP regardless of temperature. The results of the second experiment showed that spermatozoa suspended in EYT + 0.6 M glycerol containing SP exhibited the higher progressive motility before being frozen ($P<0.05$). However, frozen-thawed spermatozoa that had suspended in EYT + 0.6 M glycerol containing SP showed the similar or lower viability ($P<0.05$). In summary, although seminal plasma did not affect spermatozoa that were chilled in EYT without cryoprotectant (CPA), addition of seminal plasma to EYT containing CPA did significantly improved progressive motility of canine spermatozoa that were chilled.

***Key words*** : canine spermatozoa, seminal plasma, chilling, freezing, sperm survival.

## Introduction

Semen is composed of spermatozoa and seminal plasma. Seminal plasma (SP) is usually removed from semen during processing for cryopreservation since seminal plasma was unnecessary for cell survival (11,21). Gadella *et al.* (11) also suggested that seminal plasma had an deleterious effect on liquid-stored boar spermatozoa by deterioration of spermatozoa. These negative effects are due to a capacitation-inducing effect of the seminal plasma (12,34). Additional detrimental effects of seminal plasma on sperm survival after freezing and thawing have been reported (6,28,31) On the other hand, seminal plasma has been reported to benefit human and stallion cryopreserved spermatozoa (1,5,15,21). Head-to-head agglutination by dilution with extender can be reduced by addition of seminal plasma to medium (18). Seminal plasma has improved motility of boar, ram, and bull spermatozoa (18,25,29). Moreover, seminal plasma has beneficial effects on spermatozoa during chilling and freezing (4,16,17,21,37). Resistance to cold-shock of spermatozoa maintained by their own seminal plasma prior to its removal for cryopreservation was increased (9,33). The cold shock-damaged ram sperm membrane was reversed by incubation with a solution containing proteins in ram seminal plasma (2). Adding seminal plasma back to spermatozoa prevented premature sperm capacitation, which occurs during cryopreservation (10,21,23). Binding capacity to zona pellucida was enhanced by exposure of the sperm to seminal plasma (12).

The effects of seminal plasma on sperm fertility has been conflicting as mentioned above. The study on effect of canine seminal plasma has been limited to artificial insemination using prostatic fluid (14,24). Therefore, the final purpose

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of our study was to determine the effect of seminal plasma on survival of canine spermatozoa being cooling and freezing. The one objective was to determine if adding seminal plasma back to extender improved motility, membrane integrity (viability) and acrosome integrity of spermatozoa maintained at 4°C or 25°C. The other objective was to determine if adding seminal plasma back to extender improved survival of spermatozoa chilled or frozen-thawed.

## Materials and Methods

### Animals

Four dogs (1 mixed breed, 2 beagles, 1 schnauzer; ages ranging from 1 to 4 years) were used in this study. All males were housed at the College of Veterinary Medicine, Chonbuk National University.

The dogs were caged individually and were provided water ad libitum and dog food formulated (Puppy chow®, Purina) twice daily.

### Semen collection and seminal plasma preparation

One ejaculate from each dog was collected by manual stimulation. The first and second fraction was collected to minimize the amount of prostatic fluid (the third fraction). Ejaculates were pooled and centrifuged at 300 ×g for 10 min at 25°C. The supernatant was decanted and centrifuged at 300 ×g for 5 min to remove any remaining spermatozoa. The supernatant was used as seminal plasma for the experiment.

### Sperm extender

The Egg Yolk-Tris (EYT) solution was used as an extender. The extender was composed of EYT/1 and EYT/2. EYT/1 was prepared as described by Hermansson and Linde Forsberg (13). EYT/2 was composed of EYT/1 and 1.2 M glycerol. Respective pH of EYT/1 and EYT/2 was 7.3 and mean osmolalities of EYT/1 and EYT/2 were 389 and 989, respectively.

### Sperm freezing and thawing

A Styrofoam box (outside dimensions [l, w, h]: 52 ×34 × 47 $cm^3$; inside dimensions: 46 ×25 ×28.5 $cm^3$) and Styrofoam plate (19×14.5×4.8 $cm^2$) were used to achieve 25°C/min of cooling rate. The cooling rate was measured 5 times using a thermocouple (Model 91100, Cole-Parmer, USA) inserted into straws containing EYT/2 but no spermatozoa. The Styrofoam box was filled with Liquid nitrogen ($LN_2$) to a depth 3.5 cm and the plate was floated on the surface of the $LN_2$.

For sperm cryopreservation, hundred micrometers of diluted spermatozoa were loaded in 0.25 ml straw. Straws were aligned horizontally for 15 min on the plate and plunged into $LN_2$.

The frozen spermatozoa were thawed for 1 min in a water bath at 38°C.

### Sperm concentration

Sperm concentration was determined using a Neubauer haemocytometer.

### Sperm motility

Progressive sperm percentage was assessed subjectively by microscopic examination at a magnification of 200× using a scale of 0~4 described by Mortimer (22). Mean motility (more than a scale of 3) and viability of pooled semen at collection were 85.0 ± 1.5 (%, mean ± SEM) and 93.2 ± 2.1 (%, mean ± SEM), respectively. Mean acrosome integrity of pooled semen at collection was 98.8 ± 0.2 (%, mean ± SEM).

### Sperm membrane integrity (viability)

The integrity of sperm plasma membranes was measured by the method of Yu and Leibo (35) using the fluorescent double stain Fertilight® (Molecular Probes Inc., Eugene, OR). For each replicate sample, two slides were prepared, and appropriately 200 spermatozoa were counted for each slide. The number of spermatozoa with green or red fluorescence was counted, and the percentage of membrane-intact spermatozoa (green fluorescence on sperm head) was calculated.

### Sperm acrosome integrity

Acrosome integrity was determined using a method from that described by Yu and Leibo (35). Spermatozoa were stained with *Pisum sativum agglutinin* (PSA) conjugated to fluorescein isothiocyanate (FITC). For each replicate sample, two slides were examined, and approximately 200 spermatozoa were counted for each slide. Percentage of spermatozoa with intact acrosome (green fluorescence on sperm anterior acrosomal region) was calculated.

### Experimental designs

Experiment 1: The effect of seminal plasma on survival of spermatozoa maintained at 4°C or 25°C.

The spermatozoa were allocated into five treatment groups and each group was diluted to $2 \times 10^8$/ml in EYT/1 containing 0, 20, 40, 80 or 100% SP (V/V). Diluted spermatozoa were transferred into 1.5 ml tube and chilled to 4°C or held at 25°C for 2 h. Sperm survivals were assayed by determining motility, membrane integrity, and acrosome integrity.

Experiment 2: The effect of seminal plasma on survival of spermatozoa chilled and frozen-thawed.

The spermatozoa were allocated into nine treatment groups. The spermatozoa in the first four groups were diluted to $2 \times 10^8$/ml in EYT/1 containing 20, 40, 80 or 100% SP (V/V) and then diluted with equal volume of EYT/2 + 1.2 M glycerol. The spermatozoa in the second four groups were diluted to $2 \times 10^8$/ml in EYT/1 without SP and then diluted with equal volume of EYT/2 + 1.2 M glycerol containing 20, 40, 80 or 100% SP (V/V). Diluted spermatozoa were equilibrated at 4°C for 1 h after first dilution and second dilution, respectively. Spermatozoa chilled in EYT/1 alone and then diluted in EYT/2 without seminal plasma was regarded as control (SP 0%). The final sperm concentration was $1 \times 10^8$/ml and final SP concentrations of experimental groups were 10, 20, 40, and 50%, respectively. Spermatozoa were frozen and thawed using the method described above. Sperm survivals were assayed by

determining motility, membrane integrity, and acrosome integrity.

**Statistical analysis**

For the result of the first experiment, the mean percentages of motility, membrane integrity, and acrosome integrity between experimental groups were compared by paired t-test. The mean percentages of motility, membrane integrity, and acrosome integrity among experimental groups were compared by One-way Analysis of Variance (ANOVA). For the result of the second experiment, the mean percentages of motility, membrane integrity, and acrosome integrity among experimental groups were compared by One-way Analysis of Variance (ANOVA). The analyses were performed using GraphPad InStat (GraphPad Software, San Diego, CA, USA). Differences were considered significant when $P < 0.05$.

## Results

In the first experiment, canine spermatozoa diluted with EYT/1 containing 0, 20, 40, 80 or 100% were chilled at 4°C or held at 25°C for 2 h. Sperm motility among experimental groups did not differ significantly regardless of holding temperatures (Fig 1A).

Motility of spermatozoa in EYT/1 without containing seminal plasma was lower than those of spermatozoa in EYT/1 with containing seminal plasma at 4°C. In comparison of sperm motilities among experimental groups at 25°C, motility of spermatozoa in EYT/1 alone was similar to that in 100% seminal plasma. Sperm motilities in the rest of experimental groups at 25°C were lower than those of experimental groups mentioned above. There was no significant difference between 4°C and 25°C, although motility of spermatozoa in EYT/1 alone at 25°C was higher than that of spermatozoa at 4°C in EYT/1 without containing seminal plasma.

The results of membrane integrities between 25°C and 4°C showed no significant differences. There was no significant differences among experimental groups without regard to concentration of seminal plasma (Fig 1B).

Acrosome integrity was not significantly different among experimental groups no matter what concentration of seminal plasma and holding temperature were (Fig 1C).

In the second experiment, spermatozoa were allocated into nine groups. In four groups, the spermatozoa in EYT/1 containing 20, 40, 80 or 100% seminal plasma were chilled to 4°C for 1 h, diluted with EYT/2, and then cryopreserved. In other four groups, spermatozoa in EYT/1 alone were chilled to 4°C for 1 h, diluted in EYT/2 containing 20, 40, 80 or 100% seminal plasma, and then were frozen. Spermatozoa chilled in EYT/1 alone and then diluted in EYT/2 without seminal plasma was regarded as control.

The results of sperm survival before being frozen are shown in Fig 2. Compared among groups, in which seminal plasma was added back to spermatozoa in the first dilution and then diluted in EYT/2, motilities in all groups diluted in EYT/1 containing seminal plasma were significantly higher

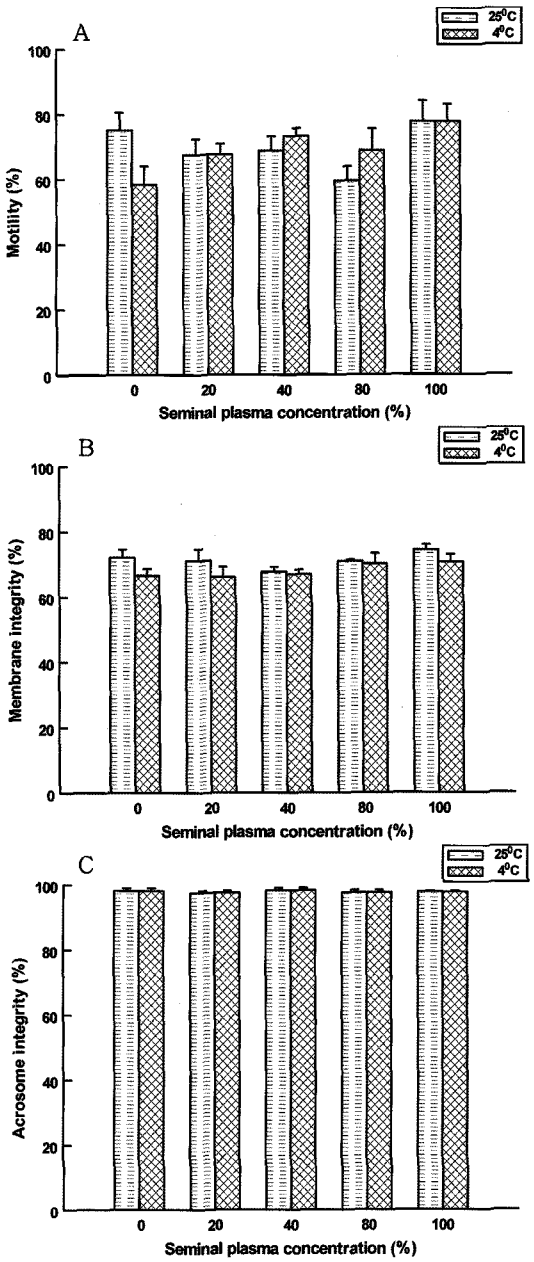


**Fig 1.** Characteristics of canine spermatozoa diluted with EYT alone or EYT containing seminal plasma with different concentration and then chilled to 4°C or held at 25°C. (A) The mean percentage of motility±SEM of spermatozoa according to seminal plasma concentration. (B) The mean percentage of membrane integrity±SEM of spermatozoa according to seminal plasma concentration. (C) The mean percentage of acrosome integrity±SEM of spermatozoa according to seminal plasma concentration.

than that in control ($P < 0.05$, Fig 2A). When seminal plasma was added back to spermatozoa in the second dilution, motilities of spermatozoa in groups treated with EYT/2 containing seminal plasma were higher than that in control. However, there was no significant difference among groups.

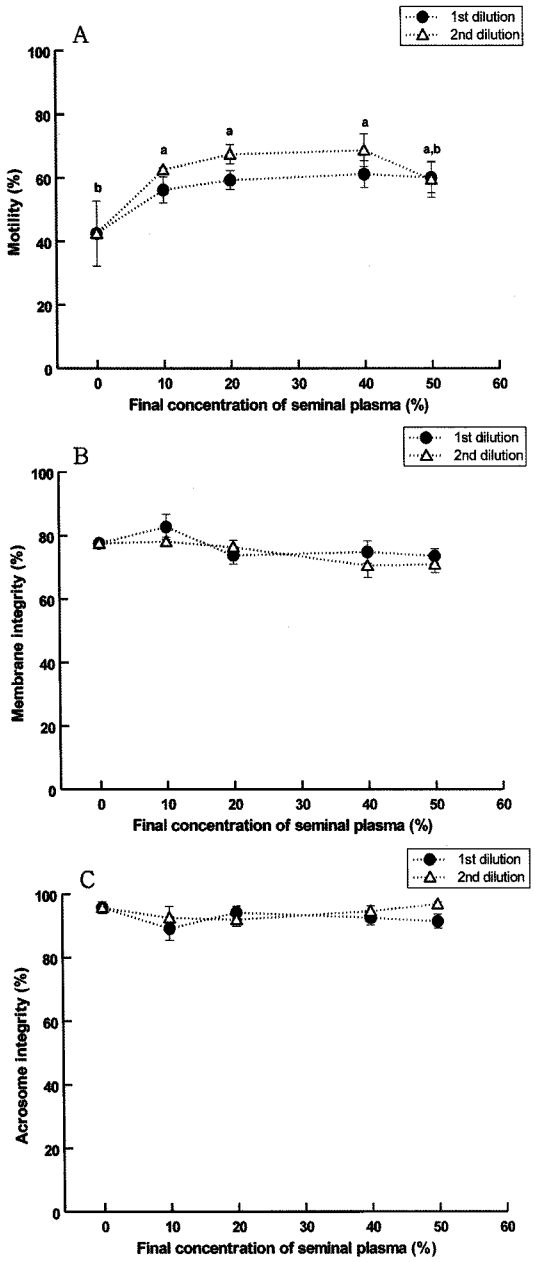


**Fig 2.** Characteristics of canine chilled spermatozoa before being freezing. (A) The mean percentage of motility ± SEM of spermatozoa according to seminal plasma concentration. (B) The mean percentage of membrane integrity± SEM of spermatozoa according to seminal plasma concentration. (C) The mean percentage of acrosome integrity± SEM of spermatozoa according to seminal plasma concentration. The first dilution and the second dilution mean the adding time, at which seminal plasma was added to extender. Differences letters indicate statistically significant differences among experimental groups ($P< 0.05$).

Membrane integrity of spermatozoa was not significantly different among groups without regard to seminal plasma being added in the first dilution or the second dilution (Fig 2B).

Acrosome integrities among groups were not significantly different either (Fig 2C).

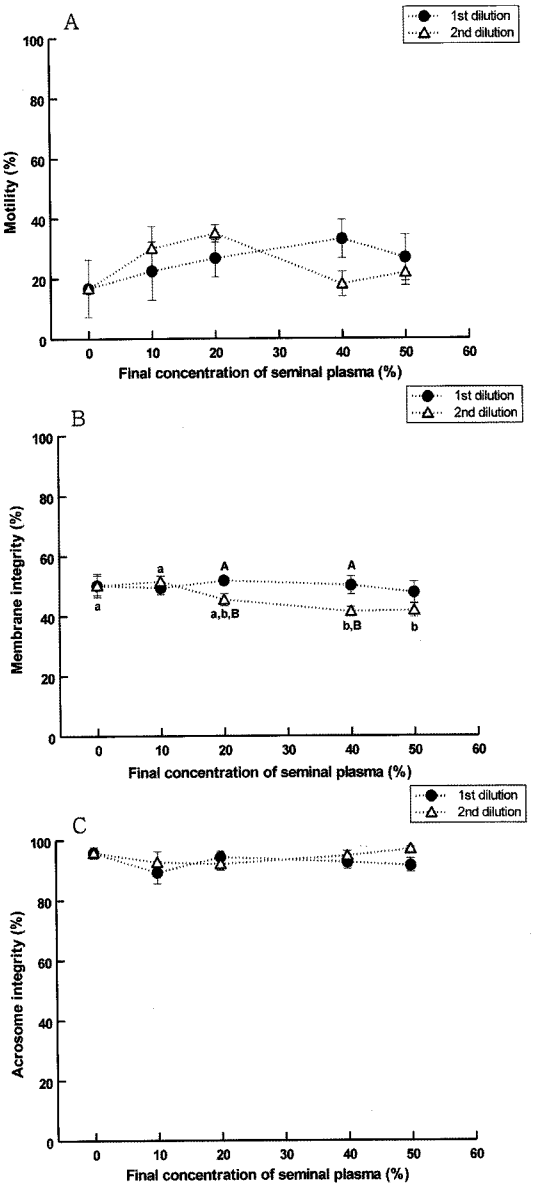


**Fig 3.** Characteristics of canine frozen-thawed spermatozoa. (A) The mean percentage of motility± SEM of spermatozoa according to seminal plasma concentration. (B) The mean percentage of membrane integrity± SEM of spermatozoa according to seminal plasma concentration. (C) The mean percentage of acrosome integrity ± SEM of spermatozoa according to seminal plasma concentration. The first dilution and the second dilution mean the adding time, at which seminal plasma was added to extender. Differences letters indicate statistically significant differences among experimental groups ($P< 0.05$).

We also determined the effect of seminal plasma on frozen-thawed spermatozoa (Fig 3). Motilities of spermatozoa diluted with EYT/1 and EYT/2 containing seminal plasma were higher than that in control regardless of the adding time of seminal plasma in extender. The highest sperm motility was

shown in the group treated with 40% seminal plasma in the first dilution while the highest sperm motility in the second dilution was shown in the group treated with 20% seminal plasma. However, there was no significant difference among groups (Fig 3A).

Membrane integrities of spermatozoa diluted with EYT/1 containing seminal plasma in the first dilution and then diluted with EYT/2 in the second dilution were higher than that in control. However, membrane integrities of spermatozoa diluted with EYT/1 without seminal plasma and then diluted with EYT/2 containing 20, 40 or 50% seminal plasma were significantly lower than that of control ($P < 0.05$, Fig 3B). Compared between the first dilution and the second dilution, twenty or forty percentages of seminal plasma in the first dilution showed significantly higher membrane integrity than those in the second dilution ($P < 0.05$).

Seminal plasma did not affect acrosome integrity with regard to seminal plasma being added in the first extender or the second extender.

## Discussion

Seminal plasma has been thought to improve the viability and motility in bull, boar, and ram (3,19). Adding seminal plasma back to spermatozoa may reverse destabilization of sperm membranes caused by removing seminal plasma in handling semen. In the first experiment, adding of seminal plasma to EYT did not significantly improve sperm viability and motility at 25°C and 4°C. However, sperm preservation with seminal plasma at 4°C showed better motility than that without seminal plasma. Martinez-Pastor *et al.* (16) indicated that seminal plasma rendered the higher sperm characteristics in the 5°C 15 min treatment than 32°C 15 min, although epididymal spermatozoa were incubated in ejaculated seminal plasma. We may infer that seminal plasma alone without any cryoprotectant could protect membrane of spermatozoa being chilled.

Compared among groups with different seminal plasma concentration in the second experiment, spermatozoa cooled in the extender containing seminal plasma showed higher motility irrespective of seminal plasma concentration ($P < 0.05$). Seminal plasma has beneficial effects on sperm motility (18). Antioxidant effect of seminal plasma protects membrane integrity from peroxidation and helps to maintain higher sperm motility parameters (8). Barrios *et al.* (2) demonstrated that ram sperm plasma membrane damaged by cold shock was reverted after incubation of seminal plasma proteins. Seminal plasma in the second experiment significantly improved sperm motility, although spermatozoa in both first and second experiments were maintained at 4°C. One of explanation may be inclusion of glycerol in the extender used in the second experiment. We infer that seminal plasma can significantly improve sperm motility if spermatozoa are exposed to seminal plasma containing cryoprotectant during cooling.

Vadnais *et al.* (37) indicated that the effect of seminal plasma appeared to be independent of the percent inclusion (10,20, or 40%). In contrast, low and higher concentration of seminal plasma appeared to be detrimental to boar survival in flow sorting. The optimal level of seminal plasma in extender improved the viability (19, 27).

Maxwell *et al.* (19) indicated that viability was different depend on what medium was used. The viability was improved if 10% seminal plasma was included in the Beltsville thawing solution (BTS) and HEPES buffer containing 0.1% bovine serum albumin (HEPES-BSA) staining extenders for boar and ram spermatozoa, and 10% and 50% seminal plasma were included in the Test buffer containing 2% yolk (TY) collection medium for boar and ram spermatozoa. Rota *et al.* (30) reported that sperm motility up to day 4 was higher in egg-yolk Tris than in egg-yolk milk and egg-yolk cream. In this study of canine spermatozoa, higher concentration of seminal plasma was, lower sperm membrane integrity was after spermatozoa being frozen and thawed (Fig 3B). We suggest that different composition of medium could affect reaction between seminal plasma and spermatozoa and then cause different effects of seminal plasma on sperm survival.

Moore *et al.* (21) suggested that incubation time of sperm in seminal plasma could affect on sperm cryosurvival: variation of seminal plasma concentration did not reduce motility and viability after cryopreservation, if spermatozoa are frozen immediately after seminal plasma addition. However, adding of 5% seminal plasma resulted in higher motility than adding of 20% seminal plasma, if spermatozoa were incubated for at least 2 h prior to freezing. In our pilot study, long-term effect of seminal plasma was observed. When dog spermatozoa were incubated in seminal plasma for 1 h, 2 h or 4 h, motility of spermatozoa incubated more than 2 h at 37°C significantly declined ($P < 0.05$). However, there was no detrimental effect on sperm viability and acrosome integrity.

Cross (10) indicated that the development of acrosomal responsiveness in human spermatozoa can be prevented by seminal plasma. Sirivaidyapong *et al.* (32) reported that acrosome integrity after freezing and thawing was not affected when seminal plasma was added. In present study, sperm acrosome integrity were not affected by seminal plasma regardless of cooling and freezing (Fig. 1 & 2 & 3).

Membrane integrity were more sensitive than acrosome, whereas motility was more sensitive than membrane integrity in the current study, In particular, sperm motility after being frozen and thawed was much sensitive (Fig 3). As a result of previous study, canine sperm motility appeared to be a more sensitive sperm characteristics to freezing (35,36).

On the other hand, the effect of seminal plasma on sperm survival has been contradictory since seminal plasma is a complex mixture containing a wide variety of components to affect sperm survival (2). In addition, differences in seminal plasma protein profiles from bulls of different fertility has been reported (20,26). In this study, semen was collected from 4 dogs and pooled without considering individual dog variation. However, we need to reconsider that volume of seminal plasma in each dog was variable depend on physical

condition of dog, although semen was collected by the same person. We infer that the effect of seminal plasma may be different depend on individual fertility.

Phospholipases in seminal plasma interact with egg yolk lecithin to produce toxic compounds to the goat spermatozoa (7). We used EYT containing egg yolk as a extender. We did not determine a direct relationship between egg yolk and phospholipases in seminal plasma. However, we can not exclude the possibility of relation between egg yolk and seminal plasma in canine. It needs to be studied in detail.

According to recently published articles, direct interactions between prostatic fluid and spermatozoa improved fertilization by enhancing migration of spermatozoa through the cervix and greater longevity of spermatozoa in the uterus (24). Likewise, damage of sperm plasma membrane by cold shock was reversed by absorption of seminal plasma protein (2). In the future study, the practical effect of seminal plasma on fertility are needed to be determined by artificial insemination and production of offspring.

In conclusion, seminal plasma was neither detrimental to dog sperm survival nor beneficial to dog sperm survival when spermatozoa were exposed in EYT containing seminal plasma in holding at 25°C or at 4°C. However, seminal plasma improved motility of cooled spermatozoa when seminal plasma was mixed with EYT containing glycerol.

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# 개 정액의 정장이 개정자의 냉각과 동결에 미치는 영향

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**요 약** : 개 정액의 정장은 정자를 동결 · 보존하기전 정액에서 제거되어진다. 그러나 근래 개정장이 정자를 냉각하고 동결하는데 유효한 효과가 있는 것으로 보고되고 있다. 그러므로 본 연구에서는 정자를 냉각과 동결하기 전 정장을 정자 희석액에 첨가하여 정장이 정자의 생존성에 미치는 영향을 알아보고자 하였다. 건강한 4마리의 수캐로부터 정액을 채취 · 혼합한 후 300 g에서 10분간 원심분리하여 얻은 상층액을 다시 원심분리하고 상층액을 회수하여 정장으로 사용하였다. 정자의 희석액으로는 Egg yolk-Tris (EYT)를 사용하였다. 다음과 같이 두 가지 실험을 실시하였다. 실험 1: 정장이 0, 20, 40, 80, 100%비율로 포함된 EYT배지에 정자를 희석하여 두 시간 동안 4°C나 25°C에서 보관하였다. 정자수는 $2\times10^8$/ml로 조정하였다. 실험 2: 전체적으로 9개 실험군으로 구분하여 4개 실험군은 정장이 20, 40, 80, 100%포함된 EYT배지에 각각 정자를 희석하여 4°C까지 냉각한 후 EYT + 1.2 M glycerol배지로 2차 희석하여 동결하였다. 나머지 4개의 실험군은 EYT배지에 정자를 희석하여 4°C까지 냉각한 후 각각 정장이 20, 40, 80, 100%포함된 EYT + 1.2 M glycerol배지로 2차 희석하여 동결하였다. 대조군으로서 정장을 포함하지 않은 EYT배지에 정자를 희석하여 4°C까지 냉각한 후 EYT + 1.2 M glycerol배지로 2차 희석하여 동결하였다. glycerol의 최종농도는 0.6 M, 정장의 최종농도는 10, 20, 40, 50%이었으며, 정자수는 $1\times10^8$/ml로 조정하였다. 정자를 straw에 충진하고 분당 25°C의 냉각율과 액체질소를 이용하여 동결하였다. 융해는 38°C에서 1분간 실시하였다. 정자 운동성, 정자 형질막 보존성(생존력), 정자 첨단체의 보존성을 검사하여 정자의 생존성을 검증하였다. 정자의 운동성은 400배율에서 현미경으로 관찰하였으며 생존력과 첨단체 보존성은 각각 이중형광염색과 *Pisum sativum agglutinin*을 이용하여 검사하였다. 실험 1의 결과는 보존온도와는 관련없이 희석액에 정장을 첨가하는 것은 정장이 포함되지 않은 희석액과 비교하여 정자의 운동성을 향상시키지 못했다. 실험 2 결과, 동결 전 냉각상태에서 정장을 포함한 EYT + 0.6 M glycerol배지에 희석되었던 정자가 정장을 포함하지 않은 EYT + 0.6 M glycerol배지보다 높은 진행운동성을 보였다 ($P<0.05$). 그러나, 동결 · 융해 후 정장을 포함한 EYT + 0.6 M glycerol배지에 희석되었던 정자의 생존력은 정장을 포함하지 않은 EYT + 0.6 M glycerol배지보다 낮거나 비슷하게 나타났다. 본 실험의 결과를 요약하면 비록 정장이 동결보호제가 포함되지 않은 EYT에 혼합되었을 경우는 냉각되어진 정자의 생존성을 향상시키지는 못했으나 동결보호제가 포함된 EYT에 정장을 혼합한 경우 정장은 정자의 냉각 후 정자의 운동성을 향상시켰다.

**주요어** : 개 정자, 정장, 냉각, 동결, 정자의 생존성