Iranian Journal of Veterinary Research, Shiraz University, 2013, Vol. 14, No. 4, Pages 305-312

The effect of seminal plasma on the quality of coated ram frozen-thawed spermatozoa

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(Received 13 Jul 2012; revised version 9 Apr 2013; accepted 13 Apr 2013)

Summary

Two experiments were designed to examine the effects of crude seminal plasma (CSP) exposure of ejaculated and epididymal spermatozoa before freezing. Experiment 1, two consecutive ejaculates were collected (n=4) within 5 min, the second one was carried out in a tube containing Fiser extender (coated spermatozoa); by centrifugation, seminal plasma was removed from coated ejaculates. The pellets were split into three parts and 0, 50 and 100% CSP (v/v) was added and they were frozen. Samples were thawed and incubated at 37°C for 6 h. The highest progressive motility (10.41%) and the highest hypo-osmotic responses (12.62%) were found for the coated spermatozoa without CSP at 6 ($P<0.05$). The maximum viability was found for coated spermatozoa without CSP at 0 (29.4%) and 6 (17%, $P<0.05$). Experiment 2, epididymal spermatozoa were recovered (n=6), pooled and split into three fractions and 0, 50 and 100% CSP and the diluents were added, after that they were frozen. Thawed epididymal spermatozoa were incubated at 37°C for 6 h. The highest (33.3%) and lowest (25%) progressive motility were found for the epididymal spermatozoa without CSP and the epididymal spermatozoa with 100% CSP at 0, respectively ($P<0.05$). The highest (19.37%) and the lowest (9.38%) viability were related to the epididymal spermatozoa with 0 and 100% CSP at 6, respectively ($P<0.05$). Under the conditions of the current study, the addition of CSP to the ram epididymal and coated spermatozoa strengthened the detrimental effect of the freezing procedure.

Key words: Cryopreservation, Sperm coating, Seminal plasma, Ram, Spermatozoa

Introduction

The effect of cooling on sperm differs depending on species (Medeiros *et al*., 2002). It is also well known that ram spermatozoa are more sensitive to cold-shock stress than those of other species (Muiño-Blanco *et al*., 2008). Freezing damage results in the impairment of sperm transport through the cervix, thus it may be contributing to the reduced fertility rates experienced for cervical insemination of frozen-thawed ram spermatozoa (Byrne *et al*., 2000).

Seminal plasma, as medium for sperm, is a complex mixture of secretions originating from testis, epididymis and accessory sex glands (Manjunath *et al*., 1993). This mixture contains numerous factors such as organic and nonorganic materials, which play an important role in the final maturation of the spermatozoa through hormonal, enzymatic and surface-modifying events (Manjunath *et al*., 1993; Muiño-Blanco *et al*., 2008; Dogan *et al*., 2009). Although, the effect of semen plasma or its proteins on the function of spermatozoa has been widely studied (Muiño-Blanco *et al*., 2008), the results are contradictory. The addition of seminal plasma proteins to ejaculated ram spermatozoa which were freed from seminal plasma and exposed to cold shock, reverted the cold-shock damage on ram sperm membrane (Barrios *et al*., 2000). Before cold treatment, the addition of semen plasma proteins (>3 kDa) to spermatozoa had an immediate beneficial effect on ram sperm survival (Pérez-Pé *et al*., 2001). On the other hand, al-Somai *et al*. (1994) demonstrated

that the cationic and anionic peptides of bovine seminal plasma had detrimental effects on sperm motility and the effects were more substantial compared with that of whole seminal plasma. It was shown that adding seminal plasma to boar spermatozoa before freezing reduced the freezability and post-thaw penetration competence of spermatozoa (Kawano *et al*., 2004). Moreover, it was demonstrated that the removal of seminal plasma had beneficial effects on the freezability of Saanen buck semen (Ustuner *et al*., 2009).

Egg yolk (EY) has been routinely used in semen diluents because of its protective action during cooling and freezing. According to one report, there are interactions between EY-LDF and seminal plasma proteins (Bergeron *et al*., 2004). De Pauw *et al*. (2003) demonstrated that the autodestructive activity of bovine seminal plasma was decreased by coating spermatozoa for less than 5 min during collection with the commercial diluent supplemented with EY. The detrimental effects of lipid efflux, which are induced by seminal plasma, may be abolished by decreasing the duration of contact between seminal plasma and sperm (Moreau and Manjunath, 2000).

The objective of this study was to determine whether the addition of crude seminal plasma to coated ejaculated and epididymal spermatozoa may be harmful for spermatozoa in the freezing and thawing process.

Materials and Methods

The effect of adding CSP to ejaculated and epididymal spermatozoa were evaluated by experiment 1 and 2, respectively. All products used in this experiment were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The same animals were used for both experiments.

Four healthy mature Taleshi rams aged 3-5 years and with an average weight of 55 kg were used. Rams were fed daily with 0.5 kg of concentrate, dried grass, salt lick and water *ad libitum*. The animals were housed at Guilan University, Faculty of Agricultural Sciences, Education Research and Practice Farm, South of Rasht (it is located at 37° 12´ North latitude and 49° 39´ longitudes).

Seminal plasma preparation

During the breeding season (September-December), ejaculates were collected from four rams using an artificial vagina; ewe was always present. Semen was centrifuged at 650 × g for 15 min at 4°C to remove sperm. The supernatant was further centrifuged at 10,000 × g for 10 min at 4°C to obtain clear seminal plasma; supernatants were collected, pooled and held at -20°C until use.

Sperm collection and preparation

Experiment 1

The semen collection was performed twice a week, over two consecutive weeks in November 2010. According to De Pauw *et al*. (2003), two consecutive ejaculates from each ram were collected within 5 min, the second of which was carried out in a tube containing 1 mL of coating medium (coated spermatozoa). Coating medium was Fiser extender [269 mM tris (hydroxymethy1) aminomethane, 52 mM D-Fructose, 89 mM citric acid, 2000 IU/ml penicillin G and 0.4 mg/mL streptomycin pH = 7.0] with 15% EY as a counteracted factor with seminal plasma (Bergeron *et al*., 2004; Leahy *et al*., 2010a). The samples were transported to the laboratory in an insulated Styrofoam box.

All ejaculates with acceptable volume (0.75 to 2 mL), concentration (>2.5 × 10^9 sperm/mL), and progressive motility (>70%) were delivered to experiment. The coated spermatozoa were pooled and then immediately centrifuged for 10 min at 700 × g at room temperature and the supernatant removed. The pellets were split into three parts. Each part was diluted 1:2 (considering ram spermatocrit ~ 32%) to 0% crude seminal plasma (CSP) + 100% Tris glucose diluents [300 mM tris (hydroxymethyl) aminomethane, 95 mM citric acid-monohydrate, 28 mMD-(+)-Glucose, 2000 IU/mL penicillin G and 0.4 mg/mL streptomycin pH = 7.0] (treatment C_0), 50% CSP + 50% Tris glucose diluents (treatment C_{50}) and 100% CSP + 0% Tris glucose diluents (treatment C_{100}), respectively; they were incubated at room temperature (22°C) for 15 min.

The samples were diluted with Tris glucose diluents to prepare 1200 × 10^6 cell/mL concentration. The aliquots were diluted 1:1 (v/v) with Tris glucose diluents containing 30% EY, as a cryoprotectant, and 10% glycerol. Final concentrations of EY, glycerol and sperm were 15, 5% and 600 × 10^6 cells/mL, respectively. All treatments were independently repeated four times.

Experiment 2

To obtain epididymis, the testes of three rams were surgically isolated by open castration under local anesthesia. After isolation, testes were placed in sterile plastic containers, including warm (37°C) normal saline (0.9% NaCl), and the lids tightly sealed. They were transported in an insulated Styrofoam box that was heated by water bottles at a temperature of 37°C. In the laboratory, epididymal spermatozoa were obtained by slicing and suspending the caudal epididymal tissue in Petri dish containing 5 mL coating medium without EY. The samples were agitated and incubated at room temperature for 10 min; liquid phase containing epididymal spermatozoa was collected. Recovered epididymal spermatozoa were pooled and centrifuged for 10 min at 700 × g at room temperature; the supernatant was removed. The pellets were divided into four parts and the following procedures were independently repeated four times. Each part was split into three fractions and 0% crude seminal plasma (CSP) + 100% Tris glucose diluents (EP_0), 50% CSP + 50% Tris glucose diluents (EP_{50}) and 100% CSP + 0% Tris glucose diluents (EP_{100}) were added, respectively. The aliquots were incubated at room temperature (22°C) for 15 min. Finally, samples were diluted the same as experiment 1.

Diluted sperm was packed in 0.25 mL plastic straws and then cooled to 5°C over 2 h. The straws were frozen in liquid nitrogen vapor, with the straws horizontally suspended 4.5 cm above the liquid nitrogen for 13 min before being plunged into liquid nitrogen for storage. After 2 weeks, six straws from each replication were thawed in a water bath at 37°C for 30 s and incubated at 37°C for 6 h. The longevity of *in vitro* incubation of spermatozoa after thawing at uterine temperature (38.5°C) for up to 6 h can be a reasonable indicator of potential fertility, as it partially mimics the *in vivo* condition inside the female genital tract. Such assessments have been found to give a better indication of fertility compared with the estimation of immediate post-thaw motility (Awad, 2011). Progressive motility, viability and membrane integrity were determined at 0, 3 and 6 h, after incubation.

Sperm assessment

Sperm concentration was determined by means of a haematocytometer. Progressive motility was determined by placing a sample aliquot on warm glass slides (38°C), covered with a cover slip and examined under a phase-contrast microscope (×400 magnification) (Olympus IX70, Olympus Optical Co., Ltd., Japan) in three random microscopic fields.

The viability was assessed by means of a one-step eosin-nigrosin staining (Björndahl *et al*., 2003). Briefly, equal volumes of semen and stain solution (0.67 g eosin Y, 0.9 g sodium chloride and 10 g nigrosin in 100 mL distilled water) were incubated for 30 s at room temperature (22°C). One drop of mixture was put on a slide, instantly smeared and air dried. A total of 200 sperm were evaluated under light microscope (×1000 magnification, oil immersion). Sperm showing partial or complete pink or red colorization were considered dead and sperm showing strict exclusion of the stain were considered to be alive.

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane. The procedure was described by Jeyendran *et al*. (1992) and adapted for ram semen by García-Artiga (1994). The hypo-osmotic swelling test was performed by incubating 5 µL of semen with 500 µL of a 100 mOsm/Kg hypo-osmotic solution (7.35 g sodium citrate dihydrate and 13.51 g fructose in 1 L distilled water) at 37°C for 30 min. One drop of the mixture was placed on a pre-warmed slide, covered with a cover slip and examined under a phase-contrast microscope (×400 magnification). A total of 200 sperm were evaluated in at least five different microscopic fields. The percentage of sperm with swollen and curled tails was recorded

as HOST-positive.

Statistical analysis

Analysis of variance was preformed to study the effects of the treatments on motility, viability and plasma membrane integrity of the coated and epididymal spermatozoa using mixed procedure of SAS (2002) with repeated measures data. There were three treatments for both experiments (C_0, C_{50} and C_{100} for Exp. 1 and EP_0, EP_{50} and EP_{100} for Exp. 2). The samples taken from pooled semen and epididymal spermatozoa were considered as subjects in these experiments. Statistical model included the concentration of CSP, time and their interaction effect. When analysis revealed a significant difference, comparison of treatment means was performed by Duncan's multiple range test. For all statistical tests the level of statistical significance was chosen at $P<0.05$.

Results

Experiment 1

There was interaction between adding seminal plasma and incubation time on sperm motility, viability and membrane integrity ($P<0.05$). The progressive motility was higher in the coated spermatozoa without CSP than the coated spermatozoa with 100% CSP at 0, 3 and 6 (Fig. 1A; $P<0.05$). There was no difference between the progressive motility of coated spermatozoa without CSP and coated spermatozoa with 50% CSP at 0 and 3. The highest progressive motility was found for coated spermatozoa without CSP at 6 ($P<0.05$).

At 3 and 6, the hypo-osmotic responses were higher in the coated spermatozoa without CSP than the coated spermatozoa with 100% CSP (Fig. 1B; $P<0.05$). The maximum viability was found for coated spermatozoa without CSP at 0 and 6 (Fig. 1C; $P<0.05$).

Experiment 2

There was interaction between adding seminal plasma and incubation time on sperm motility, viability and membrane integrity ($P<0.05$). The highest and lowest progressive motility were found for epididymal spermatozoa without CSP and epididymal spermatozoa with 100% CSP at 0, respectively (Fig. 2A; $P<0.05$). There was significant difference between progressive motility of epididymal spermatozoa without CSP and epididymal spermatozoa with 100% CSP at 3.

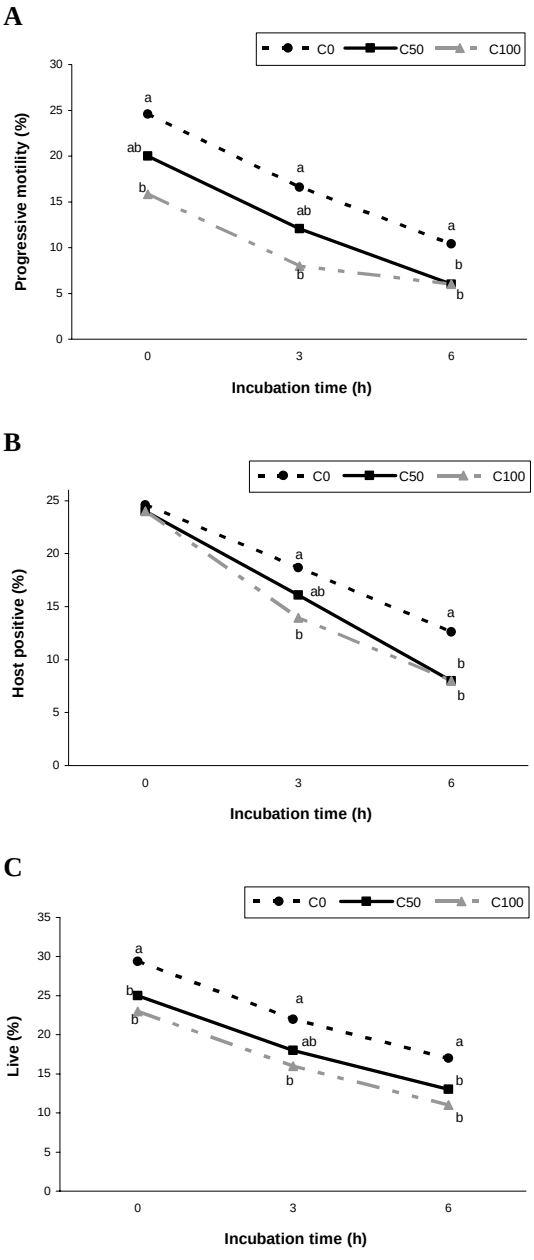

Fig. 1: The percentage of progressive motility (A), membrane integrity (B) and viability (C) of ram coated spermatozoa were treated with 0% CSP (C_0), 50% CSP (C_{50}) and 100% CSP (C_{100}) during incubation at 37°C after thawing. [a-f] Different superscripts indicate significant differences within incubation time ($P<0.05$)

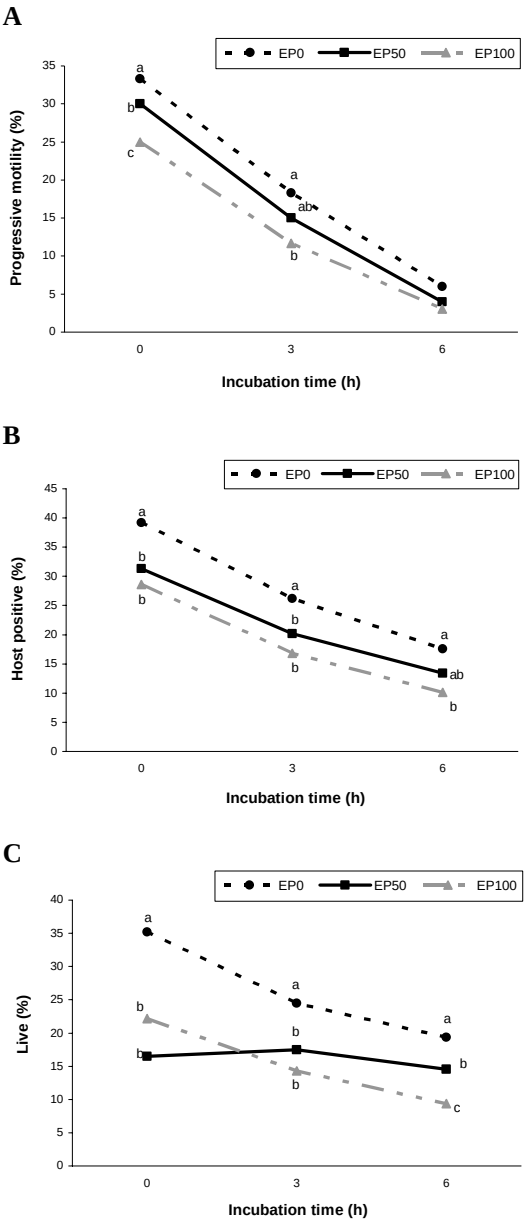

Fig. 2: The percentage of progressive motility (A), membrane integrity (B) and viability (C) of ram epididymal spermatozoa were treated with 0% CSP (EP_0), 50% CSP (EP_{50}) and 100% CSP (EP_{100}) during incubation at 37°C after thawing. [a-f] Different superscripts indicate significant differences within incubation time (P<0.05)

The maximum hypo-osmotic response was achieved by epididymal spermatozoa without CSP at 0 and 3 (Fig. 2B; P<0.05). There was significant difference between the hypo-osmotic response of epididymal spermatozoa without CSP and epididymal spermatozoa with 100% CSP at 6.

The epididymal spermatozoa without CSP presented the highest viability at 0 and 3 (Fig. 2C; P<0.05). The highest and the lowest viability were related to the epididymal spermatozoa with 0 and 100% CSP at 6, respectively (P<0.05).

Discussion

In this study, an attempt was made to answer whether the addition of CSP to spermatozoa which had been early protected by EY coating method, exert positive effect or not. Results showed that the addition of CSP to coated ram spermatozoa had a detrimental effect during freezing and thawing. Leahy *et al*. (2010a) demonstrated that supplementation with CSP at the rate of 4 mg protein per 10^8 ram spermatozoa, reduced progressive motility of frozen-thawed spermatozoa.

Some observations revealed the destructive effect of CSP on spermatozoa during sperm cryosurvival (al-Somai *et al*., 1994; Kawano *et al*., 2004; Ustuner *et al*., 2009). However, Graham (1994) reported that resuspension of washed ejaculated ram spermatozoa in seminal plasma and storage in diluent Tris containing 20% EY resulted in higher percentages of motile spermatozoa than resuspension in TALP (Tyrode's medium) after thawing (14 vs 9%). The difference may be related to different methods of semen collection, especially different concentrations of EY [20% (Graham, 1994) vs 15% in the current experiment] as cryoprotectant, different amounts of additive seminal plasma and different final sample dilution [40×10^6 (Graham, 1994) vs 600×10^6 in the current experiment]. It was demonstrated that high pre-freezing dilution improved post-thaw function of ram spermatozoa (Leahy *et al*., 2010b). According to one report, long exposure of bull sperm to BSP proteins or exposure to too large concentrations of them can be deleterious to the sperm membrane (Manjunath *et al*., 2002), whereas it has not been established whether or not RSP proteins are detrimental to ram sperm storage. Moreover, the toxic effect of seminal plasma protein on ram spermatozoa was not shown (Leahy *et al*., 2009). On the other hand, it was mentioned that cryoprotective diluents that contained EY or

milk altered seminal plasma proteins availability and also the responses of spermatozoa to the addition of seminal plasma (Leahy *et al*., 2010a). Therefore, it is important to note when seminal plasma is added to the diluted spermatozoa it must also pay attention to the concentration of EY. It is likely that using high levels of EY prevents the destructive effect of CSP during storage (Morrie *et al*., 2003); hence, positive effect of seminal plasma on sperm membrane may be exaggerated. Consequently, in the present study, the concentration of seminal plasma and EY applied high and low levels, respectively.

To the best of our knowledge, this is the first time that the coating method has been used for storing ram spermatozoa. This process and removal of seminal plasma increased freezability of ram spermatozoa. The results are consistent with a previous study in bull (De Pauw *et al*., 2003). Seminal plasma removal immediately after semen collection induced a clear improvement in ram sperm quality (Ollero *et al*., 1997). Spermatozoa and the secretions of the accessory sex glands are mixed and entered into the female genital tract during the natural mating process. Subsequently, spermatozoa swim through cervical mucus and enter the uterus within minutes (>30 min); cervical mucus acts as a barrier for seminal plasma (Manjunath *et al*., 2002). This mechanism may be activated to separate spermatozoa from seminal plasma and remove the detrimental effect of seminal plasma. It was demonstrated that some seminal plasma proteins through cholesterol and phospholipid efflux from the membrane induced negative effect on sperm storage (Bergeron *et al*., 2004).

The current results showed that semen collection within tubes containing EY-buffer and subsequent seminal plasma removal (coating method) improved sperm survival and membrane integrity during cooling and freezing procedure. In standard semen collection and preparation method, spermatozoa usually dilute to protect against destructive effect of seminal plasma within a few min after collection. In this situation, the detrimental activity of some components of seminal plasma on sperm membrane is likely decreased by EY-LDF but does not stop even after 10 times dilution (Bergeron *et al*., 2004), while coating method through early EY protection and subsequent removal of seminal plasma may minimize modification of sperm membrane. Therefore, early EY protection can be useful to keep ram sperm survival and may allow a better sperm storage.

The results from experiment 2 show that epididymal spermatozoa contact with CSP prior to freezing leads to a decrease in motility, viability and membrane integrity after thawing. Contrary to the current results, Graham (1994) reported that the addition of seminal plasma prior to freezing was beneficial to epididymal ram spermatozoa after thawing. This controversy may relate to the use of lower EY concentration, as a cryoprotectant, higher amount of supplemental CSP and higher final sample dilution in this experiment. Based on these results, seminal plasma increases sensitivity of ram epididymal spermatozoa to detrimental effects of freezing, as coated spermatozoa. These results support the hypothesis that epididymal ram spermatozoa, which are not affected by the seminal plasma auto-destructive activity, are more resistant to cryobiologically relevant stressors such as chilling, osmotic stress and adding or removing the cryoprotectants than ejaculated spermatozoa (Varisli *et al*., 2009).

The results illustrate that total seminal plasma exerts destructive effect on spermatozoa and it may be minimized by EY coating method, and hence sperm can be stored well in the frozen state. It is still unknown whether the above mentioned results will be reflected in the fertility results after AI, hence additional research is needed.

In conclusion, it can be said that continuous exposure of sperm to seminal plasma had detrimental effect on the sperm storage in frozen states (cryopreservation); in addition, the detrimental effects of cooling and freezing on epididymal and coated spermatozoa were strengthened by crude seminal plasma. Therefore, in order to improve ram sperm storage, it can be helpful to apply EY coating method.

Acknowledgements

Author would like to thank Mr. R. Rajabi and R. Motamedi for sample collection and Dr. N. Ghavi Hossein-Zadeh for his valuable help with the statistical analysis.

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