EFFECT OF SEMINAL PLASMA ON FUNCTIONAL INTEGRITY OF RABBIT SPERM MEMBRANES DURING STORAGE AT 4ºC OR FREEZING

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ABSTRACT: The effect of semen plasma removal either by simple centrifugation or by separation through a Percoll gradient on the integrity of plasma membranes of rabbit spermatozoa during storage at 4°C and freezing was evaluated in two successive experiments. A modified hypo-osmotic swelling test procedure combined with supravital staining was employed to evaluate simultaneously membrane integrity of head and tail membranes of sperm cells. In the first experiment, the impact of semen plasma on membrane integrity of sperm cells was examined in Tris-citric acid-glucose extender at 4°C for 96 h. The percentage of sperm cells with disintegrated tail and head membranes increased in all groups in correlation with the length of storage. After storage for 96 h, removal of semen plasma, irrespective of the method of removal, resulted in significant increase (*P*<0.01) in the percentage of sperm cells with disintegrated plasma membranes. The adverse effect of storage and removal of semen plasma was more prominent on the tail membranes, and especially during the first 24 h of the storage period. In the second experiment, the impact of semen plasma on membrane integrity of sperm cells undergoing freezing was examined. A total of three groups were arranged as described in the first experiment, and semen samples were frozen in straws using an extender including acetamide and methyl cellulose. Freezing of semen drastically reduced the percentage of sperm cells with intact plasma membranes in post-thaw samples. However, removal of semen plasma, irrespective of the method of removal, did not affect the proportion of sperm cells with intact plasma membranes. In conclusion, the effect of semen plasma on plasma membranes varied significantly relative to the preservation temperature of sperm cells. Although it exerted a protective influence during storage at 4°C, no protective impact was monitored during freezing.

Key words: sperm, membrane integrity, seminal plasma, rabbit, freezing, storage.

INTRODUCTION

Standard semen analysis relies on assessing a number of parameters such as sperm concentration, motility and morphology for the assessment of male fertility. However, these parameters have some limitations and, therefore, cannot be used as reliable predictors of sperm fertilizing ability. The sperm membrane appears to have particular importance in male fertility because it is involved in the metabolic exchanges of the cell with the surrounding medium. Moreover, the process of capacitation, acrosome reaction and binding of the spermatozoa to the oocyte surface requires an active and functional membrane.

Two methods have been used frequently to evaluate membrane integrity: supravital stains and hypo-osmotic swelling test (HOST), although some sensitive fluorescent staining procedures have recently been developed.

The effect of semen plasma on cryotolerance of sperm cells has been studied with contrasting results. In ram (Maxwell *et al*., 1999) and boar (Berger and Clegg, 1985), semen plasma was reported to improve resistance of sperm cells against cold shock damage. Conversely, detrimental effect of semen plasma on

sperm survival was reported in bovine (Garcia and Graham, 1987). This study was conducted to evaluate in the rabbit the effect of semen plasma on the functional integrity of sperm membranes during storage of semen at 4ºC and freezing by means of a modified HOST procedure that allows simultaneous examination of head and tail membranes.

MATERIALS AND METHODS

Animals and semen collection

Six New Zealand bucks between 1.5 and 2.5 years of age were used as semen donors. Ejaculates were collected by artificial vagina once or twice a week. Gel plugs in semen were removed immediately after collection and pooled ejaculates of 3 to 4 bucks were used in each experimental replication. Individual sperm motility was assessed under a light microscope ($\times$ 200) following 10-12 fold dilution of semen samples (v/v) in the extender containing 313.8 mM Tris, 103.1 mM citric acid and 33.3 mM glucose. Pooled ejaculates with good individual motility higher than 70.0% and abnormal sperm rate lower than 10.0% were allocated for the experiment. Bucks were kept in individual cages during the study and exposed to natural daylight. No special feeding regime was employed and water was provided *ad libitum*.

Experimental design

The study was performed in two consecutive steps. In the first, the effect of semen plasma removal either by centrifugation or by Percoll separation on the ability of sperm membranes to undergo storage at 4°C, in a refrigerator, was examined for 96 h.

In Group I, 250 µL of semen diluted in 1000 µL of Tris-citric acid-glucose extender (TCG; Roca *et al*., 2000) was centrifuged at 200 *g* for 15 min. After removal of the supernatant, the remaining part of the ejaculate was remixed and 100 µL of the sperm suspension was diluted 1:10 in TCG extender in a polyethylene tube.

In Group II, 250 µL of fresh semen was centrifuged at 200 *g* for 15 min in a 15 mL polyethylene tube through 1000 µL of Percoll column diluted 1:4 in TCG extender. After removal of the supernatant (approximately 850 µL), the remaining part of the extended semen sample was remixed in the bottom of the tube and 100 µL of this suspension was diluted 1:10 in TCG extender in a 1.5 mL polyethylene tube.

In Group C (control), fresh semen was directly diluted 1:10 (v/v) in TCG extender in a 1.5 mL polyethylene tube without removal of the semen plasma. Diluted samples in all the groups at room temperature were placed in a 1500 mL water bath at 20°C and cooled gradually to 4°C over approximately 1.5 h. In all the experimental groups extended semen samples were stored at 4°C for 96h.

In the second step of the study, the influence of semen plasma removal, either by centrifugation or by Percoll separation, on the ability of sperm membranes to undergo cryopreservation was examined. Experimental groups were organized as noted in the first step of the study. After removal of the supernatant following centrifugation in Group I and Percoll separation in Group II, sperm suspension without semen plasma was directly diluted 1:6 (v/v) with the extender including glucose (125 mM), lactose (105 mM), raffinose (91 mM), HEPES (10 mM), acetamide (6%; w/v), methyl cellulose (0.5%; w/v) and 20% (v/v) egg yolk as described by Dalimata and Graham (1997). In Group C (control), 100 µL of fresh semen were directly diluted with the extender 1:6 (v/v). Extended semen was loaded into 0.25 mL plastic straws at room temperature and cooled gradually to 5°C over 120 min in a programmable freezer (Cryocell 1200, Tiefenbach, Germany). The straws cooled to 5°C were frozen in liquid nitrogen vapor on a styrofoam boat (10×7×1 cm) covered by a metal mesh as described by Abas Mazni *et al*. (1990) for 15 min (approximately

-160°C) and, thereafter, they were directly plunged into liquid nitrogen. Thawing was achieved in a water bath at 36°C for 30 sec following at least 3 d of storage in liquid nitrogen container.

Assessment of membrane integrity in sperm cells

A modified hypo-osmotic swelling test procedure, combined with supravital staining technique described by Ducci *et al*. (2002), was used to evaluate, simultaneously, functional integrity of both head and tail membranes of sperm cells. Briefly, semen samples diluted 1:10 in 60 mOsm fructose solution including 1% (w/v) Eosin Y, were incubated in a water bath at 36°C for 15 min. Sperm smears were prepared by 10µL of mixed samples and 100 sperm cells were observed in each slide under a phase contrast microscope at 400 × magnification. Four types of sperm cells were identified in smears as presented in Figure 1.

For calculations, the number of sperm cells with unstained head (Types 1 + Type 3) was regarded as the percentage of sperms with intact head membranes. Likewise, the total count of sperm cells with curled tail (Type 1 + Type 2) was deemed the rate of sperm cells with intact tail membranes.

Statistical analysis

Experiments were replicated at least five times. The mean values were simply averaged from replications and were presented along with the standard errors throughout the study. Dead sperm rates and the percentage of sperm cells with intact tail and head membranes in experimental groups were compared with the control group using the Student's t-test. Likewise, the effect of storage duration on the spermatologic parameters in successive sampling periods were also compared using the Student's t-test. The interaction between the two factors, storage period and methods for removal of semen plasma, was analyzed by means of a two-way ANOVA. In cases where no interaction was determined between the factors, main effect means were used to evaluate the influence of the treatment.

RESULTS

Centrifugation of semen samples to remove seminal plasma, with or without a Percoll gradient, and further dilution of sperm cells in the extender significantly affected the functional integrity of head membranes of sperm cells ($P<0.01$). By contrast, the removal process of seminal plasma had no clear detrimental effect on the integrity of tail membranes compared to that observed on head membranes.

Storage at 4ºC for 24 h of the initially diluted semen samples increased ($P<0.01$) the proportion of sperm cells with intact head membranes, irrespective of seminal plasma presence, but reduced ($P<0.01$) that of sperm cells with intact tail membranes (Figure 2). The presence of seminal plasma in the extender reduced the percentage of sperm cells with deteriorated tail membranes during the first 24 h of storage ($P<0.01$). However, semen plasma did not effect the percentage of sperm cells with intact head membranes during the same period. Following the storage at 4ºC for 96 h, the percentage of sperm cells with disintegrated tail and head membranes increased gradually in all the experimental groups, and the presence of seminal plasma in the extender improved ($P<0.05$) functional integrity of both head and tail membranes of sperm
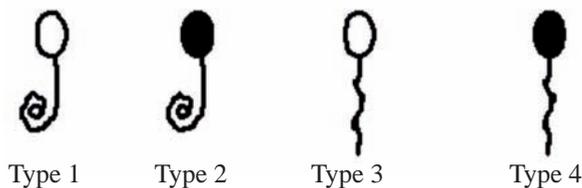

Type 1   Type 2   Type 3   Type 4

Figure 1: Four types of sperm cells identified in sperm smears. Black head sperms represent sperm cells with head stained with Eosine.

cells. No interaction was determined between the storage period and the method employed for semen plasma removal, either by centrifugation or by Percoll separation. Therefore, main effect means are presented in Table 1. Freezing of semen drastically reduced the percentage of sperm cells with intact head and tail membranes in post-thaw samples. However, removal of semen plasma, irrespective of the method, did not alter the ability of sperm membranes, neither head nor tail, to undergo cryopreservation ($P>0.05$; Table 2).

DISCUSSION

Centrifugation immediately after sperm collection is a common practice to remove seminal plasma. However, centrifugation speed is one of the important factors on the subsequent sperm loss in the supernatant and sperm viability. In the dog, centrifugation at 720 *g* for 5 min was reported as the best strategy to remove prostatic fluid before dilution of semen to be stored at 4ºC (Rijsselaere *et al.*, 2002). In this study, the relative effect of centrifugation and dilution on membrane integrity of sperm cells has not been evaluated separately due to lack of an experimental group in which semen plasma was removed without centrifugation. However, a constantly low centrifugation speed (200 *g*) was employed throughout the study to minimize any harmful effect of the process. In preliminary experiments carried out to optimize centrifugation speed it was observed that rabbit spermatozoa were very sensitive to the harmful effect of centrifugation and that a centrifugation speed higher than 500 *g* increased the number of damaged sperm cells in the pellet.
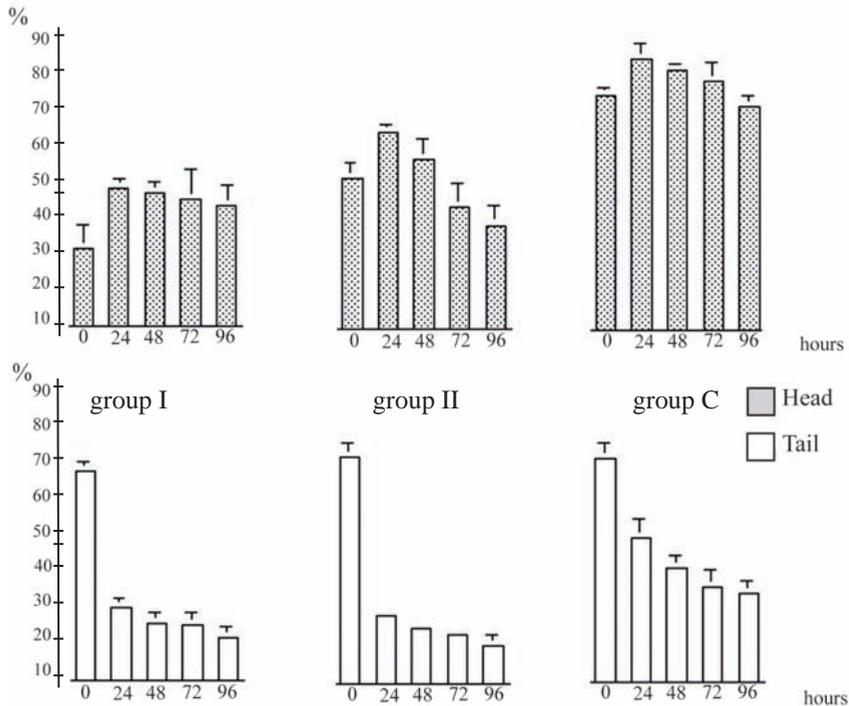

Figure 2: Effect of removal of semen plasma on the percentage of sperm cells with intact head and tail membranes during storage at 4ºC for 96 h (data, averaging 5 replications, are presented as mean±standard error; group I, group II and group C).

Table 1: Effect of seminal plasma removal by either centrifugation (I) or Percoll separation (II) on integrity of head and tail membranes of rabbit spermatozoa stored at 4˚C for 96 h (mean ± standard error).

Criteria	Groups[1]		
	I	II	C
Intact head membrane (%)	33.5±3.1[a]	40.2±3.3[a]	69.9±2.5[b]
Intact tail membrane (%)	26.0±4.0[a]	25.9±4.5[a]	39.9±3.5[b]

[1]Group I: semen plasma removal by simple centrifugation; group II: semen plasma removal separation through a Percoll gradient; group C: control where semen plasma was not removed.
Means within a raw with different superscripts differ. ($P<0.0001$).

Spermatozoa collected from many domestic animal species respond to high dilution by reducing motility, metabolic activity, and fertility. This so-called "dilution effect" is considered an indicator of cellular injury and is attributed to leaching of intracellular components or to dilution of a protective agent in seminal plasma (Watson, 1990). In the first part of this study it was interesting to see that a considerable proportion of the initially diluted sperm cells recovered functional integrity of head membranes and, thus, the percentage of the sperm cells with stained head increased following 24 h of storage at 4ºC, irrespective of the presence of seminal plasma. This observation is partly in agreement with the previous findings of Mann (1964) who reported that excessive dilution increases vital staining rate of mammalian spermatozoa. Spontaneous recovery of membrane integrity in the sperm head observed in our study indicates that sperm cells may activate internal mechanisms to re-organize membrane components by which membrane permeability is re-established after moderate dilutions, i.e. 1:10. Contrastingly, membrane damage which occurs after excessive dilutions may be irreversible (Maxwell and Johnson, 1999).

Various researchers (Dalimata and Graham 1997; Perez-Pe *et al.*, 2001; Love *et al.*, 2002) have studied the effect of semen plasma on cryoresistance of sperm cells with contradictory results. In the present study, irrespective of separation method, either by simple centrifugation or separation by a Percoll column, removal of semen plasma reduced the percentage of sperm cells with intact plasma membranes after 96 h of storage at 4°C. Likewise, Castellini *et al.* (2000) reported a beneficial influence of semen plasma even at excessive dilution rates up to 30-fold on the viability and motility parameters of rabbit sperm stored with a Tris-based extender. Moreover, in the first experiment, it was evident that tail membranes were more poorly influenced than head membranes by the storage conditions and removal of semen plasma and that a protective impact of seminal plasma on the tail membranes was more noticeable during the first 24 h of the storage period.

Male accessory gland secretions contain antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase, and free radical scavengers such as hypotaurine, taurine, uric acid, vitamins, and albumin (Twigg *et al.*, 1998; Vernet *et al.*, 2001; Chen *et al.*, 2002). Castellini *et al.* (2000) reported the role of lipid peroxidation in sperm deterioration during conservation of rabbit sperm at 37°C and

Table 2: Effect of seminal plasma removal on the functional integrity of head and tail membranes of sperm cells after dilution and freezing (mean±standard error).

Criteria	Replication	Sample	Groups		
			I	II	C
Intact head membrane (%)	5	Post-dilution	56.2±2.1	49.4±2.1	48.8±3.3
	5	Post-thaw	18.0±2.4	16.0±4.2	12.6±4.3
Intact tail membrane (%)	5	Post-dilution	59.6±3.0	59.8±4.2	58.8±4.2
	5	Post-thaw	15.8±1.6	15.6±1.4	15.0±2.3

[1]Group I: semen plasma removal by simple centrifugation; group II: semen plasma removal separation through a Percoll gradient; group C: control where semen plasma was not removed.

postulated that the beneficial effect of seminal plasma on spermatologic parameters was due to its antioxidant property. Rabbit semen plasma includes, as a unique property, numerous droplets and vesicles of various origins. Recently, a physiological role as sterol donor for the protection of spermatozoa against environmental shock and premature acrosomal reaction was proposed by Castellini *et al.* (2006) for these particles.

In the second part of the study semen plasma did not alter freezability of rabbit sperm membranes. The fact that semen plasma protected membrane integrity of rabbit spermatozoa during storage at 4°C but not at lower temperatures (-196°C) might indicate that the protective property of semen plasma may differ depending on the temperature at which sperm cells are preserved. This variation in the protective property of seminal plasma may be attributed to an altered mechanism of action of the protective substances in semen plasma at different temperatures or to the difference in the severity of cell injury triggered at different preservation temperatures.

In conclusion, this study has demonstrated that semen plasma exerts a protective impact, especially concentrated during the first 24 h of the storage period, on plasma membranes of rabbit spermatozoa stored at 4°C, although, it has no beneficial effect on membrane integrity of frozen sperm cells. The adverse effect of the storage period and removal of semen plasma on the tail membranes was more noticeable than that on the head membranes.

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