—Full Paper—

Enhanced Freezability of Goat Spermatozoa Collected into Tubes Containing Extender Supplemented with Bovine Serum Albumin (BSA)

Hideaki YAMASHIRO¹⁾, HaiFeng WANG¹⁾, Yasuhisa YAMASHITA¹⁾,
Kanako KUMAMOTO¹⁾ and Takato TERADA¹⁾

¹⁾Laboratory of Animal Reproduction, Graduate School of Biosphere Sciences, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hirosima, Hiroshima 739-8528, Japan

Abstract. The purpose of this study was to investigate the effects of semen collection into tubes containing extender supplemented with BSA on the cryosurvival of goat spermatozoa. Semen was collected from two goats into empty tubes or tubes containing 10 ml extender supplemented with 0, 0.1, 1, or 5% BSA, and the washed spermatozoa were frozen as pellets in egg yolk-trehalose extender with the addition of 0.04% SDS and 4% glycerol. Sperm motion parameters were evaluated after post-thawing and during a thermal resistance test. The acrosome status of frozen-thawed spermatozoa was also observed using FITC-PNA staining. In frozen semen that was collected into tubes containing extender supplemented with 5% BSA, the post-thawed spermatozoa exhibited a significant improvement in motion parameters and maintained high motility throughout incubation and acrosome integrity, as compared with semen collected into tubes containing extender supplemented with lower concentrations of BSA. In conclusion, semen collection into tubes with a large volume of extender containing high concentrations of BSA dramatically improves the motility and acrosome integrity of frozen-thawed spermatozoa. This suggests that the *in vitro* functional freezability of spermatozoa is abruptly modified by reducing contact with seminal plasma and by flash contact with BSA at ejaculation.

Key words: BSA, Freezability, Goat spermatozoa, Semen collection, Seminal plasma

(J. Reprod. Dev. 52: 407–414, 2006)

Through comparison of *in vitro* survival of ejaculated and epididymal spermatozoa, Blash *et al.* [1] confirmed that the motility and acrosome integrity of frozen-thawed goat epididymal spermatozoa are higher than those of ejaculated spermatozoa. Similar observations with boar epididymal spermatozoa have been reported by Kikuchi *et al.* [2] and Rath *et al.* [3]. It has also been shown that removal of seminal plasma by washing or dilution of spermatozoa immediately after collection increases the cryosurvival of

spermatozoa [4, 5], although ejaculated spermatozoa, even after washing, are incapable of attaining the post-thaw quality found in epididymal spermatozoa [6]. This evidence indicates that accessory sex gland secretions might confer extremely detrimental effects on the physiological functions of epididymal spermatozoa, i.e., the freezability of epididymal spermatozoa is dramatically altered by contact with these fluids of the glands at ejaculation. Nevertheless, until recently no attention has been paid to whether the freezability of ejaculated spermatozoa is abruptly influenced by flash contact of sperm with accessory reproductive fluids at the

moment of ejaculation.

In order to determine the effects of reducing contact between seminal plasma components and the sperm membrane, we previously examined whether the motility parameters and acrosome integrity of goat ejaculated spermatozoa were affected by collecting semen into tubes containing an extender supplemented with BSA. Our results showed that semen collection into tubes containing a large volume of tris(hydroxymethyl-aminomethane)-citric acid-glucose extender provides high sperm motility and acrosome integrity, especially extender containing high concentrations of BSA, and greatly improves the quality of ejaculated spermatozoa [7]. These findings suggest that dilution of ejaculates with a large volume of extender containing BSA in tubes reduces contact between seminal plasma components and spermatozoa, and minimizes the various kinds of damage due to exposure to seminal plasma. Therefore, it is possible that semen collection into tubes containing a large volume of extender supplemented with BSA could improve the cryosurvival of ejaculated spermatozoa.

The objective of the present study was to investigate whether semen collection into extender supplemented with BSA would improve the freezability of goat ejaculated spermatozoa in order to better understand the significance of reducing contact between seminal plasma components and the sperm membrane on the characteristics of ejaculated semen.

Materials and Methods

Semen extender and freezing extender

The basic semen extender was tris(hydroxymethyl-aminomethane)-citric acid-glucose (TCG) solution containing 375.0 mM tris (Sigma, St. Louis, MO, USA), 124.0 mM citric acid (Sigma), and 41.0 mM glucose (Sigma). This solution was adjusted to pH 7.0 with 375.0 mM tris and diluted with the milli-Q water to give an osmotic pressure of 375 mOsm [8].

The freezing extender was 0.375 M trehalose solution (Sigma). This solution was adjusted to pH 7.0 with 375.0 mM tris and to an osmotic pressure of 375 mOsm. Sodium dodecyl sulfate (SDS; Sigma) was added to a final concentration of 0.04% (w/v). Egg yolk was separated from the albumen

by blotting on filter paper. The yolk membrane was broken, and 20% (v/v) liquid yolk was added to the trehalose solution containing SDS, which was then stirred for 40 min. The extender-containing egg yolk solution was centrifuged twice at $13,500 \times g$ for 1 h to separate out the egg yolk lipoproteins as a thick, yellow, waxy plug. The plug was carefully separated from the supernatant. The supernatants were aspirated and filtered through a $0.45 \mu\text{m}$ membrane filter (Millipore SA, Molsheim, France) [9].

Semen collection and dilution

Two shiba goats (goats A and B) were subjected to semen collection two or three times weekly by artificial vagina (41 C) fixed on a dummy doe. In preliminary studies, the semen samples collected into tubes without extender from goat A had intrinsically high-quality semen (average volume was 0.75 ml, motility greater than 80%, concentrations greater than 3×10^9 sperm/ml, and teratospermia rates of less than 30%), while the semen from goat B had intrinsically low-quality semen (average volume was 0.77 ml, motility of less than 60%, concentrations greater than 3×10^9 sperm/ml, and teratospermia rates greater than 35%).

Ejaculates were collected into empty tubes (control 1) or tubes containing 10 ml extender supplemented with 0 (control 2), 0.1, 1, or 5% of bovine serum albumin ($\geq 96\%$ essentially fatty acid-free; Sigma) at 30 C. Immediately after collection, the tubes containing ejaculates were kept in a lukewarm water bottle at 30 C, transferred to the laboratory within 15 min (time elapsed before centrifugation and semen evaluation), and then immersed in a water bath at 30 C. An aliquot of the collected semen was diluted in extender to give a final concentration of $50\text{--}100 \times 10^6$ sperm/ml for evaluation of motion parameters without washing immediately after collection using a computer-assisted sperm analysis (CASA) system (Hamilton Thorne Research, Beverly, MA, USA).

Freezing procedure and thawing of spermatozoa

The semen was processed and frozen using a modified procedure based on the two-step dilution pellet freezing method originally described by Aboagla and Terada [9]. In the first step, the semen was diluted with extender to give a concentration of $50\text{--}100 \times 10^7$ sperm/ml and was centrifuged

twice at $500 \times g$ for 10 min at 30 C. The pelleted spermatozoa were resuspended in egg yolk trehalose extender and adjusted to 400×10^6 spermatozoa/ml. Then, the extended spermatozoa were allowed to cool to 5 C within a 2 h. In the second step, an equal volume of egg yolk trehalose extender containing 8% glycerol was added to the first extended spermatozoa, resulting in a final glycerol concentration of 4% and final spermatozoa concentration of 200×10^6 spermatozoa/ml. This was then equilibrated at 5 C for 15 min before freezing. Finally, the spermatozoa were frozen in pellets for 1h on a dry-ice surface by dropping 200 μ l volumes of the semen into holes engraved in the surface, and then the pellets were plunged into liquid nitrogen. Thawing was performed in a TCG solution at 37 C. Frozen semen pellets were diluted 1:3 into 0.6 ml TCG solution and allowed to equilibrate at 37 C for 10 min before post-thaw analysis.

Sperm motility assessment

The sperm motility parameters were assessed using a CASA system. The motility analyzer was set as described previously [7]. The obtained motility parameters were as follows: 1) motility was defined as the percentage of motile cells and 2) progressive motility was defined as the percentage of cells moving with VAP >25 μ m/sec and STR >80. The recovery rate was measured as the ratio of the sperm post-thawed motility parameters to the pre-freeze values.

Thermal resistance test

Tubes containing frozen-thawed spermatozoa diluted four-fold with TCG were immersed in a water bath at 37 C. Samples were evaluated 1, 2, and 3 h after incubation to determine sperm motility and progressive motility.

Evaluation of sperm acrosome integrity

The acrosome status of spermatozoa was assessed with fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; Sigma) staining, as described previously [7]. Spermatozoa were categorized into three groups as follows (Fig.3): 1) spermatozoa displaying disrupted intensively bright fluorescence of the acrosomal cap, indicating an intact acrosome; 2) spermatozoa displaying disrupted fluorescence of the acrosomal cap, indicating the process of breakdown of the

acrosomal cap (partially damaged acrosome); and 3) spermatozoa displaying no fluorescence, indicating complete loss of the outer acrosomal membrane (lost acrosome).

Statistical analysis

Experiments were repeated three times, and the data was analyzed by analysis of variance (ANOVA) and Fisher's protected least-significant difference post-hoc test using the STATVIEW Software (Abacus Concepts, Inc., Berkeley, CA, USA). All percentage data was subjected to an arcsine transformation before statistical analysis. Data are expressed as mean $\pm$ SEM. Differences in sperm quality were considered significant when P-values were lower than 0.05.

Results

When the goat A and B semen were collected into tubes containing 10 ml of extender without BSA, their motility and progressive motility were higher than those of semen collected into empty tubes (Table 1). The motility and progressive motility of semen from both goats that was collected into tubes with 5% BSA-containing extender significantly ($P < 0.05$) increased as compared with those of semen collected into empty tubes.

When semen collected from goats into tubes containing 10 ml of extender without BSA were frozen as pellets in egg yolk trehalose extender supplemented with glycerol, the post-thaw spermatozoa exhibited a small improvement in motility and progressive motility compared with those of semen collected into tubes without extender, but no significant differences were observed between them (Table 1). The semen of goat A that was collected into tubes containing extender supplemented with higher concentrations of BSA produced a slightly improved tendency in post-thaw motility and progressive motility compared with those of semen collected into tubes containing lower concentrations of BSA. There were significant ($P < 0.05$) differences in post-thaw motility between the spermatozoa of goat A that were collected into tubes with 5% BSA-containing extender and those collected in an empty tube. Semen collected from goat B into tubes with 5% BSA-containing extender showed significantly higher ($P < 0.05$) post-thaw motility and progressive

Table 1. Motion parameters and recovery rates of spermatozoa collected into tubes containing 10 ml of extender supplemented with different concentrations of BSA

Goat	Survival	Treatment	After collection	After washing	Pre-freezing ²⁾	Post-thawing ³⁾	Recovery rate(%) ¹⁾
A	Motility (%)	TCG 0 ml (control 1)	82.0 ± 2.0 ^a	91.0 ± 1.0	83.3 ± 2.8	58.6 ± 5.9 ^a	68.3 ± 9.4
		TCG 10 ml (control 2)	86.6 ± 1.8 ^{ab}	91.3 ± 0.3	83.3 ± 1.6	68.0 ± 5.5 ^{ab}	79.3 ± 7.5
		TCG 10 ml + BSA 0.1%	84.3 ± 2.8 ^{ab}	90.0 ± 2.6	80.6 ± 3.7	67.3 ± 5.4 ^{ab}	75.3 ± 1.7
		TCG 10 ml + BSA 1%	85.6 ± 0.3 ^{ab}	93.3 ± 0.8	86.0 ± 0.5	68.3 ± 4.6 ^{ab}	75.3 ± 6.3
		TCG 10 ml + BSA 5%	91.6 ± 0.3 ^b	93.6 ± 0.6	86.3 ± 3.9	77.3 ± 3.7 ^b	89.0 ± 7.0
	Progressive motility (%)	TCG 0 ml (control 1)	55.0 ± 1.7 ^a	71.3 ± 0.3	53.0 ± 0.5	42.0 ± 6.4	78.6 ± 11.6
		TCG 10 ml (control 2)	59.6 ± 1.3 ^{ab}	70.3 ± 3.1	56.3 ± 2.6	47.3 ± 2.9	83.3 ± 3.7
		TCG 10 ml + BSA 0.1%	58.0 ± 3.5 ^{ab}	66.3 ± 3.6	49.0 ± 3.7	48.6 ± 3.8	100.3 ± 12.3
		TCG 10 ml + BSA 1%	63.3 ± 2.6 ^{bc}	73.3 ± 1.8	59.6 ± 1.8	48.0 ± 3.0	80.0 ± 2.5
		TCG 10 ml + BSA 5%	67.6 ± 2.3 ^c	67.3 ± 1.3	52.3 ± 0.3	48.6 ± 2.1	97.6 ± 3.9
B	Motility (%)	TCG 0 ml (control 1)	55.0 ± 2.30 ^a	53.0 ± 2.51 ^a	40.0 ± 2.08 ^a	13.3 ± 0.33 ^a	33.2 ± 1.76 ^a
		TCG 10 ml (control 2)	75.3 ± 4.37 ^{bc}	74.6 ± 8.00 ^b	64.0 ± 3.05 ^b	17.3 ± 2.84 ^a	27.3 ± 5.45 ^a
		TCG 10 ml + BSA 0.1%	70.3 ± 1.45 ^b	82.3 ± 1.66 ^{bc}	66.3 ± 0.88 ^{bc}	28.0 ± 1.73 ^b	41.7 ± 2.90 ^b
		TCG 10 ml + BSA 1 %	85.1 ± 2.96 ^c	90.3 ± 0.88 ^c	68.3 ± 4.63 ^{bc}	53.0 ± 2.30 ^c	78.3 ± 8.35 ^c
		TCG 10 ml + BSA 5 %	86.0 ± 2.64 ^d	91.6 ± 1.45 ^c	73.0 ± 1.52 ^c	67.3 ± 4.70 ^d	91.7 ± 5.36 ^d
	Progressive Motility (%)	TCG 0 ml (control 1)	37.3 ± 2.18 ^a	41.0 ± 1.52 ^a	26.6 ± 0.88 ^a	11.3 ± 0.88 ^a	42.3 ± 2.72 ^a
		TCG 10 ml (control 2)	51.3 ± 3.93 ^b	54.3 ± 3.84 ^b	39.0 ± 2.00 ^b	11.0 ± 1.52 ^a	27.7 ± 3.75 ^a
		TCG 10 ml + BSA 0.1%	54.0 ± 0.57 ^{bc}	51.0 ± 0.57 ^b	48.3 ± 0.88 ^{bc}	20.0 ± 2.30 ^b	41.3 ± 5.48 ^b
		TCG 10 ml + BSA 1 %	61.6 ± 3.52 ^c	67.0 ± 1.52 ^c	43.3 ± 1.66 ^{bc}	40.3 ± 2.96 ^c	99.7 ± 10.80 ^c
		TCG 10 ml + BSA 5 %	61.3 ± 2.40 ^c	66.3 ± 2.40 ^c	45.5 ± 2.64 ^c	50.6 ± 3.93 ^d	112.3 ± 7.35 ^d

¹⁾Recovery rate=post-thawing value/pre-freezing value. ²⁾Pre-freezing motility and progressive motility were evaluated 170 min after collection (just before freezing). ³⁾Post-thawing motility and progressive motility were evaluated 10 min after thawing. Values are mean percentages (n=3) ± SEM. ^{a-d}Different superscripts within the same column denote significant differences (P < 0.05).

motility than the semen collected into tubes containing extender supplemented with lower concentrations of BSA.

In addition, although there was no clear tendency for the motility and progressive motility before freezing of semen collected from goat A into extender supplemented with any concentration of BSA, for the semen goat B that was collected into tubes with extenders containing various concentrations of BSA, the pre-freezing motility and progressive motility were significantly increased with elevated concentrations of BSA, which were added to the extender in the semen collection tubes (Table 1).

In the case of freezing the semen of goat A that was collected into tubes containing extender supplemented with higher concentrations of BSA, the recovery rates for both motility and progressive motility slightly increased compared with those of semen collected into tubes containing extender supplemented with lower concentrations of BSA (Table 1). Furthermore, in the case of goat B, the

recovery rates for semen collected in extenders supplemented with elevated concentrations of BSA significantly (P < 0.05) increased, with the best results being obtained when collected in the extender supplemented with 5% BSA.

When collected from goat A into tubes with 5% BSA-containing extender, frozen-thawed sperm maintained higher motility and progressive motility throughout a 3-h thermal resistance test at 37 °C than semen collected into tubes containing extender supplemented with lower concentrations of BSA (Fig. 1A). The semen of goat B that was collected into tubes with 5% BSA-containing extender also maintained significantly (P < 0.05) higher post-thaw motility and progressive motility throughout the incubation period than those collected into the lower concentrations of BSA (Fig. 1B). In contrast, freezing semen that was collected from goat B into empty tubes resulted in the lowest motility and progressive motility at all points during incubation (Figs. 1A and B).

After freeze-thawing of semen collected from

Fig. 1. Motility and progressive motility of spermatozoa collected from goats A and B into tubes containing 10 ml of extender supplemented with different concentrations of BSA, frozen-thawed, and subjected to a thermal resistance test at 37 C. Values are mean percentages ($n=3$) $\pm$ SEM. ^{a-d}Different superscripts within the same column denote significant differences ($P<0.05$).

goat A into tubes supplemented with 5% BSA-containing extender, a significantly ($P<0.05$) higher proportion of spermatozoa with intact acrosomes was found as compared with that collected into tubes without extender (Fig. 2A). In the case of goat B, there were significant ($P<0.05$) differences in the proportions of spermatozoa with intact acrosomes after freeze-thawing between semen collected into tubes with and without 10 ml of extender (Fig. 2B). When semen collected from goat B into tubes with extenders containing various concentrations of BSA was frozen, the proportions of spermatozoa with intact acrosomes were

significantly increased ($P<0.05$) with elevated concentrations of BSA, which were added to the extender in the semen collection tubes.

Discussion

In the present study, the spermatozoa of semen collected from goat A into tubes containing 10 ml of extender without BSA tended to have higher motility and progressive motility before washing than those of the semen collected into empty tubes. In the case of goat B, significantly higher motility

and progressive motility were obtained from semen collected into tubes containing 10 ml of extender than from those collected into empty tubes. These results are in agreement with our

previous studies, in which semen collected into tubes containing a large volume of extender produced higher sperm motility, progressive motility, and acrosome integrity than semen collected into tubes with a lower volume of extender [7]. Concerning the physiological role of seminal plasma on the functions of ejaculated spermatozoa, Cross [10] discovered a detrimental effect of seminal plasma on sperm membrane integrity and identified prostasome as the inhibitory factor in seminal plasma, which is expelled from the prostate gland at ejaculation. Thus, it was indicated that reduction of contact between seminal plasma components, such as prostasomes, and goat spermatozoa at ejaculation by collection in extender solution exerts a profound effect on improving the characteristics of these spermatozoa.

When semen collected into tubes containing extender supplemented with various concentrations of BSA was subjected to freezing as a pellet in egg-yolk trehalose extender including 0.04% (W/V) SDS and 4% glycerol, the post-thaw motility, progressive motility and acrosome integrity of the frozen-thawed spermatozoa were significantly enhanced with increasing concentrations of BSA, which were added to the extender in the semen collection tubes. Furthermore, using extender supplemented with 5% BSA for semen collection maintained a significantly higher motility and progressive motility for frozen-thawed spermatozoa throughout incubation for a 3-h thermal resistance

Fig. 2. Acrosome status of frozen-thawed spermatozoa collected from goats A and B into tubes containing 10 ml of extender supplemented with different concentrations of BSA. The acrosome status of spermatozoa was determined immediately after thawing. Values are mean percentages ($n=3$) $\pm$ SEM. ^{a-d}Different superscripts within the same column denote significant differences ($P<0.05$).

Fig. 3. Photographs of goat sperm acrosomes stained with FITC-PNA. The sample field of image A was obtained with phase contrast microscope. The sample field of image B was obtained with fluorescence microscope. →: Intact acrosome, ---: Partially damaged, ▲: Lost acrosome. Scale bars represent 10 μ m.

test than for those collected into tubes containing extender supplemented with lower concentrations of BSA. This data clearly shows that semen collection into tubes containing a large volume of extender supplemented with higher concentrations of BSA improves the freezability of ejaculated spermatozoa. To our knowledge, this is the first report of improving sperm freezability by means of a semen collection method.

Several researchers have provided direct evidence that BSA, which in extender adheres rapidly to the sperm membrane at the moment of dilution [11], modifies the sperm lipid composition through lipid exchange or hydrolysis [12], promotes plasma membrane protein hydrolysis [13], causes influx of Ca^{2+} ions into the cytoplasm [11], and decreases the cholesterol and phospholipid ratio in the plasma membrane of spermatozoa [14, 15]. Moreover, it is well established that cholesterol efflux from the sperm membrane induces enhanced membrane fluidity [16, 17], while elevation of membrane cholesterol is associated with decreased membrane fluidity [18–20]. Thus, it is conceivable that cholesterol efflux from sperm membranes by BSA would enhance the fluidity of membranes. These findings combined with the present results show that there is improved freezability of ejaculated spermatozoa by collecting semen into tubes with BSA-containing extender. It can therefore be concluded that use of BSA in an extender for semen collection may contribute to extraction of cholesterol from the sperm membrane and lead to high fluidity of sperm membranes before freezing, ultimately enhancing the cryosurvival of spermatozoa. However, the acrosome reaction might not occur coincidentally in cases of collection of spermatozoa into tubes containing extender supplemented with BSA. After freeze-thawing of semen collected from both goats into tubes with 5% BSA-containing extender, a significantly higher proportion of spermatozoa with intact acrosomes was found as compared with semen collected into tubes containing extender supplemented with lower concentrations of BSA. This assumption is clearly supported by the results

of Zeng and Terada [21], who conducted an interesting study of cholesterol efflux in boar sperm membranes. They provided evidence that removing the membrane cholesterol by exposure of spermatozoa to cyclodextrin results in enhanced cryosurvival of spermatozoa, as judged by intact acrosomes and motion parameters. Furthermore, our expectations were also supported by data from Aboagla and Terada [22], who showed that trehalose plays a major role in increasing membrane fluidity and imparts greater endurance to goat spermatozoa against freeze-thawing damage.

Our study also indicated that semen collection into 5% BSA-containing extender results in significantly ($P < 0.05$) higher motility and progressive motility before freezing as compared with semen collected into extender supplemented with lower concentrations of BSA. This supports the idea that membrane cholesterol is removed by BSA in extender, and that the resultant increasing membrane fluidity, as described above, may down-regulate the susceptibility of goat spermatozoa to cold shock. In agreement with this hypothesis, Zeng and Terada [23] found that exposing boar spermatozoa to 2-hydroxypropyl-beta-cyclodextrin to deplete its membrane cholesterol results in enhanced the resistance of spermatozoa to cold shock.

In summary, the results from the present study demonstrate that semen collection into tubes with extender, and especially extender containing higher concentrations of BSA, dramatically improves the motility and acrosome integrity of frozen-thawed spermatozoa. These findings provide the first experimental evidence that the *in vitro* functional ability, and especially freezability, of epididymal spermatozoa is abruptly modified by flash sperm contact with BSA at ejaculation. Furthermore, it should be noted that semen collection into tubes containing extender supplemented with BSA fulfills a strong demand to improve and expand application of this semen collection method to other domestic animals.

References

1. Blash S, Melican D, Gavin W. Cryopreservation of epididymal sperm obtained at necropsy from goat. *Theriogenology* 2000; 54: 899–905.
2. Kikuchi K, Nagai T, Kashiwazaki N, Ikeda H,

- Noguchi J, Shimada A, Soloy E, Kaneko H. Cryopreservation and ensuing in vitro fertilization ability of boar spermatozoa from epididymides stored at 4 degrees C. *Theriogenology* 1998; 50: 615–623.
3. Rath D, Niemann H. In vitro fertilization of porcine oocytes with fresh and frozen-thawed ejaculated of frozen-thawed epididymal semen obtained from identical boars. *Theriogenology* 1997; 47: 785–793.
4. Barthelemy C, Rotere D, Hammah S, Lebos C, Tharanne MJ, Lansac J. Ultrastructural changes in membranes and acrosome of human sperm during cryopreservation. *Arch Androl* 1990; 25: 29–40.
5. Ritar A J, Salamon S. Effects of seminal plasma and of its removal and of egg yolk in the diluent on the survival of fresh and frozen-thawed spermatozoa of the Angora goat. *Aust J Biol Sci* 1982; 35: 305–312.
6. Leboeuf B, Restall B, Salamon S. Production and storage of goat semen for artificial insemination. *Anim Reprod Sci* 2000; 62: 113–141.
7. Yamashiro H, Kumamoto K, Wang HF, Yamashita Y, Terada T. Effect of semen collection in extender solution on the characteristics of goat spermatozoa. *J Reprod Dev* 2006; 52: 397–406.
8. Aboagla ME, Terada T. Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. *Theriogenology* 2004; 62: 1160–1172.
9. Aboagla ME, Terada T. Effects of the supplementation of trehalose extender containing egg yolk with sodium dodecyl sulfate on the freezability of goat spermatozoa. *Theriogenology* 2004; 62: 809–818.
10. Cross NL, Mahasreshti P. Prostate fraction of human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist progesterone. *Arch Androl* 1997; 39: 39–44.
11. Blank M, Soo L, Britten JS. Adsorption of albumin on rabbit sperm membranes. *J Membr Biol* 1976; 29: 401–409.
12. Davis BK, Byrne R, Hungund B. Studies on the mechanism of capacitation. II. Evidence for lipid transfer between plasma membrane of rat sperm and serum albumin during capacitation in vitro. *Biochim Biophys Acta* 1979; 558: 257–266.
13. Davis BK, Gergely AF. Studies on the mechanism of capacitation: changes in plasma membrane proteins of rat spermatozoa during incubation in vitro. *Biochem Biophys Res Commun* 1979; 88: 613–618.
14. Davis BK, Byrne R, Bedigian K. Studies on the mechanism of capacitation: albumin-mediated changes in plasma membrane lipids during in vitro incubation of rat sperm cells. *Proc Natl Acad Sci USA* 1980; 77: 1546–1550.
15. Singleton CL, Killian GJ. A study of phospholipase in albumin and its role in inducing the acrosome reaction of guinea pig spermatozoa in vitro. *J Androl* 1983; 4: 150–156.
16. Grunze M, Deuticke B. Changes of membrane permeability due to extensive cholesterol depletion in mammalian erythrocytes. *Biochim Biophys Acta* 1974; 356: 125–130.
17. Cooper RA, Leslie MH, Fischkoff S, Shinitzky M, Shattil SJ. Factors influencing the lipid composition and fluidity of red cell membranes in vitro: production of red cells possessing more than two cholesterol per phospholipid. *Biochemistry* 1978; 17: 327–331.
18. Vanderkooi J, Fischkoff S, Chance B, Cooper RA. Fluorescent probe analysis of the lipid architecture of natural and experimental cholesterol-rich membranes. *Biochemistry* 1974; 13: 1589–1595.
19. Shattil SL, Cooper RA. Membrane microviscosity and human platelet function. *Biochemistry* 1976; 15: 4832–4837.
20. Hartel S, Diehl HA, Ojeda SF. Methyl-beta-cyclodextrins and liposomes as water-soluble carriers for cholesterol incorporation into membranes and its evaluation by a microenzymatic fluorescence assay and membrane fluidity-sensitive dyes. *Anal Biochem* 1998; 258: 277–284.
21. Zeng WX, Terada T. Effects of methyl-beta-cyclodextrin on cryosurvival of boar spermatozoa. *J Androl* 2001; 22: 111–118.
22. Aboagla ME, Terada T. Trehalose enhanced fluidity of the goat sperm membrane and its protection during freezing. *Biol Reprod* 2003; 69: 1245–1250.
23. Zeng WX, Terada T. Protection of boar spermatozoa from cold shock damage by 2-hydroxypropyl-beta-cyclodextrin. *Theriogenology* 2001; 55: 615–627.