Effects of Seminal Plasma Components on Motility and Acrosomal Integrity of Meishan Boar Spermatozoa after Cooling Treatments

Hiroshi HARAYAMA, Akira IMANO¹,
Masashi MIYAKE and Seishiro KATO¹

Graduate School of Science and Technology, Kobe University,
Nada-ku, Kobe-shi 657-8501

¹ Faculty of Agriculture, Kobe University, Nada-ku, Kobe-shi 657-8501

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Abstract This study was conducted to determine effects of seminal plasma components on maintaining motility and acrosomal integrity in Meishan boar spermatozoa during cooling treatments. Ejaculates were collected from a Meishan boar by a manual method. Seminal plasma components were separated by dialysis and anion-exchange column chromatography. Spermatozoa resuspended in TCG solution containing seminal plasma or its components were cooled slowly to 4°C, or rapidly to 0°C, after which sperm motility and acrosomal integrity were examined. For those samples that were cooled slowly, the addition of seminal plasma (5–20%, v/v) exerted protective effects on maintaining sperm motility, but most spermatozoa were immobile at a high concentration (80%). However, the deleterious effects on sperm motility were not detected in seminal plasma dialyzed against TCG solution. When seminal plasma components were separated into four fractions (Fractions 1–4) by anion-exchange column chromatography, the protective effects were detected in Fractions 2 and 4. However, seminal plasma and its components hardly affected acrosomal integrity in the spermatozoa that were cooled slowly. For sperm samples that were cooled rapidly, the protective effects on acrosomal integrity as well as on sperm motility were observed in Fractions 2 and 4. These results imply that Meishan boar seminal plasma contains both beneficial and detrimental components for maintaining sperm motility during the cooling treatments. The formers are characterized by the affinity to the anion-exchange column, *i.e.*, molecules with isoelectric points in acidic and/or neutral ranges, and the latters are passable through the dialysis tubing, *i.e.*, low-molecular-mass molecules.

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Seminal plasma is originated from epididymal plasma and accessory genital gland fluids, and contains a variety of elements²⁹. Some components of seminal plasma have been considered to promote or inhibit sperm fertilization, such as caltrin⁹, acrosome stabilizing factor¹⁰, fertilization-associated proteins²⁰, fertilization promoting polypeptide¹², BSP proteins³¹, and spermadhesin²⁸. Indeed, different aspects have been observed in

the fertilizing process between cauda epididymal and ejaculated spermatozoa³².

Boar epididymal spermatozoa are mixed with a large quantity of fluids from the accessory glands at ejaculation²⁹, and they become more sensitive to cooling immediately after ejaculation^{7,22}. Many studies on the effects of seminal plasma on motility and acrosomal integrity of spermatozoa during cooling have been made in boars of European and American

breeds. The conclusions, however, are still subject to controversy^{19,23,25}. To our knowledge, no report is available on Meishan boars in spite of their unique reproductive characteristics^{8,13}. The aim of the present study is to determine effects of seminal plasma components separated by dialysis and anion-exchange column chromatography on maintaining motility and acrosomal integrity in Meishan boar spermatozoa during cooling treatments.

Materials and Methods

Preparation of sperm samples and seminal plasma

Sperm-rich fractions from ejaculates were obtained from one mature Meishan boar by a manual method using a dummy. The portion (30 ml) of each sperm-rich fraction was centrifuged at 400 g for 10 min at room temperature and then the spermatozoa were separated from the supernatant. The resulting sperm pellet was resuspended in approximately 1 ml of TCG solution composed of 111.0 mM Tris (hydroxymethyl) aminomethane, 34.7 mM citric acid, and 185.0 mM glucose¹⁸) and the diluted aliquot was layered on a discontinuous gradient consisting of 1 ml of 80% Percoll (Pharmacia Biotech AB, Uppsala, Sweden) and 3 ml of 55% Percoll in TCG. After centrifugation at 700 g for 20 min, the sperm-concentrated portion (bottom) was recovered and then washed three times with TCG solution by centrifugation at 700 g for 10 min each. After the washing treatments, the sperm pellet was resuspended in an equal volume of TCG solution.

Whole ejaculates were collected as described above and centrifuged at 14,250 g for 15 min at 4°C to remove spermatozoa. The obtained seminal plasma was stored at -80°C before use.

Cooling treatments

The suspensions of washed spermatozoa were mixed with a four-fold volume of TCG solution containing seminal plasma and its

components (see below), and used for the cooling treatments. For the slow cooling procedure, the routine procedures for liquid storage of spermatozoa were performed. Briefly, glass test tubes (volume size: 22 ml) containing approximately 2 ml of the sperm samples were placed into about 3 l water (20–25°C) and left overnight in a cold room (4°C). The samples were cooled to 4°C over 4 h by this treatment. For the rapid cooling procedure, glass test tubes containing sperm samples were placed into wet ice (0°C) and left for 20 min.

Assessment of sperm motility and acrosomal integrity

Approximately 0.5 ml of each sperm sample after the cooling treatment was shaken at 37°C for 15 min, and then the percentages of motile spermatozoa were estimated using a bright-field microscope equipped with a heated stage (37°C). The remaining samples were used for the examination of acrosomal integrity¹⁷. One drop of each sperm sample was smeared on a glass slide and air-dried on a hot plate (37°C). The slide was rinsed in water, blotted, and fixed for 45 min in the fixative composed of 10% (v/v) formalin in 6.8% potassium dichromate solution. After fixation, the slide was rinsed in water and stained in a phosphate-buffered solution of Giemsa stain (pH 7.4, Merck, Darmstadt, Germany) for 90 min. Approximately 200 spermatozoa for each preparation were counted at random under a light microscope at 400× or 1,000× to determine the percentages of spermatozoa with a normal acrosome.

Separation of components from seminal plasma

Dialysis of seminal plasma

Dialysis tubing (Seamless Cellulose Tubing, cut-off 12,000–14,000 Da) was purchased from Wako Pure Chemical Corp. (Osaka, Japan). Seminal plasma (30 ml) was dialyzed against 3 l TCG solution at 4°C twice for 24 h each (referred to as dialyzed seminal plasma).

Anion-exchange column chromatography

Seminal plasma (200 ml) was treated with 80% ammonium sulfate at 4°C. The resulting precipitate was separated by centrifugation at 14,500 g for 15 min at 4°C. The precipitate was dissolved in 200 ml 5 mM Tris-HCl (pH 7.4). The solution was dialyzed against 3 l 20 mM Tris-HCl (pH 8.0) for 24 h and then against 40 mM Tris-HCl (pH 9.0) for 24 h, followed by centrifugation at 14,500 g for 15 min at 4°C to remove insoluble constituents (referred to as precipitated seminal plasma components). When the components were used for the cooling treatment, the aliquot (30 ml) was dialyzed against 3 l TCG solution at 4°C twice for 24 h each and then added to the sperm suspension. The remaining solution was applied to a DEAE-Sephacel column (2.2 cm × 26.5 cm) (Pharmacia) equilibrated in 40 mM Tris-HCl (pH 9.0). Elution of components bound to the column was performed with a linear gradient obtained with 200 ml 40 mM Tris-HCl and 200 ml 40 mM Tris-HCl containing 1 M NaCl at a flow rate of 14 ml/h. The fractions of 5 or 10 ml were collected. Prior to undergoing cooling treatments, the fractions were dialyzed against 3 l 10 mM NH_4HCO_3 buffer at 4°C for 24 h, loaded on a prepacked disposable column PD-10 (Pharmacia) equilibrated in distilled water to remove low-molecular-mass (less than 1,000 Da) materials, concentrated by freeze-drying, and diluted with an equal volume of double-strength TCG solution.

Protein concentrations

Protein concentrations in seminal plasma components were determined according to a dye-binding assay using bovine serum albumin as a standard (Bio-Rad protein assay kit, Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

The percentages of motile spermatozoa and of cells with a normal acrosome were subjected to analysis of variance (one-way ANOVA). When F-test was significant, individual means were further tested by Tukey's multiple range test²⁴⁾.

Results

Figure 1 shows effects of seminal plasma on sperm motility after the slow cooling treatment. The percentages of motile spermatozoa were increased by the addition of the intact seminal plasma in a dose-dependent manner between 0 and 20% (v/v), but decreased markedly at 80%. However, such adverse effect was not observed in dialyzed seminal plasma.

The precipitated seminal plasma component solution was as effective as the dialyzed seminal plasma in the maintenance of sperm motility during the slow cooling treatment (data not shown). The typical pattern of fractionation of precipitated seminal plasma components by the DEAE-Sephacel column

Fig. 1. Effects of intact (A) and dialyzed seminal plasma (B) on motility of ejaculated boar spermatozoa after the slow cooling treatment. Values are means $\pm$ SEM of the data which were obtained in the sperm samples shaken at 37°C for 15 min after the cooling treatment. Values with different superscripts differ significantly, $P < 0.05$.

Fig. 2. Chromatography of boar seminal plasma components on DEAE-Sephacel column. Fractions of 10 ml (unbound portion : left figure) and of 5 ml (bound portion : right figure) were collected at a flow rate of 14 ml/h. The absorbance was monitored at 280 nm. The column (2.2 cm $\times$ 26.5 cm) was equilibrated in 40 mM Tris-HCl (pH 9.0). Bound components were eluted with a linear gradient obtained with 200 ml 40 mM Tris-HCl (pH 9.0) and 200 ml 40 mM Tris-HCl containing 1 M NaCl (pH 9.0).

Table 1. Effects of seminal plasma components separated by anion-exchange column chromatography (Fractions 1-4) on motility of ejaculated boar spermatozoa after the slow cooling treatment (n=5)

Fractions (μ g protein/ml) ^a	Control (0)	1 (500)	2 (500)	3 (500)	4 (352)
% of motile sperm ^b	38 $\pm$ 5 ^c	48 $\pm$ 5 ^{de}	65 $\pm$ 3 ^c	48 $\pm$ 3 ^{de}	58 $\pm$ 3 ^{cd}

^a Final concentration of protein in sperm suspension.

^b The percentages of motile sperm were obtained in the sperm samples shaken at 37 °C for 15 min after the cooling treatment.

^{cde} Values within the same line with different superscripts differ significantly, $P < 0.05$.

Values are means $\pm$ SEM.

chromatography is illustrated in Fig. 2. The effects of Fractions 1-4 on sperm motility after the slow cooling treatment are shown in Table 1 and Fig. 3. Significantly higher percentages of motile spermatozoa were obtained by the addition of the Fractions 2 (500 μ g protein/ml at the final concentration in sperm suspension) and 4 (352 μ g protein/ml), compared to control. The increases in the percentages were dependent on the concentrations of these fractions. Similar effects of the Fractions 2 and 4 on sperm motility were observed in the samples after the rapid cooling treatment (Table 2). Moreover, the percentages of spermatozoa with a normal acrosome after the rapid cooling

treatment were significantly higher in the sperm samples containing these fractions (Table 2).

Discussion

The effects of seminal plasma components on the motility of ejaculated spermatozoa have been extensively studied. Low-molecular-mass basic components have deleterious effects on motility of spermatozoa from boars³⁰⁾ and bulls^{1,2)}. Also, a 50 kDa boar seminal vesicle basic protein composed of 14, 16, and 18 kDa subunits reduces sperm motility by inhibiting motility-dynein ATPase^{15,16)}. By contrast, a supplement of ram seminal plasma (10%, v/v)

Fig. 3. Effects of increasing concentrations of Fractions 2 and 4 of seminal plasma components separated by anion-exchange column chromatography on motility of ejaculated boar spermatozoa after the slow cooling treatment ($n=5$). Values are means $\pm$ SEM of the data which were obtained in the sperm samples shaken at 37°C for 15 min after the cooling treatment. Values with different superscripts differ significantly, $P<0.05$.

or of the 5–10 kDa fractions from it to a saline medium produces protective effects on the survival of spermatozoa incubated *in vitro*⁸⁾. Moreover, bull seminal plasma contains <0.5 kDa motility-stimulating factors for spermatozoa inactivated by washing with Ficoll⁹⁾. These observations indicate that seminal plasma contains both detrimental and beneficial components for motility of ejaculated spermatozoa. On the other hand, detrimental components of seminal plasma for the spermatozoa cooled or stored below 15°C have been characterized. The reduction of motility in boar spermatozoa coincident with cooling seems to be associated with basic proteins from seminal vesicles “haemagglutinin”²⁶⁾. Moore *et al.*²²⁾ have reported that the addition of seminal vesicle basic proteins causes damage in plasma membrane integrity and the releasing of enzymes at the sperm head and mid-piece when boar spermatozoa were cooled. Possibly these events may be due to the increased permeability of the membrane^{14,27)}. However, Berger and Clegg⁷⁾ have indicated seminal vesicle secretions are not important in sensitizing the acrosomal membrane to the rapid cooling treatment. By contrast, only limited data are available regarding the characterization of components enhancing sperm cooling

Table 2. Effects of seminal plasma components separated by anion-exchange column chromatography (Fractions 1–4) on motility and acrosomal integrity of ejaculated boar spermatozoa after the rapid cooling treatment ($n=5$)

Fractions (µg protein/ml) ^a	Control (0)	1 (500)	2 (500)	3 (500)	4 (352)
% of motile sperm ^b	19 $\pm$ 5 ^d	32 $\pm$ 4 ^{cd}	40 $\pm$ 5 ^c	36 $\pm$ 7 ^{cd}	44 $\pm$ 4 ^c
% of sperm with normal acrosome ^b	4 $\pm$ 0 ^e	6 $\pm$ 1 ^{de}	10 $\pm$ 1 ^{cd}	6 $\pm$ 2 ^{de}	12 $\pm$ 2 ^c

^a Final concentration of protein in sperm suspension.

^b The percentages of motile sperm were obtained in the sperm samples shaken at 37 °C for 15 min after the cooling treatment. The percentages of sperm with a normal acrosome were assessed by the observation of sperm preparations stained with a phosphate-buffered solution of Giemsa stain.

^{cd} Values within the same line with different superscripts differ significantly, $P<0.05$.

Values are means $\pm$ SEM.

resistance, although the presence of them has been assumed^{4,11,19,26}.

In the present study, the addition of Meishan boar seminal plasma at a concentration of 80% (v/v) adversely affected sperm motility after the slow cooling treatment (Fig. 1), although more than 70% of spermatozoa were motile immediately before the cooling treatment (data not shown). However, dialysis against TCG solution (cut-off, 12,000–14,000 Da) abolished the deleterious effects (Fig. 1). These findings suggest that boar seminal plasma contains damaging factors for sperm motility during the slow cooling treatment. Moreover, the factors can be removed from seminal plasma by the dialysis. This is supported by the previous results obtained with boars of European and American breeds⁶) that motility of boar spermatozoa was maintained at the high level for 7 days by dialyzing semen in cellulose tubing at 15°C. However, it remains to be determined the factors which exert deleterious effects on cooling resistance or motility system in spermatozoa.

Our results revealed that the addition of seminal plasma (5–20%) to TCG solution increased the percentages of motile spermatozoa after the slow cooling treatment (Fig. 1). This is in agreement with the previous results obtained with Landrace boar spermatozoa stored at 4°C for 5 days in TCG solution containing egg yolk¹⁹), although aggregation of egg yolk occurred by the seminal plasma components²¹). When Fractions 2 and 4 were added to sperm samples, the percentages of motile spermatozoa after the slow cooling treatment increased in a dose-dependent manner (Table 1 and Fig. 3). Although the addition of these fractions had little influence on the percentages of motile spermatozoa immediately before cooling treatments (data not shown), they had protective effects on maintaining the motility of spermatozoa during the rapid cooling treatment (Table 2). These fractions also exerted protective effects on the acrosomal integrity of

spermatozoa cooled rapidly (Table 2). These findings imply that Meishan boar seminal plasma contains components enhancing cooling resistance in spermatozoa.

Our data suggest that Meishan boar seminal plasma contains both beneficial and detrimental components for maintaining sperm motility during the cooling treatment. The formers are characterized by the affinity to the anion-exchange column, *i.e.*, molecules with isoelectric points in acidic and/or neutral ranges, and the latters are passable through the dialysis tubing, *i.e.*, low-molecular-mass molecules. It could be also suggested that protective effects of seminal plasma components should be taken into consideration when developing protocols for liquid storage of Meishan boar spermatozoa. However, these suggestions remain to be confirmed for spermatozoa ejaculated from boars of other breeds.

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冷却処理後の梅山豚射出精子の運動性および先体の形態に 及ぼす精漿成分の影響

原山 洋・今野 彰¹・三宅正史・加藤征史郎¹

神戸大学大学院自然科学研究科, 神戸市灘区 657-8501

¹ 神戸大学農学部, 神戸市灘区 657-8501

冷却処理中の梅山豚精子の運動性および先体の正常性の維持に及ぼす精漿成分の影響について検討した。精漿成分は透析および陰イオン交換カラムクロマトグラフィーにより分離した。ついで、洗浄した射出精子を浮遊させた TCG 液に精漿成分を添加した後、4℃まで緩慢に、または 0℃まで急速に冷却し、冷却処理後の精子の運動性および先体の正常性を観察した。緩慢冷却される精子浮遊液に 5~20% (v/v) の濃度で精漿を添加した場合、精子の運動性は無添加区に比べて良好に維持されたが、添加濃度を 80% にすると大部分の精子が運動性を失った。しかし、透析処理後の精漿では添加濃度 80% 区においても精子の運動性は良好に維持された。陰イオン交換カラムクロマトグラフィーにより精漿成分を 4 分画 (分画 1~4) に分離した場合、緩慢冷却後の精子の運動性維持には分画 2 および 4 が好適に作用した。しかし、これらの精漿成分は緩慢冷却された精子での先体の正常性維持にはほとんど影響しなかった。他方、急速冷却される精子浮遊液に分画 2 および 4 の精漿成分を添加した場合には、精子の運動性および先体の正常性の両方が無添加区よりも良好に維持された。以上の結果より、梅山豚の精漿には冷却処理中の精子の運動性の維持に有害な成分だけでなく好適な成分も含まれ、これらの成分はそれぞれ低分子マス成分、および酸性域または中性域に等電点をもつ成分と推定される。

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