Premature Capacitation of Frozen-Thawed Spermatozoa from Subfertile Japanese Black Cattle

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Abstract. Artificial insemination (AI) subfertility is an indication of failure of AI with frozen-thawed sperm classified as normal by conventional semen examination. Recently, 8 AI-subfertile Japanese Black cattle (S1–S8) were identified using the routine AI test or in vivo fertilization test, which included AI with frozen-thawed sperm of superovulated females and subsequent non-surgical recovery of presumptive zygotes. In the present study, we assessed capacitation states and in vitro oocyte penetration of frozen-thawed sperm from these bulls to estimate causal factors of AI subfertility. Frozen-thawed sperm from 8 AI-subfertile (S1–S8) and 9 fertile (F1–F9, control) bulls were washed and then used for a chlortetracycline (CTC) staining assay and in vitro fertilization test. The CTC staining assay revealed that approximately 50% of the sperm from 4 of the AI-subfertile bulls (S5–S8) were prematurely progressing into the capacitation state immediately after washing and resuspension in a CaCl2-lacking medium. In contrast, most of the sperm from the fertile bulls and other AI-subfertile bulls (S1–S4) remained uncapacitated. Addition of CaCl2 to the medium effectively promoted a spontaneous acrosome reaction in the sperm samples from the AI-subfertile bulls (S5–S8). Moreover, the in vitro fertilization test showed that rates of sperm penetration into oocytes were significantly lower in sperm samples from the AI-subfertile bulls (S5–S8) than in the control sperm samples from the fertile bulls (F2–F4 and F7–F9). It has previously been suggested that prematurely capacitated sperm undergo spontaneous acrosome reaction possibly due to uncontrolled influx of calcium ion, and consequently they possess relatively lower in vitro fertilizing ability. It is therefore possible that premature capacitation of sperm used for AI is a causal factor of subfertility of male Japanese Black cattle and a potentially good marker for identification of subfertile bulls for removal from AI programs.

Key words: Bull, Capacitation, Chlortetracycline (CTC), In vitro fertilization (IVF), Subfertility


The subfertility derived from male factors is of concern in cattle artificial insemination (AI) because it could cause disintegration of the breeding system and large economic losses, particularly when the subfertility affects a genetically superior sire. Therefore, determination of the causal factors of male subfertility in AI programs is urgently required. The reproductive performance of bulls is usually estimated by conventional examination of sperm characteristics, including total number, concentration, viability, motility and morphology of spermatozoa. In the actual examination, in vivo sperm fertilizing ability is assessed by observation for pregnancy in females artificially inseminated...
with the bull’s frozen spermatozoa [1, 2]. An increasing number of AI-subfertility cases have recently been found in the representative breed of Japanese beef cattle, Japanese Black cattle [3, 4]. Surprisingly, many cases of male AI subfertility in Japanese Black cattle are barely detectable by conventional examination of sperm characteristics, because the sperm characteristics of AI-subfertile bulls are classified as normal. However, artificial insemination with frozen spermatozoa from these AI-subfertile bulls fails to produce pregnancy in female cattle [3]. Thus, we hypothesize that spermatozoa from these AI-subfertile bulls might have abnormal characteristics in relation to the process of expression of fertilizing ability in the female reproductive tract after AI.

Mammalian spermatozoa do not exhibit fertilizing ability immediately after ejaculation. The spermatozoa undergo a variety of modifications for fertilization, including rearrangement of the membrane, alteration of motility pattern and change of metabolic activity, during the several hours they remain in the female reproductive tract. These physiological changes are collectively called capacitation [5]. Capacitation is apparently promoted by the action of fluid components of the female reproductive tract, including bicarbonate and calcium ions [6]. Consequently, spermatozoa become capable of undergoing the acrosome reaction, acrosomal exocytosis, in response to physiological factors, such as progesterone and zona pellucida glycoprotein [7, 8]. This exocytotic event results in release of enzymes (principally a trypsin-like acrosin) from the acrosome. As a result of these events, spermatozoa become capable of penetrating into the zona pellucida. For successful fertilization, spermatozoa must reach the site of fertilization where an oocyte is present, complete capacitation and subsequently undergo the acrosome reaction on the surface of the oocyte. However, it has been reported that cryopreservation affects the proteins and lipids of the sperm plasma membrane. These molecular alterations lead to premature capacitation and subsequently a spontaneous acrosome reaction prior to arrival at the ampulla of the oviduct when prematurely capacitated spermatozoa are used for AI [9]. Therefore, a laboratory assay for sperm capacitation state should be used to evaluate the normality of spermatozoa after freezing and thawing. The capacitation state can be evaluated using a chlortetracycline (CTC) staining assay that is valid for bull spermatozoa [10] as well as mouse spermatozoa [11]. Moreover, the in vitro fertilization test is a good laboratory assay for evaluation of sperm penetration into oocytes in vitro [2].

The purpose of this study was to estimate causal factors of AI subfertility in male Japanese Black cattle. We comparatively assessed the fertilization-related parameters of frozen spermatozoa from AI-subfertile and fertile (control) bulls after thawing and subsequent washing. Initially (as a supplemental experiment), sperm motility was subjectively observed under a light microscope. Sperm capacitation state was then determined using the CTC staining assay. Finally, sperm penetration into oocytes was examined using the in vitro fertilization test.

Materials and Methods

Preparation of frozen semen

Frozen semen samples from 17 Japanese Black cattle, 8 AI-subfertile (S1–S8) and 9 fertile (F1–F9, control) bulls, were produced by the straw method using an egg yolk-Tris-citrate extender at the Northern Center of Agricultural Technology, General Technological Center of Hyogo Prefecture for Agriculture, Forest and Fishery (Wadayama Hyogo, Japan). Eight AI-subfertile bulls (S1–S8) were identified by routine AI test (the no. of AIs and non-return rates were 42 and 2% for S1, 23 and 0% for S2, 70 and 4% for S5, 191 and 19% for S6 and 142 and 1% for S7 and 21 and 5% for S8) or by in vivo fertilization test, which included AI of superovulated females with frozen-thawed spermatozoa and subsequent nonsurgical recovery of presumed zygotes (the no. of tests, no. of recovered zygotes and rate of A- or B-ranked zygotes were 4, 8 and 0% for S3 and 4, 33 and 6% for S4). The fertility of each control bull (F1–F9) was also confirmed by AI test (the no. of tests and non-return rates for F1–F9 were greater than 100 and 40%, respectively) and by in vivo fertilization test (the no. of recovered zygotes and rate of A- or B-ranked zygotes for S8 were 46 and 76%, respectively).

Evaluation of sperm motility and capacitation state

Frozen semen samples were thawed in 38.5 C water and layered on 9 ml of phosphate-buffered saline (PBS) containing 0.1% polyvinyl alcohol (PVA; Sigma-Aldrich, St. Louis, MO, USA; PBS-
PVA) in a 15-ml tube. The tube was centrifuged at 700 × g for 10 min. After aspiration of the resultant supernatant, the spermatozoa were resuspended in 10 ml PBS-PVA and washed twice by centrifugation at 700 × g for 5 min. The washed spermatozoa were finally resuspended in Hepes (37 mM, Dojindo, Kumamoto, Japan)-buffered Bracket & Oliphant (BO) medium (pH 7.4) [12] containing 0.1% PVA instead of bovine serum albumin (BSA) and lacking NaHCO₃ and CaCl₂ (BO-H (Ca-)) to adjust the final sperm concentration to 1.0 × 10⁸ cells/ml. We believe that this medium is rarely or only slightly effective for promotion of sperm capacitation, since it does not contain any capacitation-supporting components, such as bicarbonate, calcium and bovine serum albumin.

Immediately after resuspension in a BO-H (Ca-) medium, aliquots of the samples were evaluated for sperm motility under a light microscope on a 38.5°C heater. The percentages of progressive motility and non-progressive motility were estimated subjectively. The balances of the samples were used for the CTC staining assay to evaluate the sperm capacitation state. Staining was performed as described previously [13] with a minor modification. Briefly, the CTC solution was composed of 750 μM CTC (Sigma-Aldrich), 5 mM cysteine (Sigma-Aldrich), 130 mM NaCl and 20 mM tris(hydroxymethyl)aminomethane (pH 7.8). The sperm suspension (45 μl) was treated with the CTC solution (45 μl) for 30 sec and subsequently mixed with 12.5% paraformaldehyde in 0.5 M Tris buffer (8 μl, pH 7.4). One drop of the sperm suspension was placed on a glass slide with one drop of 0.22 M 1,4-diazabicyclo[2.2.2]octane (Sigma-Aldrich) dissolved in glycerol and PBS (9:1, v/v) and was covered with a coverslip. One hundred cells were assessed for each preparation under a differential interference microscope equipped with epifluorescence (EFD2 and BV filter set containing a 400–440 nm excitation filter, 455 nm dichroic mirror and 470 nm emission filter, Nikon, Tokyo, Japan). Spermatozoa were classified into the following three patterns. The F pattern consisted of uniform fluorescence over the entire head (uncapacitated spermatozoa). The B pattern consisted of dark fluorescence in the postacrosome and relatively bright fluorescence in the intact acrosome (capacitating and capacitated spermatozoa). The AR pattern consisted of almost no fluorescence over the entire head except for a thin band of fluorescence in the equatorial segment (acrosome-reacted spermatozoa) or dark fluorescence in the postacrosome and relatively bright fluorescence in the disintegrated acrosome (acrosome-reacting spermatozoa).

Evaluation of sperm penetration into oocytes in vitro

Thawed spermatozoa were loaded onto a two-step gradient of 3 ml and 4 ml of 60% and 45% isotonic Percoll (GE Healthcare Bio-Sciences, Piscataway, NJ, USA), respectively, that was prepared with a modified synthetic oviduct fluid medium (mSOF) [14] containing 6 mg/ml BSA (Wako Pure Chemical Industries, Osaka, Japan), 5.0 mM taurine (Sigma-Aldrich), 1.0 mM L-glutamine (Sigma-Aldrich), 0.91 mg/ml mannitol (Sigma-Aldrich) and 2 units/ml heparin (Wako) (mSOF+) in a 15-ml plastic centrifugation tube and centrifuged at 700 × g for 15 min. The recovered spermatozoa were resuspended in mSOF+ medium, washed twice by centrifugation at 2,500 × g for 20 sec and finally resuspended in mSOF+ medium to adjust the sperm concentration to 1.0 × 10⁷ cells/ml.

Bovine ovaries were obtained at a local slaughterhouse and transported to our laboratory. The ovaries were washed once in 0.2% cetyltrimethylammonium bromide and twice in PBS-PVA. Oocytes with cumulus cells were aspirated with an 18-G injection needle from healthy antral follicles with a diameter of 3–6 mm and washed three times in Hepes-buffered medium 199 (Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% PVA, 25 mM Hepes, 0.85 mg/ml sodium bicarbonate and 0.08 mg/ml kanamycin sulphate (Sigma-Aldrich). The recovered oocytes with cumulus cells were cultured in a 0.5 ml drop of culture medium covered with oil (Wako) in a 4-well dish (Nunc A/S, Roskilde, Denmark) at 38.5°C in 5% CO₂ in air for 21–23 h. The culture medium was a bicarbonate-buffered medium 199 supplemented with 10% (v/v) fetal bovine serum (FBS, Dainippon Sumitomo Pharma, Osaka, Japan), 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulphate and 2.2 mg/ml sodium bicarbonate.

After maturation culture, oocytes with cumulus cells were inseminated with 2 × 10⁶/ml spermatozoa in mSOF+ medium (5% CO₂ in air at 38.5°C). After insemination for 3–5 h or 9 h, the spermatozoa attached to cumulus cells were removed by gentle pipetting in PBS-PVA. The presumptive zygotes after 9-h insemination (experimental series #1) were mounted onto a glass slide, fixed with ace-
tic acid and ethanol (1:3, v/v) and stained with 1% aceto-orcein (Wako). The stained zygotes were examined by Nomarski interference microscopy (Olympus Optical, Tokyo, Japan). Additionally, the presumptive zygotes after 3–5-h insemination (experimental series #2) were cultured in mSOF+ medium for 20 h, including the insemination period, and then treated as described above.

**Statistical analysis**

The percentages of motile spermatozoa, progressively motile spermatozoa and spermatozoa classified into each pattern by the CTC staining assay and the sperm penetration rates were analyzed using the two-tailed unpaired t-test after arcsine transformation [15].

**Results**

**Progressive motility of spermatozoa from AI-subfertile (S1–S8) and fertile (F1–F4) bulls**

Fig. 1 shows the percentages of progressively motile spermatozoa in the samples that were frozen-thawed, washed and then resuspended in BO-H (Ca-) medium. There were large variations in the percentages of progressively motile spermatozoa among the AI-subfertile bulls. Specifically, comparison with the average value of F1–F4 revealed that the values of S3, S4 and S5 were almost equal (P=0.3587 to 0.7592 vs. the F1–F4 average value); however, the values of S1, S2 and S7 were significantly lower (P=0.0130 to 0.0379 vs. the F1–F4 average value). Moreover, the value for S6 was intermediate between the above-mentioned groups (P=0.0663 vs. the F1–F4 average value), and the value of S8 was relatively (not significantly, P=0.0826) higher than the average value of F1–F4. In addition, approximately 70% of the spermatozoa from all bulls exhibited flagellar movement (data not shown).

**Capacitation state of spermatozoa from AI-subfertile (S1–S8) and fertile (F1–F6) bulls**

The CTC staining patterns were determined for spermatozoa that were frozen-thawed, washed and then resuspended in a BO-H (Ca-) medium (Fig. 2). The obtained results were classified into three groups and subjected to statistical analyses (two-tailed unpaired t-test). There was a much larger difference in the percentages of F-pattern and B-pattern spermatozoa between the F1–F4 and S5–S8 averages than between the F1–F4 and S1–S4 averages. In addition, the percentages of AR-pattern spermatozoa were very low in all groups (data not shown).

Fig. 3 shows the CTC staining patterns of spermatozoa from 4 AI-subfertile bulls (S5–S8) that were frozen-thawed, washed and then resuspended in BO-H medium (containing 2.25 mM CaCl₂). The spermatozoa from all bulls progressed into the capacitation state as a result of the action of CaCl₂. In fertile bulls (F3–F6), the average percentages of F-pattern, B-pattern and AR-pattern spermatozoa were 19, 66 and 15%, respectively. In the AI-subfertile bulls (S5–S8), the average percentage of AR-pattern spermatozoa was significantly higher (42%) than that of the fertile bulls. Furthermore, the percentage of F-pattern spermatozoa for these 4 AI-subfertile bulls (3%) was significantly lower than that of fertile bulls.
In vitro fertilization test using spermatozoa from AI-subfertile (S5–S8) and fertile (F2–F4) bulls

The spermatozoa from 4 AI-subfertile bulls (S5–S8) were used for this test because many of the spermatozoa from these bulls underwent premature capacitation and a subsequent acrosome reaction before insemination (see Figs. 2 and 3). Table 1 shows the results of the in vitro fertilization test (series #1) for these AI-subfertile bulls, in which oocytes were inseminated with spermatozoa for 9 h. The average sperm penetration rate of the fertile bulls (F2–F4) was 77%. The lowest rate (10%) was obtained for the samples from S7, which exhibited significantly lower progressive motility immediately after thawing (Fig. 1). In the samples from S5, S6 and S8, the rates were significantly lower than the average rate of the fertile bulls. These results were supported by the data obtained in the other test (Table 2, series #2), in which oocytes were cultured for 20 h, including sperm insemination for 3–5 h. These results indicate lower penetration into oocytes in vitro for prematurely capacitated spermatozoa from the AI-subfertile bulls.

Discussion

In this experiment, we washed spermatozoa in PBS-PVA before the motility examination. The washing procedure was necessary to prepare the sperm samples for the in vitro fertilization test and CTC staining. Immediately after resuspension in BO-H (Ca-) medium after washing, the spermatozoa of 3 AI-subfertile bulls (S1, S2 and S7) exhibited significantly lower progressive motility than those of fertile bulls (Fig. 1). This suggests that the frozen-thawed spermatozoa from some of the AI-subfertile bulls used in this study had lower tolerance for changes in the extracellular environment. Many previous reports [16] have indicated that the cAMP/PKA pathway regulates sperm motility via phosphorylation of flagellar proteins. Indeed, a study using PKA catalytic subunit Ca2-null mice
reported that the spermatozoa from these mice exhibited hardly any progressive movement [17]. It seems necessary to compare the sperm PKA activity among individual bulls in order to reveal the causes for the above-mentioned low progressive motility.

An elevation of the intracellular calcium level is essential for capacitation. The first event in sperm capacitation is an increase of intracellular calcium and bicarbonate ions to activate soluble adenylyl cyclase (sAC), which is a synthetase of cAMP [18, 19]. It has previously been shown that cAMP signaling plays a central role in capacitation via protein phosphorylation [6]. In our experiment, addition of CaCl$_2$ (2.25 mM) to the medium accelerated the progress of the capacitation process in spermatozoa from the fertile bulls (Figs. 2 and 3). This result suggests that extracellular calcium enters the sperm cytoplasm and that the resultant increased intracellular calcium may enhance both

**Fig. 3.** CTC staining patterns of frozen-thawed spermatozoa from AI-subfertile (S5–S8) and fertile (F3–F6) Japanese Black cattle resuspended in medium containing CaCl$_2$. Frozen spermatozoa (from AI-subfertile bulls S5–S8 and fertile control bulls F3–F6) were thawed, washed and then resuspended in a Hepes-buffered BO containing 0.1% PVA and CaCl$_2$ (BO-H). Aliquots of the samples were used for the CTC staining assays as described in the legend of Fig. 2. The values for each bull are means, and the average values for F3–F6 and S5–S8 are means ± SEM. Statistical analyses (two-tailed unpaired t-test) were performed for the average values of F3–F6 and S5–S8 and the values of each AI-subfertile bull (S5–S8). * Values with an asterisk are significantly different from the F3–F6 average value (P<0.05). **Values with an asterisk are significantly different from the F3–F6 average value (P<0.01). The numbers in the parentheses indicate the numbers of repeated experiments.

**Table 1.** In vitro fertilization test (series #1) for frozen-thawed spermatozoa from AI-subfertile (S5–S8) and fertile (F2–F4) Japanese Black cattle

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<tr>
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<th>Fertile bulls (control)</th>
<th>AI-subfertile bulls</th>
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<tbody>
<tr>
<td></td>
<td>F2</td>
<td>F3</td>
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<tr>
<td>Sperm insemination period (h)</td>
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<td>9</td>
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<tr>
<td>Culture period after sperm insemination (h)</td>
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<td>0</td>
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<td>No. of repeated experiments</td>
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<td>2</td>
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<tr>
<td>No. of examined oocytes</td>
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<td>52</td>
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<tr>
<td>Sperm penetration rates (%)</td>
<td>67 ± 6*</td>
<td>73 ± 7*</td>
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* Values are means ± SEM. Statistical analyses (two-tailed unpaired t-test) were performed for the values indicated by this superscript. * The sperm penetration rates of S5, S6, S7 and S8 are significantly lower than the average rate (77%) of F2–F4 (control, P<0.05).
cAMP-signaling and calcium-signaling pathways, which lead to progress of sperm capacitation state. One of the causes for this calcium influx may be associated with membrane disintegration. In fact, it has been shown that bull spermatozoa contain more intracellular calcium after cryopreservation than before cryopreservation; this indicates the presence of impaired membrane selective permeability mechanisms [20]. Moreover, it has been reported that the membrane of the entire spermatozoa is impaired during the cryopreservation procedure [21].

Murase et al. [3] reported that sperm sensitivity to calcium ion and calcium ionophore A23187 is different for fertile and subfertile Japanese Black cattle. In our experiment using medium without CaCl₂, the percentages of B-pattern spermatozoa were significantly higher in the 4 AI-subfertile bulls (S5–S8, Fig. 2). Moreover, addition of CaCl₂ (2.25 mM) to the medium increased the number of AR-pattern spermatozoa in these AI-subfertile bulls (Fig. 3). These results suggest that the spermatozoa from these AI-subfertile bulls prematurely initiated capacitation-related alterations and that these spermatozoa spontaneously underwent an acrosome reaction due to exposure to extracellular CaCl₂. Thus, it is possible that prematurely capacitated spermatozoa undergo a spontaneous acrosome reaction due to uncontrolled influx of calcium ion as a result of membrane disintegration. Furthermore, since these spermatozoa have released their acrosomal contents immediately after resuspension in medium containing CaCl₂, they may not have had enough acrosomal enzymes to penetrate the zona pellucida when they reach an in vitro-matured oocyte during the in vitro fertilization test. Thus, this sperm abnormality might account for the low penetration into oocytes of spermatozoa from the AI-subfertile bulls used in this study (S5–S8, Tables 1 and 2).

Conclusions

Premature capacitation was found in the spermatozoa of half of the bulls in the AI-subfertile group (Fig. 2). Prematurely capacitated spermatozoa underwent a spontaneous acrosome reaction possibly due to an uncontrolled influx of calcium ion (Fig. 3), and consequently they exhibited significantly lower penetration into oocytes in vitro compared with spermatozoa from the control fertile bulls (Tables 1 and 2). It is therefore possible that premature capacitation is a candidate causal factor for the AI subfertility of male Japanese Black cattle and a potentially good marker for identification of AI-subfertile bulls. However, it is apparent that other sperm abnormalities also affect the fertility of bulls because spermatozoa from the other half of the AI-subfertile group rarely exhibited premature capacitation (Fig. 2). Thus, examination of multiple sperm parameters is necessary for exact prediction of the reproductive performance of sperm donors in relation to Japanese Black cattle used for AI.

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