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Opinions and Hypotheses

Necessity of exact examination of sperm characteristics to assess artificial insemination-subfertile bulls

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Abstract. Conception rates of artificial insemination (AI) have gradually been decreasing in the cattle. In order to overcome this problem, AI centers need supply high-quality frozen semen whose insemination makes cow pregnant efficiently. Semen quality is conventionally assessed under the light microscope with cell biological methods, and only high-quality frozen semen straws are used for AI. However, lower conception rates are occasionally recorded in AI with frozen semen straws from some bulls (AI-subfertile bulls). In this paper, we introduce new methods to assess sperm molecular characteristics to find AI-subfertile bulls.
Key words: Cattle, Sperm, Subfertility

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Introduction

Many factors influence the reproductive performance of cattle, including the state of cows, rearing management conditions, season, and semen quality [1–4]. To improve reproductive performance, contribution from the male side, the supply of frozen semen with high ability to make females conceive through artificial insemination (AI) on commercial farms is especially important. The reproductive ability of sires is still evaluated by examination of semen characteristics using cell biological techniques established several decades ago, and sire candidates producing sperm with a low conception rate after AI (AI-subfertile bulls), despite general characteristics and motility after freezing and thawing being normal at the light-microscopic level, are occasionally observed. If frozen semen straws of these sire candidates are supplied, the open period of breeding cows is prolonged, causing economic loss to farm producers. However, these sire candidates cannot be found by conventional examination of semen characteristics. This report outlines the examination of semen characteristics we have been working on to identify sire candidates producing sperm with a low conception rate after AI (AI-subfertile bulls) before being selected and offered for semen production as Japanese Black sires, and proposes introduction of a detailed sperm examination method at a sire center.

1. *In-vivo* fertilization test by AI to superovulated cows

Frozen semen straws of sire candidates were experimentally used for AI in cows. Although confirming conception is the most reliable method to judge frozen-thawed sperm fertility, to calculate a statistically evaluable conception rate, it is necessary to secure a considerable number of cows. Thus, we performed AI in superovulated cows, and investigated the association between the development rate of recovered *in-vivo* fertilization-derived embryos and actual conception rate after AI, in addition to the possibility of establishing a bioassay method to estimate the fertility potential of frozen-thawed spermatozoa of sire candidates. The development rate of *in-vitro* fertilization (IVF)-derived embryos was also compared.

Frozen semen straws of 13 sire candidates were assessed. These frozen semen straws passed the general characteristics test of fresh semen and frozen-thawed sperm motility test, and the rate of conception acquired by AI in cows reared in Hyogo Prefecture including our institute has been determined. In superovulation treatment to collect *in-vivo* fertilization-derived embryos, 20 AU of follicle stimulating hormone (FSH) preparation (Antolin®, Kyoritu Seiyaku, Tokyo, Japan) was administered at a tapering dose (5, 5, 3, 3, 2, then 2 AU) and 750 μg of prostaglandin F2alpha ($PGF_{2\alpha}$) analog (Estrumate®, Intervet, Tokyo, Japan) was intramuscularly administered to breeding cows. AI was performed after detecting standing estrus, the development stage and quality of non-surgically collected embryos were judged on day 7.5 after the onset of standing estrus, and the transferable embryo (excellent and good embryos) rate was measured. Three or more embryo donor cows were used per sire candidate, and embryos were collected targeting recovery of 40 or more embryos. IVF-derived embryos were prepared according to the previously reported method [5]. Meat processing center-derived immature

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ova were prepared by *in-vitro* maturation, IVF, and *in-vitro* culture, and the embryo development rates at 3 and 7 days after IVF were measured. Seventy or more ova were used per sire candidate.

The conception rate after AI was within a range of 1.4–70.5%, demonstrating large variation among individual animals (Table 1). As the results of AI were acquired from 40 or more cows, 4 sire candidates with a low conception rate (J, K, L and M) may have produced spermatozoa with a markedly low conception ability. In the *in-vivo* fertilization test, 614 embryos were recovered from 65 embryo donor cows and the transferable embryo rate was measured. The rate ranged from 0 to 93.9%, demonstrating large variation among individual animals. The transferable embryo rate involving the 4 sire candidates (J, K, L and M) considered to produce spermatozoa with a low conception ability was 0–22.2%, being markedly low compared with that involving other sire candidates. When Spearman's rank correlation between the transferable embryo rate and conception rate after AI was analyzed, a significant positive correlation was detected ($r = 0.60$, $P < 0.05$). It has been reported that premature capacitation occurred immediately after thawing frozen semen in animals with a poor conception outcome of AI and these spermatozoa did not reach fertilization [6] and semen quality influenced embryo production [7]. It may be possible to evaluate semen of poor quality (unlikeliness of fertilization) by investigating the embryo development rate because there is a process of AI in preparation of *in-vivo* fertilization-derived embryos.

Regarding the development rate of IVF-derived embryos acquired using sperm of 7 sire candidates, the rate of embryos in the 4-cell or later stage was 0–63.9% and the rate of transferable embryos in the late morula or later stage was 0–15.3%, demonstrating variation among individual animals (Table 2). Significant correlation was not confirmed between results of the development of IVF-derived embryos (rates of embryos showing normal development to 4-cell stages or to the stage of transferable embryos) and results of AI (conception rates after AI) by statistical analyses owing to insufficiency in the number of examined sire candidates [8]. However, we have a hypothesis that a possible relationship may exist between rates of IVF-derived embryos showing normal development to 4-cell stages and conception rates after AI.

This study clarified that the fertility of spermatozoa of sire candidates can be estimated by *in-vivo* fertilization of superovulated cows and the reproductive ability of sire candidates from which frozen semen was able to be prepared was judged using this method.

Table 1. Transferable embryo rate after internal fertilization and conception rate after artificial insemination

Seed bull candidate	Number of embryo donor cows	Number of test embryos	Number of transferable embryos (%)	Number of cows for artificial insemination	Number of conceptions (%)
A	3	37	29 (78.4)	61	43 (70.5)
B	4	42	29 (69.0)	81	54 (66.7)
C	7	45	28 (62.2)	117	77 (65.8)
D	9	99	63 (63.6)	829	518 (62.5)
E	3	46	33 (71.7)	860	530 (61.6)
F	5	53	39 (73.6)	146	85 (58.2)
G	4	33	31 (93.9)	186	107 (57.5)
H	3	65	39 (60.0)	487	280 (57.5)
I	4	46	35 (76.1)	232	103 (44.4)
J	4	37	0 (0.0)	191	11 (5.8)
K	8	33	2 (6.1)	117	5 (4.3)
L	6	36	8 (22.2)	42	1 (2.4)
M	5	42	0 (0.0)	142	2 (1.4)

Table 2. Transferable embryo rate after *in vitro* fertilization and conception rate after artificial insemination

Seed bull candidate	Number of test embryos	Number of 4-cell stage embryos (%)	Number of transferable embryos (%)	Number of cows for artificial insemination	Number of conceptions [a] (%)
A	90	23 (25.6)	3 (3.3)	61	43 (70.5)
C	36	23 (63.9)	4 (11.1)	117	77 (65.8)
G	72	43 (59.7)	11 (15.3)	186	107 (57.5)
I	117	38 (32.5)	8 (8.6)	232	103 (44.4)
J	103	0 (0.0)	0 (0.0)	191	11 (5.8)
K	123	9 (7.3)	4 (3.3)	117	5 (4.3)
M	102	3 (2.9)	0 (0.0)	142	2 (1.4)

[a] Data was reproduced in Table 1.

2. Evaluation of spermatozoa by observation of acrosomal conditions

In the *in-vivo* fertilization test described above, many factors of semen leading to a poor conception outcome on commercial farms can be identified because of the presence of the actual AI process, being an excellent method for the evaluation of sperm fertility. However, when many sire candidates are introduced yearly, it is necessary to secure many superovulated cows and the test is costly and laborious, which is problematic; therefore, development of a new simple evaluation method is desired. It was recently reported that when frozen semen straws of sires with a markedly low rate of conception by AI were observed on commercial farms, the acrosome was damaged in many spermatozoa and the acrosomal protein distribution in the sperm head was abnormal [9]. In this report, in which immunostained sperm smears were observed and judged under a fluorescence microscope, the time to judgment was 1–2 days. If frozen-thawed sperm fertility potential can be judged using this method, labor and cost required for the test can be reduced because it is not necessary to secure cows to superovulate and the cost of judgment per sire candidate is only several hundred yen. Thus, aiming at establishing a new evaluation method of frozen-thawed spermatozoa, we investigated the correlation between the results of the method observing the sperm acrosomal morphology and intra-acrosomal protein and that of the conventional *in-vivo* fertilization test using superovulated cows.

1) Evaluation by observing FITC-PNA/PI-stained sperm acrosomal morphology

Peanut lectin (peanut agglutinin (PNA)) isolated from *Arachis hypogaea* (peanuts) specifically recognizes a sugar chain structure, β-D-Gal (1→3)-D-GalNAc, and it specifically binds to the outer acrosomal membrane and acrosomal content of spermatozoa [10]. Utilizing this principle, the sperm acrosome was classified into 7 patterns by staining of the acrosome with fluorescein isothiocyanate (FITC)-labeled PNA (FITC-PNA) followed by propidium iodide (PI), a cationic fluorescent dye for nucleic acid staining classified as a phenanthridium dye (FITC-PNA/PI staining) [9].

In this test, frozen semen straws of 16 sire candidates were used. In FITC-PNA/PI staining, frozen semen straws were thawed in 38.5°C warm water. The resultant frozen-thawed spermatozoa were washed three times with 0.1% polyvinyl alcohol-containing phosphate-buffered saline (PBS) by centrifugation for 5 min, and fixed with 3% paraformaldehyde (PFA)-containing PBS for 15 min under protection from light. After removal of PFA-containing PBS by centrifugal separation, the precipitate was washed twice with blocking buffer (1% bovine serum albumin (BSA), 100 mM glycine-containing PBS) by centrifugation, followed by membrane permeation treatment with 1% (Vol/Vol) Triton X-100-containing PBS for 5 min. Then, after washing three times with BSA-PBS by centrifugation, the precipitate was reacted with 20 μg/ml of FITC-PNA-containing PBS for 30 min and washed, followed by nuclear staining with 25 μg/ml of PI-containing PBS for 5 min. After washing with BSA-PBS by centrifugation, the stained sperm suspension was mounted with VECTASHIELD on a slide glass and sealed with a cover glass. The preparation was observed under an epifluorescence microscope and the acrosome was morphologically evaluated. Spermatozoon with an acrosome exhibiting staining pattern I or II was defined as normal in this test [9] (I: the acrosome is stained homogenously and strongly, II: the acrosome is mostly stained homogenously and strongly, but partially stained heterogeneously and markedly strongly). Spearman's rank correlation between the rate of spermatozoa with normal acrosomal morphology and transferable embryo rate on the *in-vivo* fertilization test (using data of cases with 10 or more recovered embryos) was analyzed.

On the sperm acrosomal morphology test using FITC-PNA/PI staining, the rate of normal spermatozoa ranged from 22 to 75%, demonstrating large variation among individual animals. On the *in-vivo* fertilization test, 57 embryo donor cows were used and the number of recovered embryos was 358 in total. The number of transferable embryos was 13–39 and the transferable embryo rate ranged from 0 to 100%, demonstrating large variation among individual animals. A significantly high positive correlation was noted between the 2 rates on Spearman's rank correlation analysis (Fig. 1, $r = 0.838$, $P < 0.01$) [11]. In frozen-thawed spermatozoon judged as having abnormal acrosomal morphology

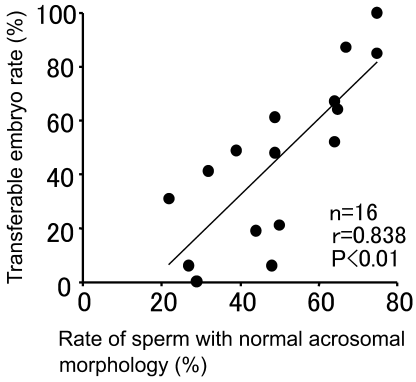

Fig. 1. Relationship between acrosomal conditions of frozen-thawed spermatozoa and the *in-vivo* fertilization-derived transferable embryo rate [11].

using FITC-PNA/PI staining, the acrosome was heterogeneously and strongly stained in entirety. In addition, acrosome stainability was markedly reduced or the acrosome was partially not stained, suggesting that the acrosome was damaged and the content had leaked or was lost. Sperm acrosome includes many functional molecules which are required for the accomplishment of fertilization with oocytes in the ampulla of the oviduct [12]. Moreover, a recent report [13] reveals that mouse sperm with intact acrosomes can enter the oviduct and undergo the acrosomal exocytosis in the upper region of the oviduct during a normal process prior to fertilization with oocytes, suggesting necessity of sperm acrosomal integrity for the transportation from the uterus to the oviduct. These may account for low conception rates after AI with cryopreserved semen containing many spermatozoa with damaged acrosomes.

2) Evaluation of sperm intra-acrosomal tyrosine-phosphorylated protein distribution

The sperm acrosome is abundant in fertilization-related sperm-specific proteins [14] and phosphorylation of these proteins is involved in the promotion of mammalian sperm fertilization [15]. In addition, abnormal tyrosine-phosphorylated (pY) protein distribution was noted in the sperm acrosome of sires with a markedly low AI-induced conception rate on commercial farms [9]. Thus, using the frozen semen described above, the phosphorylation state of sperm acrosomal tyrosine protein was examined and its correlation with

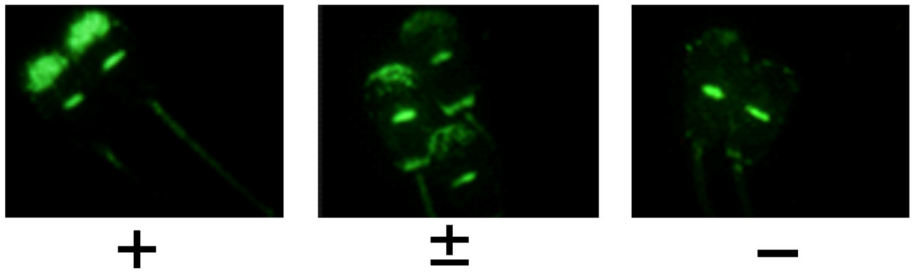

Fig. 2. Classification of tyrosine-phosphorylated protein detection patterns in the main part of the acrosome of bovine frozen-thawed spermatozoa using the indirect fluorescence antibody method. +: The antigen protein distribution was extensive and clear, ±: the antigen protein distribution was narrow or mottled, –: almost no antigen protein was observed.

the transferable embryo rate on the *in-vivo* fertilization test was investigated.

After washing frozen-thawed spermatozoa, sperm smears were prepared and fixed with methanol for 10 min. After washing twice with PBS and blocking with 5% BSA-containing PBS for 1 h, the smear was reacted with primary antibody (mouse anti-phosphorylated tyrosine antibody-containing BSA-PBS (1,000-times dilution)) at 4°C overnight. After washing with PBS, the smear was reacted with secondary antibody (FITC-labeled rabbit anti-mouse immunoglobulin polyclonal antibody-containing BSA-PBS (100-times dilution)) under protection from light. After washing with PBS, the smear was sealed with VECTASHIELD and the pY protein distribution was evaluated under an epifluorescence microscope. In the evaluation, 100 or more acrosomes per preparation were classified into the 3 staining patterns shown in Fig. 2. In this test, regarding staining pattern (+) as spermatozoon with a normal distribution, Spearman's rank correlation between the rate of spermatozoa with normal pY protein distribution and transferable embryo rate on the *in-vivo* fertilization test was analyzed.

The rate of spermatozoa with normal acrosomal pY protein distribution ranged from 14 to 93%, demonstrating large variation among individual animals, and a significantly high positive correlation with the *in-vivo* fertilization-derived transferable embryo rate was noted (Fig. 3, r = 0.721, P < 0.01) [11].

As the above 2 methods were highly correlated with the current *in-vivo* fertilization test using superovulation treatment, considering that both tests are capable of evaluating the fertility potential of frozen-thawed spermatozoa, we evaluated the fertility potential of spermatozoa of sire candidates that passed

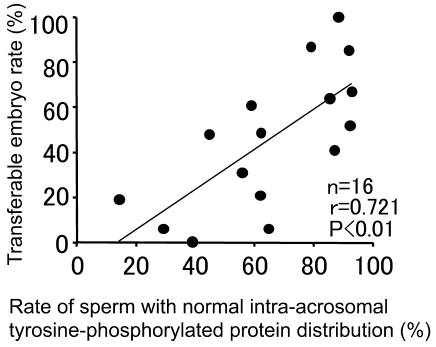

Fig. 3. Relationship between the intra-acrosomal tyrosine-phosphorylated protein distribution in frozen-thawed spermatozoa and *in-vivo* fertilization-derived transferable embryo rate[11].

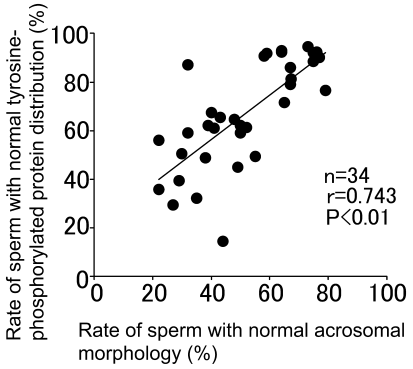

Fig. 4. Relationship between acrosomal conditions of frozen-thawed spermatozoa and the intra-acrosomal tyrosine-phosphorylated protein distribution [11].

the general characteristics test of semen and motility test of frozen-thawed spermatozoa using the 2 methods, and performed the *in-vivo* fertilization test only for animals requiring detailed confirmation.

3. Association between sperm acrosomal conditions and intra-acrosomal pY protein distribution

Significantly high positive correlations were found between the rate of frozen-thawed spermatozoa with normal acrosomal morphology or normal intra-acrosomal pY protein distribution and *in-vivo* fertilization-derived transferable embryo rate. Thus, the number of cases was increased, and the correlation between the sperm acrosomal morphology and intra-acrosomal pY protein distribution was analyzed. A significantly high positive correlation was observed (Fig. 4, r = 0.743, P < 0.01, n = 34) [11], suggesting that the intra-acrosomal pY protein distribution is involved in acrosomal normality. Furthermore, although the correlation of the rate of frozen-thawed spermatozoa with abnormal intra-acrosomal pY protein distribution and of frozen-thawed spermatozoa with abnormal acrosomal morphology is high, 80% or more spermatozoa possess a normal acrosome in fresh semen after ejaculation [16]. Based on these findings, spermatozoon with abnormal acrosomal morphology is likely to be damaged in the freezing and thawing processes, and intra-acrosomal pY protein may have function in the tolerance of the acrosomal membrane to freezing.

Conclusion

Improvement of Japanese Black cattle is

promoted mainly in sires, and the selection of meat production traits rapidly progressed over the last 10 or more years due to advances in statistical genetics and molecular biology. However, although the characteristics targeted by selection are excellent, bulls are not selected as sire unless they meet specific criteria for reproductive ability. We became able to perform highly accurate sire selection by identifying sire candidates with poor reproduction by performing the detailed examination introduced in this study in addition to the current general property test of semen. To improve the reproductive performance on commercial farms, we propose production of frozen semen with a high conception outcome of AI by introducing our detailed sperm examination.

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