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Differential Attachment of Bovine Y Chromosome-bearing Sperm to the Zona Pellucida

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Abstract: *The theoretical rate of Y chromosome-bearing sperm (Y-sperm) and X chromosome-bearing (X-sperm) in ejaculate is 50:50 in mammals, therefore the sex ratio of the embryos following fertilization is expected to be 50:50. The sex ratio of embryos produced in vitro, however, is skewed towards either males or females. The primary aim of the present study was to investigate the factors of in vitro fertilization (IVF) that affect hte attachment or binding rate of bovine Y chromosome-bearing sperm (Y sperm rate) to the zona pellucidae (ZPe). Oocytes collected from bovine ovaries were cultured for 21 h in vitro under various conditions. ZPe were collected from the oocytes. After frozen-thawed bovine semen and ZPe were co-incubated, the ZPe were mounted on glass slides. Then, sperm attached or bound to the ZPe were subjected to in situ hybridization with a Y chromosome-specific probe to determine the Y-sperm rate. In a preliminary experiment, the Y-sperm rate of frozen-thawed sperm was 50.2% without deviation from the theoretical rate (50%), and the Y sperm rate in Y sperm sorted semen was 93.4% as expected. In Experiment 1, the effect of the sperm-ZP co-incubation period on the Y-sperm rate was investigated. Short coincubation periods (5 min and 8 min) deviated the Y-sperm rate (55.1% and 54.9%) from the theoretical rate, but long coincubation periods (300 min) did not affect the Y-sperm rate (49.2%). In Experiment 2, a combination of preincubation of sperm for 3 h prior to sperm-ZP coincubation and extension of the maturation period to 36 h did not change the skewed Y-sperm rate of coincubation of untreated sperm with ZPe derived from 21-h-matured COCs (53.0%, 53.9%, and 54.5%, respectively). In conclusion, the ability of frozen-thawed sperm to attach to the ZPe during the first 5 min or 8 min of coincubation was slightly higher in Y sperm than X sperm although neither sperm preincubation nor extension of the maturation period affected the Y-sperm rate.*

Key words: *Bovine, Zona pellucida, Sex ratio, Y chromosome bearing sperm, In vitro fertilization*

Introduction

In vitro-produced embryos are widely used in the beef and dairy industries, and the sex of the calves is an important factor for profits in both industries. Thus, the factors affecting the sex ratio of either embryos or calves have attracted a great deal of interest among researchers.

The theoretical rate of Y chromosome-bearing sperm (Y sperm) and X chromosome-bearing sperm (X sperm) in ejaculate is 50:50 in mammals. Therefore, the sex ratio of mammalian zygotes at fertilization is expected to be 50:50. Some reports, however, indicate that the sex of *in vitro*-produced embryos is skewed towards either males or females [1–4], although the sex ratio of *in vivo*-derived embryos is 1:1 in mammals [5].

The sperm-oocyte coincubation period profoundly affects the *in vitro* fertilization rate and short bovine sperm-oocyte incubation periods skew the embryo sex ratio towards males [1, 6]. Bovine oocyte maturation periods and preincubation of sperm before fertilization also skews the sex ratio of the resultant embryos [2, 7]. These earlier reports suggest that there is a differential ability to access or fertilize oocytes between X and Y sperm. Neither the cause of these phenomena nor the validity of these hypotheses, however, has been clarified.

In mammals, the sex of the embryos depends on whether a Y or X sperm reaches the oocyte, penetrates the zona pellucidae (ZPe), and fuses with the oocyte.

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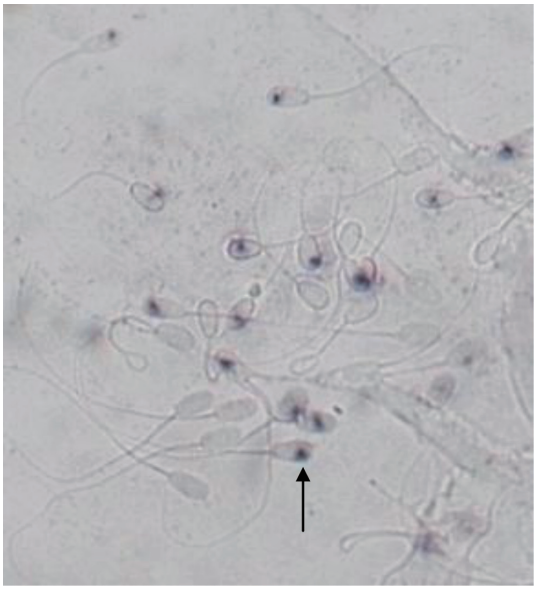

Fig. 1. Images of Y and X sperm bound to a zona pellucida determined by *in situ* hybridization. The arrow indicates a Y sperm with a signal.

Successful fertilization comprises primary attachment of the sperm to the ZPe, tight binding between the sperm and ZPe, sperm penetration into the ZPe, and sperm-oolemma fusion. In this context, to elucidate the cause of the skewed sex ratio originating from sperm-oocyte coincubation periods and the states of the sperm and oocyte, the discrete steps of fertilization with X and Y sperm need to be compered. The number of sperm attached to the ZPe significantly correlates with the frequency of oocyte penetration after *in vitro* fertilization [8]. In the present study, we focused on the primary attachment of the sperm to the ZPe, and examined the possibility that the skewed sex ratio of *in vitro*-produced embryos depends on differences in the abilities of Y and X sperm to attach to the ZPe. Experiments with varying sperm-ZP coincubation times, oocyte maturation durations, and sperm preincubation periods were performed to examine their effects on the sex ratio. Our results demonstrate the effects of sperm-oocyte coincubation periods, oocyte maturation periods, and sperm preincubation on the rate of Y sperm attaching or binding to the ZPe.

Materials and Methods

Chemicals

All chemicals were purchased from Nacalai (Kyoto, Japan) unless otherwise indicated. The media used for *in vitro* maturation and sperm-ZP coincubation were based on synthetic oviduct fluid (SOF) [9] with slight modifications: *in vitro* maturation medium (IVM medium) comprised SOF containing 10% fetal bovine serum (5703H, ICN, Costa Mesa, CA), 5.56 mM glucose, and amino acids (Sigma-Aldrich, St. Louis, MO); sperm-ZP coincubation medium comprised SOF containing 5 mg/ml fatty acid-freebovine serum albumin (Sigma-Aldrich) and 10 IU/ml heparin (Sigma-Aldrich).

Ovary collection and ZP preparation

Bovine ovaries were collected at the local abattoir and transported to the laboratory within 4 h. Cumulus-oocyte complexes (COCs) were collected from 3 to 6 mm antral follicles using a syringe with an 18G needle. The COCs were washed and matured in IVM medium for 21 or 31 h under 5% CO_2 and 95% air. After the maturation period, oocytes were denuded from the enclosed cumulus cells by vortexing for 5 min and the ooplasm was removed using narrow-pulled Pasteur pipettes (diameter, 120 μm). The collected ZPe with an intact morphology (spherical shape with only one slit) were washed and placed in 50-μl drops of coincubation medium (10 ZPe/drop).

Sperm preparation and sperm-ZP coincubation

Frozen-thawed semen of one Japanese Black bull was mixed and washed with a 45% to 60% discontinuous Percoll (Amersham Co., Ltd., Uppsala, Sweden) gradient solution by centrifugation (800×g for 10 min). Sperm were diluted with coincubation medium and an equal volume of sperm suspension was added to the drops containing ZPe (5×10^5 cell/ml, 10 ZPe/100 μl drop). Sperm and ZPe were then coincubated for 5 min or 300 min. After the coincubation period, ZPe were washed loosely (for 5 min or 8 min coincubation) or tightly to remove the loosely attached sperm (for 300 min coincubation) in phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin, then mounted on glass slides. Frozen-thawed semen was also mounted on a slide as a control.

DNA in situ hybridization probes

The *in situ* hybridization probes were produced as described previously [10]. Briefly, the satellite repeat on the Y chromosome (locus DYZ1; Gene Bank Accession no. M 26067) was amplified by polymerase chain reaction (PCR) with the primer set (5′CAACCAGTATCTGTATGCCT3′ and 5′AAACATACGCAATCTGCTT3′) to produce a 510-bp fragment and labeled with a digoxigenin labeling kit (Roche Diagnostics, Indianapolis, IN). The PCR conditions were 3 min at 95 °C, 35 cycles of 45 s at 93 °C, 45 s at 60 °C, and 45s at 72 °C. The labeled PCR

products were purified using High Pure PCR Cleanup Micro Kit (Roche, Barcelona, Spain) and were diluted in standard hybridization buffer [5.5 ml formamide (Amresco, Solon, OH), 1 g dextran sulfate (Sigma), 0.5 ml 20 × saline sodium citrate (SSC, 300 mM NaCl, 30 mM sodium cirate, pH7.0), and distilled water in a total volume of 7 ml]. Sperm DNA was denatured by immersing the slides in PBS (-) (bovine serum albumin-free PBS) for 5 min followed by 6 mM EDTA in PBS for 5 min, and then in 5 mM dithiothreitol and 100 IU/ml heparin (Sigma, St. Louis, MO) in PBS for 20 min at room temperature. The slides were then dehydrated through a graduated ethanol series (70%, 80%, 90%, and 100%). Sample slides were pre-heated for 10 min to denature the DNA. The probe mixture was denatured at 75 °C for 5 min and applied to the preheated dry slides at 85 °C for 10 min, followed by hybridization overnight at 38 °C. High stringency washing was performed for 5 min in 0.4 × SSC at 75 °C and for 2 min in PN buffer (phosphate buffer containing 0.1% Nonidet-P 40; Amresco, pH. 8.0) at room temperature. Slides were blocked with PNB buffer (PN buffer containing 2% Block Ace: DS Pharma Biomedical, Osaka, Japan). Hybridization of the digoxigenin-labeled Y-chromosome probe was visualized with monoclonal mouse anti-digoxigenin AP (Roche, Barcelona, Spain). Sperm that attached to the ZPe were examined under a microscope (×400) and the numbers of sperm with signal (Y sperm) and that of sperm without signal (X sperm) were counted. Disrupted or overlapping sperm heads were excluded from the analysis. Images of Y sperm and X sperm following *in situ* hybridization are shown in Fig. 1.

Experimental design

A preliminary experiment was performed to examine the accuracy of *in situ* hybridization. Frozen-thawed semen was washed as described above and mounted on a glass slide and subjected to *in situ* hybridization. Frozen-thawed semen of one bull was used for the experiments. In addition, Y sperm-sorted semen from another bull (Genetics Hokkaido Association Sapporo, Japan) was used for the *in situ* hybridization.

In Experiment 1, COCs were matured in the IVM medium for 21 h and ZPe collected from these oocytes were used. ZPe and sperm were coincubated for 5 min, 8 min or 300 min. The effect of the coincubation period on the rate of Y sperm to the total number of sperm attached or bound to the ZPe was evaluated in comparison with that of control sperm.

In Experiment 2, the effect of preincubation of semen prior to sperm-ZP coincubation and prolongation of the maturation period on the rate of Y sperm to the total number of sperm attached to the ZPe in 5 min of coincubation was assessed. COCs were matured in IVM medium for 21 or 36 h and the ZPe collected from these oocytes were used. Frozen-thawed semen was washed and the sperm concentration was adjusted to 1×10^6 cell/ml. The sperm were then preincubated for 0 or 3 h in IVF medium.

For each replicate, sperm that were mounted on glass slides were used as internal controls and subjected to *in situ* hybridization (data not shown). One thousand cells were counted on the sperm mounted on a glass slide and when the rate of Y sperm to totally counted sperm did not significantly differ from the theoretical rate (50%), the data for the replicates were included in the analysis. None of the replicates failed to qualify for analysis. For each replicate, 30 ZPe were used in each group and each trial was repeated 4 times with a different series of oocytes. Therefore, a total of 120 ZPe were used in each experimental group. All observations of Y sperm following *in situ* hybridization were conducted under blind conditions.

Statistical analysis

The chi square test was conducted to determine whether the percentage of Y sperm in the internal control sample differed significantly from the expected value of 50:50. One-way analysis of variance followed by Tukey's post hoc test was conducted to compare experimental groups. Before the analysis, the percentage of Y sperm was arcsine transformed. When the *P* value was less than 0.05, the result was considered to be statistically significant.

Results

In the preliminary experiment, the percentage of Y sperm per total sperm mounted on the glass slide was assessed. After *in situ* hybridization, a Y chromosome-bearing sperm was detected as a Y sperm with a signal (Fig. 1). After 10 trials, the Y sperm rate was 50.2 ± 0.16% (10050/20022), which was not significantly different from the expected theoretical rate (10011:10011). In addition, when Y sperm-sorted semen was assessed, the Y sperm rate was 93.4% (934/1000, one trial).

The effects of the coincubation period on the rate of Y sperm per total number of sperm attached or bound to the ZPe (Y-sperm rate) are shown in Table 1. In Experiment 1, the Y sperm rate of the control was 50.3%. When frozen-thawed sperm and ZPe were coincubated for 5 min or 8 min, the Y sperm rates were 55.1% and 54.9%, respectively both of which were significantly higher than

Table 1. Effect of coincubation period on sperm and zona pellucida interaction

Coincubation periods (min)	No. trial	No. ZP	No. sperm	Y sperm rate (%) ± SE
Internal control	4	–	4227	50.3 ± 0.0a
5	4	120	6931	55.1 ± 0.1b
8	4	120	7663	54.9 ± 0.2b
300	4	120	6824	49.2 ± 0.3a

a–b; Different letters indicate significant differences ($P<0.05$).

Table 2. Effect of sperm preincubation and maturation periods of oocyte on interaction between sperm and zona pellucida coincubation for 5 min

Maturation periods (h)	Preincubation period (h)	No. Trial	No ZP	No. sperm	Y-sperm rate (%) ± SE
21	–	4	120	6196	54.5 ± 0.7
21	3	4	120	6785	53.0 ± 0.6
36	–	4	120	6196	53.9 ± 1.2

that of the control. Prolonging the coincubation period to 300 min reduced the Y sperm rate to 49.2%, which was not significantly different from that of the control.

In Experiment 2, when the maturation period was prolonged to 36 h, the Y sperm rate was 53.9% and preincubation of the semen prior to sperm-ZP coincubation resulted in a Y-sperm rate of 53.0%. These data did not significantly differ from that of untreated sperm which attached to ZPe (54.5%, Table 2).

Discussion

The results of the present study demonstrate that Y chromosome-bearing sperm have a selective advantage in their ability to attach to the ZPe in short coincubation periods (5 min and 8 min). However, this advantage was not observed in the binding to ZPe in a long coincubation period (300 min). Also, neither the prolongation of the preincubation of sperm nor the oocyte maturation period had an effect on the Y sperm rate.

The duration of the sperm-oocyte coincubation period is a primary factor affecting the efficiency of *in vitro* fertilization and blastocyst production [1, 6] and short-term sperm-oocyte coincubation increases the proportion of male embryos, whereas a prolonged coincubation period tends to reduce the sex ratio of embryos to the theoretical rate [1]. In addition, Iwata *et al.* demonstrated that a short coincubation period of either sperm and COCs or sperm and denuded oocytes skewed the sex ratio of 8-cell stage embryos towards more males compared with longer coincubation periods, suggesting that there is a selective advantage for Y sperm in binding to the ZPe. In the present study, when sperm were co-incubated with ZPe for short periods (5 min and 8 min), the Y-sperm rates were 55.1% and 54.9%, which were significantly higher than that of the control. However, in the coincubation period of 300 min, the Y-sperm rate (49.2%) was not different from that of the control. The results suggest that Y sperm might have a selective advantage in attachment to ZPe during short coincubation periods.

The skew of the Y-sperm rate resulting from ZP-attached sperm in short coincubation periods disappeared when sperm bound to ZPe in a long coincubation period. Sperm attachment to the ZPe is mediated by an interaction between carbohydrate chains on the surface of the ZPe and the lectin-like protein on the sperm head. In addition, not only the carbohydrate structures, but also the position of the carbohydrate structure in the molecules and the three dimensional structure of the ZPe affect binding functionality [11, 12]. Recent observations by electron microscopy have revealed that the ultrastructure of the pig and cat ZPe changes during oocyte maturation [12–14]. The elasticity of the ZPe also changes in mouse oocytes during maturation [15]. Matured mouse oocytes begin to change the surface structure of the zona pellucida 6 h after ovulation [16, 17]. Moreover, both the ability of the ZPe to induce the acrosome reaction of the attached sperm and the acidity of the ZPe increase during oocyte maturation in the pig [8]. We reported that the number of porcine sperm attaching to the ZPe increased during oocyte maturation and then decreased in association with oocyte aging [18], and preincubation of bull

semen increased the number of sperm attaching to the ZPe compared with semen that was not preincubated [3]. Based on these findings, we expected that the maturation period and preincubation of semen would affect sperm-ZP attachment properties, possibly skewing the Y-sperm rate. The Y-sperm rates following these treatments were slightly lower than those without treatment, but the difference between them was not significant because of the large deviation. Accordingly s we suggest that previously reported the skewed sex ratio [2, 7] is not due to different attachment properties of Y sperm and X sperm. In addition, it is noteworthy that there is no conclusive evidence as to whether sperm which attach to the ZPe earlier contribute more to successful fertilization than those that attach to the ZPe later. Therefore, further studies are needed to elucidate the causes behind previous reports of skewed sex ratios in *in vitro* produced embryos.

In conclusion, the ability of frozen-thawed sperm to attach to the ZPe during the first 5 or 8 min of coincubation is slightly higher for Y sperm than X sperm, although neither sperm preincubation nor extension of the maturation period affects the Y-sperm rate.

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