Changes in the Sperm-zona Pellucida Binding Properties during Porcine Oocyte Maturation

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Abstract: *Sperm-zona pellucida (ZP) binding is the initial step in fertilization. In this study, to examine sperm-ZP binding properties, we collected ZPs from oocytes obtained from gilt ovaries after 0, 20, 44, and 68 h of maturation culture, and counted the number of sperm bound to the ZP (NSBZ) after 2 min of sperm-ZP co-incubation. Culture of cumulus oocyte complexes (COCs) for 44 h produced significantly higher NSBZ than the other culture durations. Culture of denuded oocytes (DO) produced the same result. To examine the effect of cumulus cells (CCs) and the maturity of oocytes on the NSBZ after maturation culture for 44 h, three oocyte culture conditions (COCs, DO, and combination of DO and CCs) were established, and oocytes were categorized according to the presence of a polar body (PB). The NSBZ did not differ among the culture conditions. NSBZ of oocytes with a PB was greater than that of oocytes without a PB. The addition of tunicamycin, a potent inhibitor of N-linked glycosylation, to the COC maturation medium significantly decreased the NSBZ, although the maturation rate was not affected. In conclusion, NSBZ increases during oocyte maturation and oocytes play a key role in increasing the NSBZ via modification of N-linked glycosylation.*

Key words: *Porcine, Sperm, Zona pellucida, Tunicamycin, Initial binding*

Introduction

The zona pellucida (ZP) is a transparent membrane that envelops mammalian oocytes, and the binding of sperm to the ZP is a first step toward successful fertilization [1]. The ability of the ZP to bind to sperm and to induce the acrosome reaction may be a key ZP property. Porcine ZP is comprised of three glycoprotein families (ZPA, ZPB, and ZPC). N-linked and O-linked chains on the surface of the ZP are key factors for sperm-ZP binding [2], although the precise recognition molecules are not known. In the pig, the oocyte and surrounding cumulus cells synthesize the ZP during oocyte growth [3]. Although it has been assumed that ZP synthesis is almost complete at the antral follicle stage [4], changes in the characteristics of the ZP during *in vitro* oocyte maturation are evidenced by changes in the architecture of porcine ZP and by the ability of porcine ZP to induce an acrosome reaction [1, 5, 6]. In addition, changes in the ZP properties during oocyte maturation, such as in the ultra-structure and ZP elasticity, are reported in other species [7, 8]. The cause and effect of these changes in ZP properties are unclear. Other changes in ZP properties during oocyte maturation are likely, e.g., the sperm binding ability of the ZP during *in vitro* maturation.

The main aim of the present study was to investigate sperm-ZP binding properties during oocyte culture *in vitro* and to examine the contribution of cumulus cells and N-linked glycosylation to the changes in sperm-ZP binding ability.

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Materials and Methods

Chemicals

Unless otherwise indicated, all chemicals were purchased from Nacalai Tesque, Kyoto, Japan.

Oocyte collection and in vitro maturation

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in phosphate-buffered saline (PBS) containing 10 IU/ml

penicillin G potassium and 0.1 g/ml streptomycin sulfate at 37ºC within 1 h. The oocytes were aspirated from the follicles (3–6 mm in diameter) using an 18-gauge needle connected to a 10 ml syringe. Only cumulus oocyte complexes (COCs) with compact cumulus cells were selected and used for the experiment. For *in vitro* maturation, oocytes were cultured in *in vitro* maturation medium at 38.5°C under 5% CO_2 in air with maximal humidity (10 oocytes/100 μl). The maturation medium was TCM-199 (Gibco BRL, Paisley, UK) supplemented with follicular fluid (10%, v/v), follicle stimulating hormone (1 mAU/ml), and 0.5 mM pyruvate acid (Sigma-Aldrich, St. Louis, MO, USA).

Cumulus cell preparation

Just after collection, oocytes were removed from the cumulus cells by 0.2% hyaluronidase treatment followed by 5 min of vortexing. The medium containing cumulus cells was centrifuged for 3 min (300 g) and the resulting pellets were re-suspended in maturation medium to a final concentration of 10^6 cells/ml. Denuded oocytes were cultured in drops of these suspensions.

ZP collection

At 0, 20, 44, or 68 h after culture, oocytes were removed from the cumulus cells by 0.2% hyaluronidase treatment followed by 5 min of vortexing. ZPs were collected from the oocytes using a narrow-pulled Pasteur pipette. ZPs with an intact morphology (spherical shape with only one slit) were used for the experiments.

Sperm preparation

A frozen-thawed semen mixture prepared from three boars was used for the sperm-ZP binding experiments. The medium used for sperm preparation was calcium- and magnesium-free PBS or modified Tris-buffered medium (mTBM; 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2$, 20 mM Tris [T-1410; Sigma-Aldrich], 11 mM glucose, 5 mM sodium pyruvate, 1 mM caffeine, and 0.8% [w/v] bovine serum albumin [A-3311, Fraction V, Sigma-Aldrich]) [9]. PBS for sperm washing consisted of 136.9 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.5 mM sodium pyruvate, 5.6 mM glucose, and 0.1% (w/v) bovine serum albumin. Frozen semen was thawed at 38ºC in a water bath and then diluted in 10 ml PBS. The sperm suspension was centrifuged twice at 350 g for 3 min, and the final sperm pellet was suspended in mTBM at a concentration of 1 × 10^6 sperm/ml. ZPs were washed twice in mTBM, and transferred into 50 μl of mTBM. An equal volume of the sperm suspension (50 μl) was added to the drops of mTBM containing 5 ZPs and co-incubated for 2 min (final sperm concentration 5 × 10^5 sperm/ml). After co-incubation, ZPs were carefully washed twice in PBS using pipettes (approximately 200 μm in diameter) and mounted on a slide. Samples were air-dried overnight and then PBS containing Hoechst 33342 (5 mg/ml) was applied to the slide. The number of sperm bound to each ZP was counted under a fluorescence microscope (Olympus, Tokyo, Japan) (see Fig. 1).

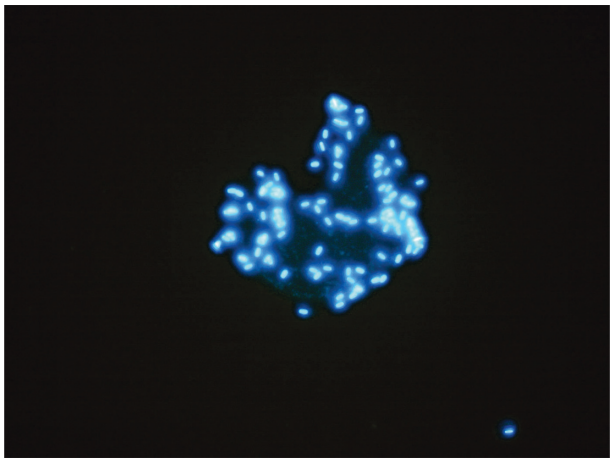

Fig. 1. Image of sperm bound to ZP. Sperm were stained with Hoechst 33342 and the number of sperm bound to ZP was counted under a fluorescence microscope.

Experimental design

In a preliminary experiment, ovaries were collected from the slaughterhouse every other day. The effect of culture duration on sperm-ZP binding properties was investigated. Oocytes were collected and cultured for 0 and 44 h and the ZP were collected from the oocytes on the same day. Twenty ZPs were selected from each pool and co-incubated with sperm for 2 min, and the number of sperm bound to the ZP was counted. Experiments were conducted 3 times with each oocyte collection series and a total of 60 ZPs was used for the experiments unless otherwise indicated.

In Experiment 1, ovaries were collected from the slaughterhouse on three consecutive days, and the number of sperm bound to the ZP was counted. Oocytes were cultured for 20, 44, or 68 h. ZPs were collected on the same day from oocytes that were maturation-cultured for either 20 h, 44 h, or 68 h, yielding three groups of ZP pools.

In Experiment 2, denuded oocytes were used instead

Table 1. Effect of maturation periods of oocytes cultured in cumulus-oocytes complexes on the number of sperm bound to the zona pellucida

Maturation period (h)[1)]	No. of ZP	No. of sperm bound to the ZP[2)]
		Mean ± SE
20	60	69.0 ± 1.5[a]
44	60	90.0 ± 1.2[b]
68	60	72.1 ± 1.6[a]

[1)]ZP were collected from oocytes that were cultured for 20, 44, and 68 h in cumulus-oocytes complexes. [2)]ZP were co-incubated with sperm for 2 min and the number of sperm bound to the ZP was counted. [a–b]Values with different superscripts differ significantly ($P < 0.05$).

Table 2. Effect of maturation periods of oocytes cultured after removal of cumulus cells on the number of sperm bound to the zona pellucida

Maturation period (h)[1)]	No. of ZP	No. of sperm bound to the ZP[2)]
		Mean ± SE
20	60	77.2 ± 3.1[a]
44	60	89.0 ± 3.3[b]
68	60	75.5 ± 2.5[a]

[1)]ZP were collected from oocytes that were cultured for 20, 44, and 68 h after removal of cumulus cells. [2)]ZP were co-incubated with sperm for 2 min and the number of sperm bound to the ZP was counted. [a–b]Values with different superscripts differ significantly ($P < 0.05$).

of COCs and the same experimental procedure as described for Experiment 1 was followed to examine the effect of culture duration on the sperm-ZP binding property. Oocytes were freed from cumulus cells immediately after collection from ovaries.

In Experiment 3, the effect of the presence of cumulus cells during oocyte maturation on the sperm-ZP binding property was examined. In addition, the effect of oocyte maturity, based on polar body extrusion, on the sperm-ZP binding was also examined. COCs, denuded oocytes, or a combination of denuded oocytes and cumulus cells (1×10^6 cells/ml) were cultured for 44 h. One hundred oocytes were cultured for each experimental group. After the culture period (44 h), oocytes were divided into two groups according to the presence or absence of a polar body. Six groups in all were examined and the rate of polar body extrusion was recorded. The ZP were then collected and pooled. Sets of 20 ZPs were randomly selected from the pool of ZP and used for the sperm-ZP binding assay. Each experiment was conducted 3 times with each oocyte collection series and total of 300 oocytes and 60 ZPs were used for each group.

In Experiment 4, the effect of N-linked glycosylation during oocyte maturation on sperm-ZP binding was examined. Forty COCs were matured in medium containing 0 or 3 mM tunicamycin for 44 h. After 44 h, oocytes were divided into two groups according to the presence or absence of a polar body, and the rate of polar body extrusion was recorded. ZPs were then collected and pooled from only oocytes that extruded a first PB. Sets of 20 ZPs were randomly selected from the pool of ZP and used for the sperm-ZP binding assay. Each experiment was conducted 3 times with each oocyte collection series and a total of 120 oocytes and 60 ZPs were used for each group.

Statistical analysis

The number of sperm attached to the ZP and the rate of oocyte maturation were analyzed using a one-way ANOVA followed by Tukey's post-hoc test. The data from Experiment 3 were analyzed using two-way ANOVA. The percentage of oocytes with a polar body was arcsin-transformed before analysis. A *P* value less than 0.05 was considered to be significant.

Results

The mean number of sperm bound to the ZP increased significantly from immediately after collection (63.5 ± 1.5 [mean ± se]) to 44 h of maturation culture (91.2 ± 2.6; $P < 0.001$). The number of sperm bound to the ZP increased during oocyte maturation (69.0 at 20 h and 90.0 at 44 h) and the number of sperm bound to the ZP was significantly decreased ($P < 0.05$) when COCs were cultured for a further 24 h (Table 1). In Experiment 2, oocytes were denuded and cultured for 20 to 68 h. The number of sperm bound to the ZP also increased from 77.2 to 89.0 during oocyte maturation (from 20 h to 44 h). When the oocytes were incubated for an additional 24 h, the number of sperm bound to the ZP decreased ($P < 0.05$, Table 2). In Experiment 3, oocytes were cultured for 44 h under three conditions: COCs, denuded oocytes, or a combination of denuded oocytes and cumulus cells. The percentage of oocytes with an extruded first polar body was significantly higher among COCs than among the other groups (66.7% vs. 50.0% and 53.4%, respectively, $P < 0.05$). There was no difference between the numbers of sperm bound to the ZP among the three culture conditions (93.7, 98.0, and 96.6, respectively). The number of sperm bound to the ZP was greater in ZP collected from oocytes with a polar body than in ZP collected from oocytes without a

Table 3. Effect of maturation conditions on the number of sperm bound to the zona pellucida

Maturation conditions[1)]	No. of oocytes	Rate of PB extrusion (%)[2)] Mean ± SE	PB extrusion[2)]	No. of ZP	No. of sperm bound to the ZP[3)] Mean ± SE
COCs	300	66.7 ± 3.3[a]	+	60	93.7 ± 2.8[a]
			–	60	70.2 ± 1.8[b]
Denuded	300	50.0 ± 2.9[b]	+	60	98.0 ± 2.7[a]
			–	60	71.1 ± 1.9[b]
Denuded and CC	300	53.3 ± 1.7[b]	+	60	96.6 ± 1.8[a]
			–	60	75.3 ± 2.2[b]

[1)]COCs, denuded oocytes, or a combination of denuded oocytes and cumulus cells (1×10^6 cells/ml) were cultured for 44 h. [2)]After 44 h, oocytes were divided into two groups according to the presence or absence of a polar body, and the rate of PB extrusion was recorded. ZP were then collected from each group of oocytes. [3)]ZP were co-incubated with sperm for 2 min and the number of sperm bound to the ZP was counted. [a–b]Values with different superscripts differ significantly ($P < 0.05$).

Table 4. Effect of tunicamycin in the maturation medium on the number of sperm bound to the zona pellucida

Tunicamycin (3 mM)[1)]	No. of oocytes	Rate of PB extrusion (%)[2)] Mean ± SE	No. of ZP	No. of sperm bound to the ZP[3)] Mean ± SE
–	120	63.0 ± 1.0	60	93.4 ± 3.0[a]
+	120	60.8 ± 1.5	60	75.8 ± 2.0[b]

[1)]COCs were cultured in medium containing 0 or 3 mM tunicamycin for 44 h. [2)]After culture for 44 h, oocytes were divided into two groups according to the presence or absence of a polar body and the rate of the 1st PB extrusion was recorded. Then ZP were collected from only oocytes that extruded the 1st PB. [3)]ZP were co-incubated with sperm for 2 min and the number of sperm bound to the ZP was counted. [a–b]Values with different superscripts differ significantly ($P < 0.05$).

polar body ($P < 0.05$, Table 3). There was no interaction between the maturation-culture conditions and polar body extrusion with regard to the number of sperm bound to ZP. Adding tunicamycin to the maturation medium did not affect the maturation ratio, but decreased the number of sperm bound to the ZP ($P < 0.05$, Table 4).

Discussion

The present study showed that the sperm-binding ability of ZP increased during oocyte maturation, and that oocytes, but not cumulus cells, played a key role in the sperm-binding ability. The number of sperm bound to the ZP during co-incubation with sperm for 2 min was first compared between ZPs just after collection and ZPs after 44 h of maturation. The number of sperm bound to the ZP after 44 h of maturation was significantly greater than that just after collection. In porcine oocytes, 40 to 48 h is the optimal culture period [10]. We thus evaluated 3 time points (20, 44, and 68 h) to examine the kinetics of ZP-sperm binding around the optimal maturation period. In addition, we collected ZPs from oocytes cultured for different durations, because sperm-ZP binding characteristics change during ZP preservation for 24 to 48 h in PBS at 4 C. The number of sperm bound to the ZP also increased from 20 h to 44 h of maturation-culture, and decreased from 44 h to 68 h of additional culturing, demonstrating that there may be two distinct phases of ZP sperm-binding ability. In porcine oocytes, exceeding the optimal maturation period induces oocyte aging. Premature partial cortical granule exocytosis and ZP hardening occur in aged mouse oocytes [11], suggesting that ZP characteristics also change according to oocyte age in the pig. Based on this, oocyte aging is likely to be the cause of the

decrease in the number of sperm bound to the ZP. The ZP ultrastructure and the ability to induce an acrosome reaction are reported to change during *in vitro* maturation of porcine oocytes [1, 5, 6]. In the present study, the number of sperm bound to the ZP increased from 0 to 44 h and from 20 to 44 h of maturation culture. It is plausible that the increased sperm-binding is due to modifications such as proteins secreted by either oocytes or cumulus cells. Pig ZP, similar to related domestic animals, is comprised of three glycoproteins: ZPA, ZPB, and ZPC [12], and the spatio-temporal expression/secretion of the three glycoproteins is differentially regulated during folliculogenesis [3]. In the pig, these proteins are secreted by both oocytes and the surrounding somatic cells [3]. In the present study, the sperm-ZP binding properties also changed during oocyte maturation when oocytes were denuded and cultured for 44 h, suggesting that the changes in the ZP properties are affected by the oocytes themselves, or by changes in the ZP. To further evaluate this hypothesis, oocytes in three different conditions (COCs, denuded oocytes, and both denuded oocytes and cumulus cells) were cultured for 44 h. The number of sperm bound to the ZP was similar among the three groups, suggesting that the cumulus cell-oocyte connection or that proteins secreted from cumulus cells have a very small role in the changes in sperm-ZP binding. In this experiment, the oocytes were classified into two groups, oocytes with or without a polar body. The aim of this classification was to investigate the effect of oocyte maturity or oocyte quality on sperm-ZP binding. In all groups, ZPs collected from oocytes with an extruded polar body had greater sperm binding ability than ZPs from oocytes without a polar body. This finding suggests that the oocyte quality and maturation state affect sperm-ZP binding. Therefore, in the next experiment we classified oocytes into two groups according to the presence or absence of a polar body. Most of the carbohydrate structures of the pig ZPs proteins have been identified, and the N- and O-glycosylation sites of ZPB and ZPC are established. Furthermore, the structural significance of the ZP N-glycans in species-selective recognition of the spermatozoa between pig and cattle are also known [13]. The initial binding between sperm and ZP depends on the carbohydrate structure itself as well as on its position in the molecule and the three-dimensional architecture of the ZP affects the binding function [1]. Fléchon *et al.* [14] reported that incubation of porcine oocytes with tunicamycin decreases the amount of ^{3}H-fucose labeled molecules in the ooplasm and perivitelline space. This finding suggests that N-linked modification of proteins occurs during oocyte maturation. In the present study, the addition of 3 μM tunicamycin to the maturation medium did not affect the oocyte maturation rate, consistent with a previous report [14], and the number of sperm bound to the ZP was significantly decreased. These results strongly suggest that N-linked modification of some proteins on the surface of the ZP is one of the causes of the increase of sperm-ZP binding during oocyte maturation.

Many researchers are interested in developing methods to prevent polyspermic fertilization in porcine oocytes, and the effects of artificial ZP modifications, such as pre-fertilization ZP treatment with a Ca-ionophore and amine reactive cross-linker on polyspermic fertilization, have been reported [15–17]. In the present study, the relationship between the number of sperm bound to ZP during a short co-incubation period and fertilization outcome is still unclear, because fertilization comprises many steps (i.e., initial sperm-ZP binding, induction of the acrosome reaction, tight sperm-ZP binding, penetration, and sperm-oolemma fusion). We previously reported that adding N-acetyl-D-glucosamine to the sperm-ZP co-incubation medium reduces the number of sperm bound to ZP but increases the rate of polyspermic fertilization [18]. Moreover, N-acetyl-D-glucosamine reduced postfertilization ZP hardening [18]. Furthermore, adding tunicamycin to the maturation medium changes porcine ZPs modification and increases polyspermic fertilization [14], whereas tunicamycin decreased ZP-sperm attachment in the present study. Further studies are required to determine the significance of changes in ZP-sperm binding properties.

In conclusion, sperm-ZP binding increased during oocyte maturation and oocytes play a key role in the changes in the sperm-ZP binding properties.

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