Journal of Reproduction and Development, Vol. 57, No. 6, 2011

—Original Article—

Role of Acidification Elicited by Sialylation and Sulfation of Zona Glycoproteins During Oocyte Maturation in Porcine Sperm-zona Pellucida Interactions

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Abstract. The porcine zona pellucida (ZP) undergoes biochemical changes during the final phase of maturation prior to fertilization. The present study was conducted to elucidate whether the acidification of ZP glycoproteins during porcine oocyte maturation influences sperm-ZP interactions. Two-dimensional gel electrophoresis clearly demonstrated that ZP acidification occurred in accordance with the sialylation and sulfation of ZP glycoproteins in oocytes matured for 44 h. The increases in the incidences of sperm penetration and polyspermy with the progress of the IVM culture period were significantly suppressed by ZP desialylation on treatment with neuraminidase as a consequence of reductions in the number of sperm bound to ZPs and the acrosome reaction (AR) in ZP-bound sperm ($P<0.05$). In contrast, the blocking of ZP sulfation by $NaClO_3$ treatment during IVM markedly reduced the incidence of polyspermy with no inhibitory effect on penetration, but the number of sperm bound to ZPs and the rate of AR-inducing sperm were decreased to the same level as in desialylated oocytes. The results indicate that ZP sulfation influences sperm-ZP interactions in a ZP sialylation-independent manner. Moreover, sialylation and sulfation were not associated with a protective proteolytic modification of the ZP matrix before fertilization. These findings suggest that ZP acidification elicited by the sialylation and sulfation of ZP glycoproteins during oocyte maturation contributes to the porcine ZP acquiring the capacity to accept sperm.
Key words: Acidification, Porcine oocytes, Sialylation, Sulfation, Zona pellucida

(**J. Reprod. Dev. 57:** 744–751, 2011)

All mammalian eggs are surrounded by a relatively thick, insoluble extracellular coat called the zona pellucida (ZP) [1–3]. During fertilization, the ZP mediates species-selective recognition between the oocyte and spermatozoon [4]. There is evidence that the sperm-ZP interaction is a carbohydrate-mediated event [5–8] that triggers a signal transduction pathway that results in the fenestration and fusion of the sperm plasma membrane and the outer acrosomal membrane (acrosome reaction, AR) [3].

The porcine ZP is composed of three glycoprotein families, ZP1 (ZPA; 92 kDa), ZP3α (ZPB; 55 kDa) and ZP3β (ZPC; 55 kDa) [9, 10]. ZP1 is split into two smaller molecules, ZP2 (69 kDa) and ZP4 (23 kDa), under reducing conditions [11]. Very recently, we reported that the increase in the amount of terminal *N*-acetylglucosamine (GlcNAc) residues in porcine ZP3 glycoproteins through new *N*-glycosylation for periods in excess of 20–24 h after meiotic maturation played a critical role in sperm-ZP interactions [12]. This new *N*-glycosylation is responsible for significant increases in the sperm penetration rate, the polyspermic fertilization rate, the number of sperm bound to ZPs and the number of AR-inducing sperm. These findings clearly imply that ZP glycoproteins undergo biochemical changes during oocyte maturation prior to fertilization. In this context, it is of interest that the acidity of porcine ZP glycoproteins increases in mature oocytes compared with immature oocytes as indicated by an acidic shift of pI units in two-dimensional (2D) gel electrophoresis elicited by increases in the sialic acid residues and sulfate of ZP glycoproteins [13–15]. Porcine ZP3 glycoproteins are highly heterogeneous mainly as a results of the differences in the amount of sulfated *N*-acetyllactosamine and sialic acid in the acidic chains [16] and have been shown to be required for the binding of sperm to the intact ZP leading to completion of AR [17]. It is reported that the sialic acid and sulfate of ZP glycoproteins are physiologically involved in regulating the binding of sperm to ZPs and the partial activation of proacrosin to form α- and β-acrosin in mouse [18], bovine [19] and porcine [20] oocytes. Contrary to these reports, Liu *et al.* [21] suggest that neither the sulfate nor sialic acid present on mouse ZP3 oligosaccharides is directly involved in sperm receptor or AR-inducing activity. Additionally, there are apparently no reports regarding whether the acidification of ZP glycoproteins elicited by sialylation and sulfation during *in vitro* maturation (IVM) of porcine oocytes has crucial effects on sperm-ZP interactions.

Therefore, in the present study, experiments were undertaken 1) to examine whether porcine ZP glycoproteins are acidified with the advance of sialylation and sulfation during oocyte maturation

Received: April 27, 2011
Accepted: August 8, 2011
Published online in J-STAGE: September 6, 2011

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and 2) to evaluate the effects of sialylation and sulfation of ZP glycoproteins during IVM on fertilization responses including sperm penetration and polyspermy, sperm binding to ZPs, induction of AR in ZP-bound sperm and ZP hardness.

Materials and Methods

All chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Collection of oocytes and in vitro maturation

Ovaries were collected from prepubertal gilts (85–110 kg, mean age of 6 months) at a local slaughterhouse and transported to the laboratory in 0.9% (w/v) NaCl at 30 C. Within 2 h post slaughter, the visible small antral follicles (2–6 mm in diameter) on the ovarian surface were excited and recovered by using a razor blade. Cumulus-oocyte complexes (COCs) were collected by scraping the inner surface of the follicle walls with a disposable surgical blade. They were washed three times with HEPES-buffered Tyrode medium containing 0.01% (w/v) PVA (H-TL-PVA). The basic medium for oocyte maturation was tissue culture medium 199 (TCM 199, HEPES-buffered) supplemented with 0.57 mM cysteine, 0.04 units/ml ovine FSH, 0.02 units/ml ovine LH and 10% (v/v) porcine follicular fluid. Porcine follicular fluid, aspirated from follicles 2–6 mm in diameter, was centrifuged (10 000 × *g* for 15 min at 4 C) to remove cellular debris and stored at −30 C until used. After being washed in IVM medium, groups of 15–20 COCs were transferred to 100-µl droplets of IVM medium, covered with light weight mineral oil and incubated for various periods at 39 C in an atmosphere of 5% CO_2 in air. Since germinal vesicle breakdown and oocyte maturation at the metaphase II (M-II) stage were achieved at 28 and 44 h of IVM, respectively, under our culture conditions, oocytes cultured in IVM medium for 0, 28 and 44 h were employed for various experiments to determine sperm-ZP interactions associated with meiotic progression.

Removal of sialic acid residues and blocking of sulfation residues

To remove the sialic acid residues from the ZP, some oocytes were treated with 1 IU/ml of neuraminidase, a hydrolase specific to neuraminic acid, for 1 h following 0, 28 and 44 h of IVM, and to block the sulfation of ZP glycoproteins, other oocytes were cultured in IVM medium supplemented with 50 mM sodium chlorate ($NaClO_3$), which acts as a sulfation inhibitor of carbohydrate. This dose of $NaClO_3$ had no deleterious effect on oocyte maturation at the M-II stage (73.3 ± 4.0%, n=120) compared with the control (75.8 ± 3.7%, n=132).

Isolation and biotinylation of ZPs and assessment of ZP modifications by two-dimensional gel electrophoresis

After 0 and 44 h of IVM, COCs were stripped of cumulus-granulosa cells by passage through a narrow-bore pipette in H-TL-PVA containing 0.1% (w/v) hyaluronidase. The intact ZP was isolated from oocytes and washed as described by Kurasawa *et al*. [22]. Briefly, ZPs were ruptured and removed mechanically with a narrow micropipette (about 60 µm in diameter) in Dulbecco's phosphate buffered saline (PBS) containing 1 mg/ml ethylenediaminetetraacetic acid (EDTA), 10 µg/ml lima bean trypsin inhibitor and 0.1 mM phenylmethylsulfonyl fluoride and washed four times. The ZPs were transferred to 1 M NaCl containing 1% Triton X-100 and 1 mM benzamidine for 5 min and then washed three times in H-TL-PVA. The intact ZPs were biotinylated with water-soluble succinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin II; Thermo Scientific Pierce Protein Research Products, Rockford, IL, USA) for 4 h at room temperature as described previously [23, 24]. Twenty biotinylated ZPs were dissolved in 20 µl of lysis buffer consisting of 60 mM Tris, pH 8.8, 5 M urea, 1 M thiourea, 1% (v/v) CHAPS, 1% (v/v) Triton X-100 and 66 mM dithiothreitol for 1 h at room temperature and applied to 5-cm-long agar gel strips (pH 3–10; ATTO Corporation, Tokyo, Japan) according to the manufacturer's instructions. Under reducing conditions, isoelectric focusing was carried out on a discRun Unit (ATTO) at 300 V for 150 min at room temperature. For the second dimension, the strips were equilibrated for 10 min in 50 mM Tris-HCl, pH 6.8, containing 2% SDS and then placed onto a 10% SDS-polyacrylamide gel. The electrophoresis was carried out under reducing conditions, and the products were transferred to PVDF membranes (Hybond-P; GE Healthcare, Buckinghamshire, U.K.). The membranes were blocked for 2 h at room temperature with 3% (v/v) teleostean skin gelatin in Tris-buffered saline (pH 7.4) containing 20 mM Tris-HCl, 150 mM NaCl and 0.1% (v/v) Tween-20 (TBS-T-gelatin), incubated for 50 min at room temperature with 0.1% (v/v) streptavidin-horseradish peroxidase conjugate (GE Healthcare) in TBS-T-gelatin and then washed with TBS-T. The bound peroxidase was detected using ECL detection kits and an ECL Mini-camera (GE Healthcare) according to the manufacturer's instructions. The amount of each ZP glycoprotein in the pI range of 5.5–7.0 was quantified using UN-SCAN-IT gel (Silk Scientific, Orem, UT, USA). The data were expressed in terms of the fold increase in intensity of the biotinylated-ZP band present in oocytes freshly isolated from follicles.

Detection of sialic acid residues by lectin blotting

Ten intact ZPs were subjected to SDS-PAGE on a 7.5% polyacrylamide gel under reducing conditions and transferred to PVDF membranes as described previously [12]. After blocking for 2 h in TBS-T-gelatin, the membranes were treated with 2.5 µg/ml of biotin-labeled SSA (Sambucus sieboldiana; Seikagaku Corporation, Tokyo, Japan) lectin in TBS-T for 40 min. As mentioned above, lectin-binding ZP glycoproteins were detected by the ECL method, and the lectin blotting intensity was quantified using UN-SCAN-IT gel. The lectin blotting intensity in oocytes freshly isolated from follicles was taken to be 1.0, with data reported as relative values.

In vitro fertilization

After IVM, COCs were sucked through a narrow-bore pipette to remove their cumulus cells in H-TL-PVA containing 0.1% (w/v) hyaluronidase. The oocytes were rinsed with modified Tris-buffered medium (mTBM; [25]) designated as *in vitro* fertilization (IVF) medium and supplemented with 2 mM caffeine sodium benzoate and 0.1% (w/v) BSA. Groups of 15–20 oocytes were ultimately transferred to 50-µl droplets of IVF medium that had been covered with warm mineral oil. The droplets were kept in an incubator containing 5% CO_2 in air at 39 C for 1 h until spermatozoa were added

for fertilization.

After thawing and washing, frozen ejaculated boar spermatozoa were resuspended at 4×10^8 sperm/ml in mTBM supplemented with 4 mM caffeine sodium benzoate and 0.4% (w/v) BSA and then incubated for 90 min at 39 C in a 5% CO_2 incubator. After preincubation, 50 µl of the diluted sperm suspension in IVF medium was added to a droplet containing oocytes at a final concentration of 1×10^6 sperm/ml, and the gametes were coincubated for 10 h under the conditions described above.

Assessment of fertilization parameters

After 10 h of *in vitro* insemination, groups of 30–40 cumulus-free oocytes were mounted on slides, fixed, stained with 1% (v/v) lacmoid in 45% (v/v) acetic acid and examined under a phase-contrast microscope at × 400 magnification. Oocytes were designated as penetrated when one or more sperm heads and/or male pronuclei (MPN) and corresponding sperm tails were present. The sperm penetration rate was determined using all inseminated oocytes, and the rates of polyspermy and MPN formation and mean number of sperm per oocyte were determined from the oocytes penetrated.

Sperm-ZP binding assay

After 2 h of coincubation, the oocytes and bound sperm were gently pipetted 10 times in H-TL-PVA with a wide-bore pipette to remove loosely bound sperm and fixed with 2% formaldehyde for 40 min at room temperature. The oocytes were then placed into 50-µl drops of H-TL-PVA containing 10 µg/ml of *bis*-benzimide Hoechst 33342 and incubated for 10 min. The oocytes were washed in H-TL-PVA and mounted, and sperm tightly bound to the ZP were counted under a fluorescence microscope (Nikon, Tokyo, Japan).

Evaluation of sperm acrosomal status

To examine the induction of AR in sperm bound to the ZP, the preincubated sperm were coincubated with cumulus-free oocytes in IVF medium for 2 h, since the penetration of ZPs was observed from 2 h after the start of insemination under our experimental conditions. After several rinses with PBS using a wide-bore pipette to remove loosely attached sperm, sperm-oocyte complexes were immediately stained with 2 µg/ml of Alexa-labeled PNA (peanut agglutinin; Invitrogen, Carlsbad, CA, USA), 1 µg/ml of 4'6-diamidino-2-phenylindole-2HCl (DAPI), and 10 µg/ml of propidium iodide (PI) in PBS at 39 C for 20 min, fixed with 4% formaldehyde for 15 min at room temperature and mounted on glass slides. For evaluation of sperm acrosomal status using a fluorescence microscope, sperm showing partial or complete green fluorescence (Alexa-PNA staining) in the acrosomal region were evaluated as AR-inducing sperm, and sperm showing partial or complete red fluorescence (PI staining) were excluded from the data set as nonviable sperm.

Assessment of ZP solubility

After the maturation culture, COCs were stripped of expanded cumulus-granulosa cells by passage through a narrow-bore pipette in H-TL-PVA containing 0.1% (w/v) hyaluronidase. After three washes in H-TL-PVA, 15–20 cumulus-free oocytes were transferred to 50-µl droplets of 0.1% (w/v) protease solution in H-TL-PVA. Dissolution time was observed at room temperature with an inverted microscope (magnification, × 200). For each oocyte, the time required for dissolution was recorded as the ZP dissolution time.

Experimental design

In Experiment 1, to examine whether porcine ZP glycoproteins were acidified with the advance of sialylation and sulfation during oocyte maturation, 2D gel electrophoresis and lectin blotting assays were performed for ZPs of oocytes freshly isolated from their follicles and IVM oocytes cultured for 44 h in the absence or presence of neuraminidase or $NaClO_3$.

Experiment 2 was designed to evaluate the effects of the sialylation and sulfation of ZP glycoproteins during oocyte maturation on subsequent sperm-ZP interactions in porcine oocytes. After 0, 28 and 44 h of IVM, a large portion of oocytes treated with neuraminidase, $NaClO_3$ and neuraminidase plus $NaClO_3$ were inseminated with preincubated sperm to examine fertilization parameters, the binding of sperm to ZPs and AR induction in ZP-bound sperm, and the remaining oocytes were used to assess ZP dissolution. The oocytes removed of sialic acid residues or with blocked sulfation during IVM were coincubated with preincubated sperm in IVF medium containing 1 IU/ml neuraminidase and 50 mM $NaClO_3$, respectively, with attention paid to the blocking of sialylation and sulfation during the IVF procedure.

Statistical analysis

Data from four replicates are expressed as the mean ± SEM. All statistical analyses were performed using the R statistical analysis system software package (http://www.R-project.org/). To evaluate the differences between groups, the percentage data (nonparametric data) were analyzed by a generalized linear model (GLM, in accordance with a binomial distribution) and ANOVA procedures followed by the Tukey test for nonparametric multiple comparisons [26]. Analyses of other data (parametric data) were carried out with the Shapiro-Wilk normality test and the GLM (in accordance with the Gaussian distribution) and ANOVA procedures followed by the Tukey-Kramer test. A probability of $P<0.05$ was considered statistically significant.

Results

Acidification of ZP glycoproteins during oocyte maturation

Two-dimensional gel electrophoresis demonstrated an obvious shift in the mobility of ZP1 + ZP2 and ZP3 glycoproteins in response to acidity during the oocyte maturation process and a slight change to the ZP4 glycoprotein (Figs. 1A and 1B). Accordingly, at a pI of 5.5–7.0, the intensity of the ZP1 + ZP2 and ZP3 bands in oocytes cultured for 44 h in IVM medium declined dramatically to 0.2 ± 0.1 and 0.1 ± 0.1, respectively, compared with that in oocytes freshly isolated from follicles (1.0 ± 0.1 and 1.0 ± 0.1, respectively, $P<0.05$; Fig. 2). However, the mobility shift caused by the acidification of ZP glycoproteins in IVM oocytes was broadly prevented by the desialylation treatment, and the intensity of the ZP1 + ZP2 and ZP3 bands (0.5 ± 0.1 and 1.2 ± 0.2, respectively) at a pI of 5.5–7.0 was maintained in IVM oocytes treated with neuramini-

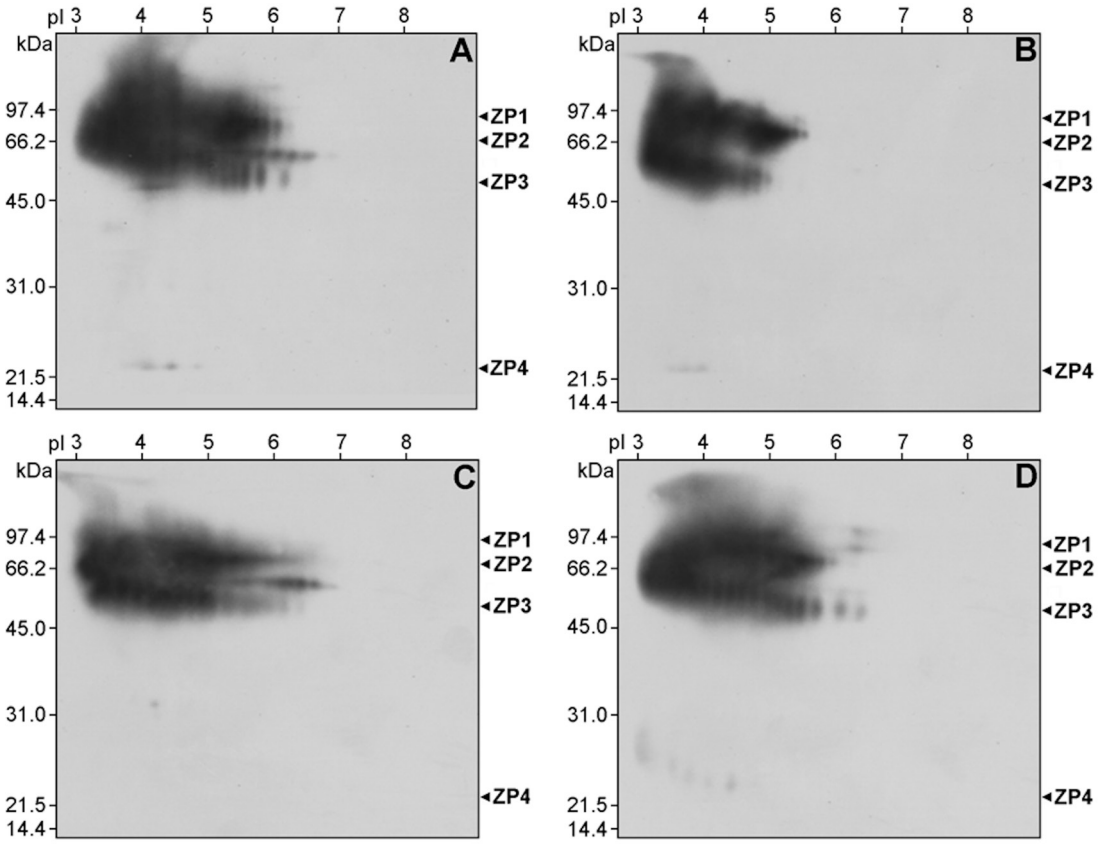

Fig. 1. Two-dimensional (2D) gel electrophoresis of porcine ZP glycoproteins analyzed by biotinylation and ECL in oocytes freshly isolated from follicles (A) and after IVM for 44 h (B). After the IVM, some oocytes were treated with 1 IU/ml of neuraminidase to remove sialic acid residues in ZP glycoproteins before the collection of ZPs (C). Other oocytes were treated with 50 mM $NaClO_3$ to block ZP sulfation during IVM (D). Twenty biotinylated ZPs were subjected to 2D gel electrophoresis under reducing conditions. The first dimension (left to right) is isoelectric focusing (pI of 3.0–10.0) and the second dimension (top to bottom) is SDS-PAGE in 10% gels.

dase (Figs. 1C and 2). The same phenomenon was also detected in the oocytes treated with $NaClO_3$ to block sulfation (Figs. 1D and 2). Moreover, the relative intensity of sialic acid residues in ZP glycoproteins detected by SSA blotting increased significantly to 1.4 ± 0.1 in oocytes cultured for 44 h compared with that in oocytes freshly isolated from follicles (1.0 ± 0.1, $P<0.05$), but this increase was completely repressed in oocytes treated with neuraminidase (Fig. 3).

Effects of sialylation and sulfation of ZP glycoproteins during oocyte maturation on sperm-ZP interactions and ZP solubility

The incidences of sperm penetration, polyspermy and MPN formation (Table 1), the number of sperm bound to ZPs (Fig. 4A) and the proportion of AR-inducing sperm (Fig. 4B) were significantly increased in untreated oocytes with the progress of the IVM culture period ($P<0.05$). However, the rates of penetration and polyspermy were significantly decreased in oocytes treated with neuraminidase after 28 and 44 h of culture compared with those in untreated oocytes as a result of significant reductions in the number of sperm bound to ZPs and the percentage of AR in sperm bound to ZPs ($P<0.05$). On the other hand, the blocking of sulfation during oocyte maturation had no inhibitory effect on the progressive increase in the penetration rate, despite the fact that the number of sperm bound to ZPs and the rate of AR-inducing sperm were reduced to the same level as in desialylated oocytes. In particular, the addition of $NaClO_3$ during IVM for 28 and 44 h to block the sulfation of ZP glycoproteins significantly reduced the incidence of polyspermy compared with the control ($P<0.05$). Treatment with neuraminidase plus $NaClO_3$ significantly decreased the rates of penetration, polyspermy and acrosome-reacting sperm and the number of sperm bound to ZPs in oocytes cultured for 28 and 44 h compared with those in untreated oocytes ($P<0.05$). However, the ZP dissolution time in 0.1% protease was not altered by treatment with neuraminidase, $NaClO_3$ or neuraminidase plus $NaClO_3$ and significantly decreased with the progress of IVM in all treatment groups ($P<0.05$; Fig. 5).

Discussion

In the present study, the acidity of ZP glycoproteins significantly

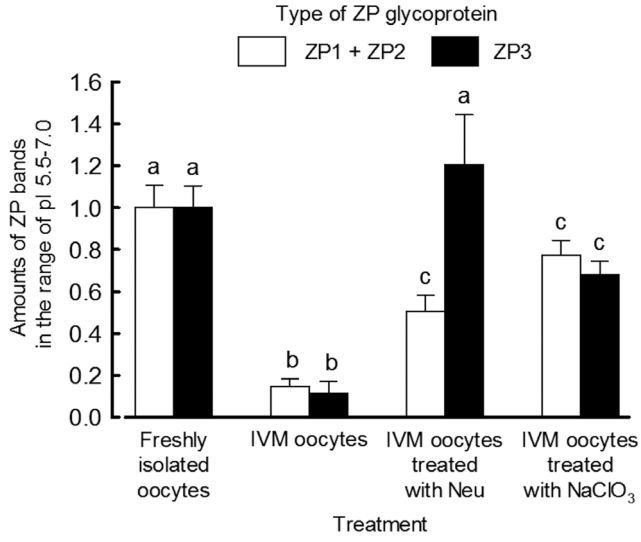

Fig. 2. Changes in amounts of porcine ZP1 + ZP2 and ZP3 glycoproteins (in the range of pI 5.5–7.0) subjected to 2D gel electrophoresis under reducing conditions. After 44 h of IVM, some oocytes were treated with 1 IU/ml of neuraminidase (Neu) before collection of ZPs. Other oocytes were treated with 50 mM $NaClO_3$ during IVM. Values are expressed as the mean ± SEM. The value for each ZP glycoprotein of freshly isolated oocytes was taken to be 1.0. [a-c] Values with different superscripts within each ZP glycoprotein are significantly different (P<0.05).

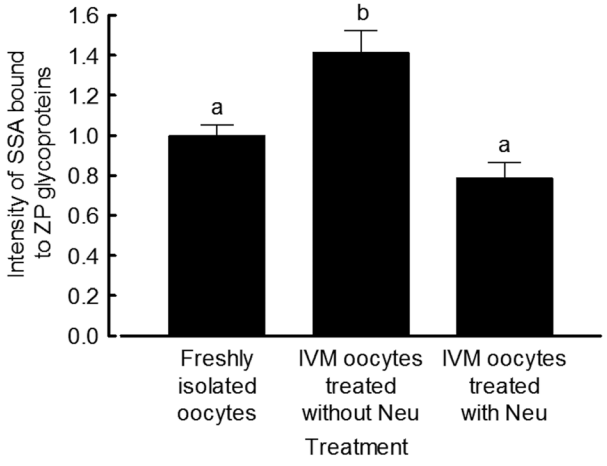

Fig. 3. Changes in the affinity of SSA lectin for glycoproteins of porcine ZPs collected from IVM oocytes. After 44 h of IVM, oocytes were treated with 1 IU/ml of neuraminidase (Neu) before collection of ZPs. Values are expressed as the mean ± SEM. The values for ZP glycoproteins of freshly isolated oocytes are taken to be 1.0. [a,b] Values with different superscripts are significantly different (P<0.05).

Table 1. Effects of treatment with neuraminidase (Neu), $NaClO_3$ and Neu plus $NaClO_3$ in porcine oocytes matured for 0, 28 and 44 h on the fertilization parameters

Treatment	IVM culture period (h)	No. of oocytes examined	Oocytes (%; mean ± SEM)			No. of sperm per penetrated oocyte (mean ± SEM)
			Penetrated	Polyspermic [a]	Male pronucleus [a]	
None (control)	0	120	16.7 ± 3.4 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	1.0 ± 0.0 [b]
	28	148	48.7 ± 4.1 [c]	26.4 ± 5.2 [c]	37.5 ± 5.7 [c]	1.4 ± 0.1 [c]
	44	136	69.9 ± 3.9 [d]	62.1 ± 5.0 [d]	65.3 ± 4.9 [d]	1.8 ± 0.1 [d]
1 IU/mL Neu	0	151	9.3 ± 2.4 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	1.0 ± 0.0 [b]
	28	123	32.5 ± 4.2 [c,*]	5.0 ± 3.5 [b,*]	22.5 ± 6.6 [b]	1.1 ± 0.1 [b, *]
	44	122	50.8 ± 4.5 [d,*]	45.2 ± 6.3 [c, *]	66.1 ± 6.0 [c]	1.5 ± 0.1 [c]
50 mM $NaClO_3$	0	141	18.4 ± 3.3 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	1.0 ± 0.0 [b]
	28	130	38.5 ± 4.3 [c]	10.0 ± 4.2 [b, *]	28.0 ± 6.4 [c]	1.1 ± 0.1 [b, *]
	44	127	70.9 ± 4.0 [d]	40.0 ± 5.2 [c, *]	60.0 ± 5.2 [d]	1.6 ± 0.1 [c]
1 IU/mL Neu + 50 mM $NaClO_3$	0	153	11.8 ± 2.6 [b]	0.0 ± 0.0 [b]	0.0 ± 0.0 [b]	1.0 ± 0.0 [b]
	28	131	33.6 ± 4.1 [c,*]	4.6 ± 3.1 [b, *]	27.3 ± 6.7 [c]	1.1 ± 0.1 [bc, *]
	44	128	58.6 ± 4.4 [d,*]	34.7 ± 5.5 [c, *]	60.0 ± 5.7 [d]	1.7 ± 0.3 [c]

[a] Percentage of oocytes that were penetrated. [b-d] Values with different superscripts in the same column are significantly different within each treatment group (P<0.05). * Values are significantly different as compared with the control in the same IVM culture period (P<0.05).

increased in IVM oocytes compared with immature oocytes as indicated by 2D gel electrophoresis, which was consistent with the sialylation and sulfation of ZP glycoproteins during oocyte maturation, because acidification was prevented in the desialylated oocytes and the sulfation-blocked oocytes (Figs. 1 and 2). These findings strongly suggest that porcine ZP glycoproteins undergo acidification in the final maturation phase of oocytes prior to fertilization, as reported by Rath *et al*. [13].

The incidences of penetration and polyspermy (Table 1), the number of sperm bound to ZPs (Fig. 4A) and the proportion of AR in sperm bound to ZPs (Fig. 4B) increased significantly in IVM oocytes with the progress of the maturation procedure. In addition, it is clear from the present study that the sialic acid residues labeled with SSA lectin on ZP glycoproteins were significantly increased

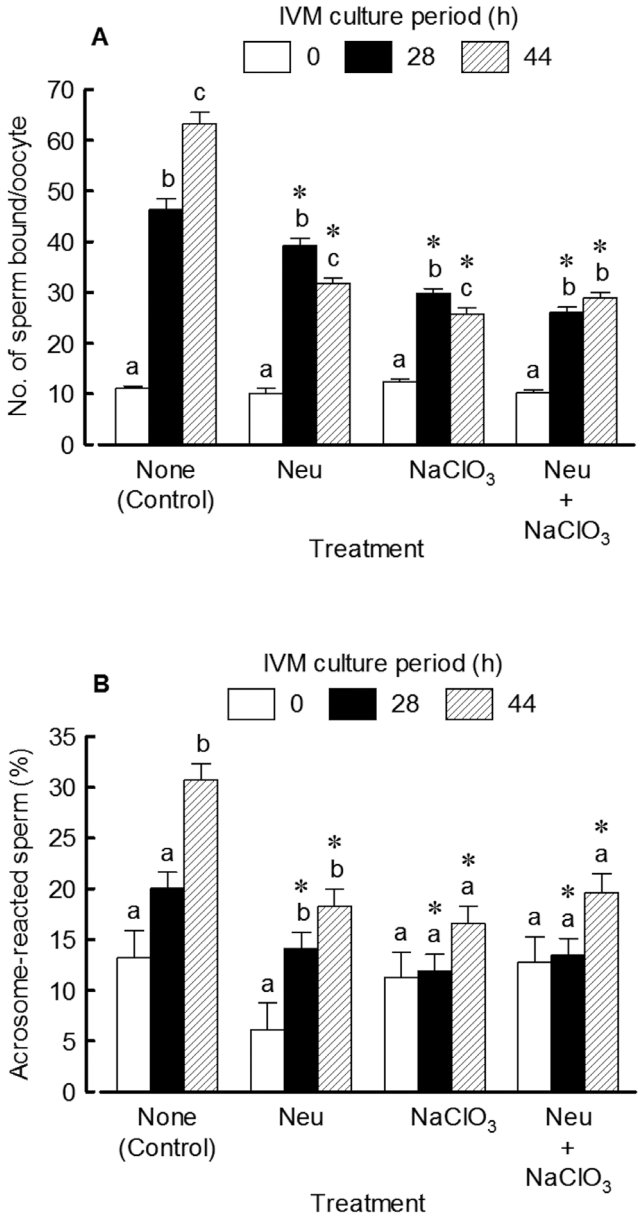

Fig. 4. Effects of treatment with 1 IU/ml neuraminidase (Neu), 50 mM $NaClO_3$ and Neu plus $NaClO_3$ in porcine oocytes matured for 0, 28 and 44 h on the binding of sperm to ZPs (A) and the AR induction in ZP-bound sperm (B). Treated oocytes were coincubated with preincubated sperm in IVF medium for 2 h. Values are expressed as the mean ± SEM. For each IVM culture period, the total number of oocytes examined was 35–50 for determination of the number of sperm bound to ZPs, and the total number of live sperm examined was 2931 to 3225 for determination of the number of acrosome-reacted sperm. [a-c] Values with different superscripts are significantly different within each treatment group ($P<0.05$). * Values are significantly different as compared with the control in the same culture period ($P<0.05$).

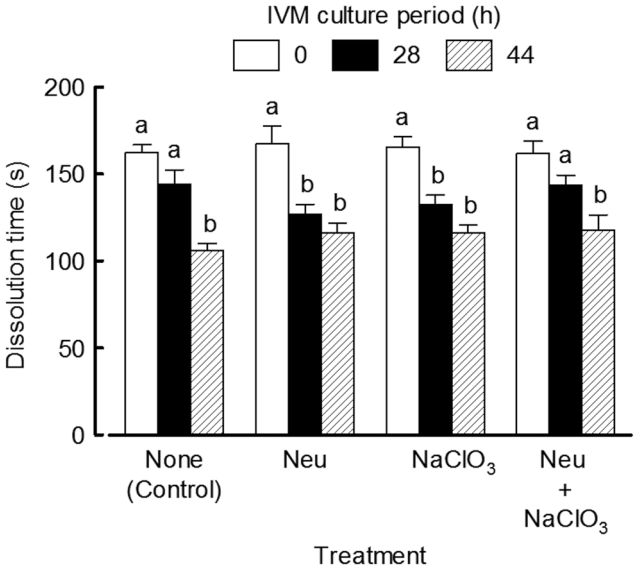

Fig. 5. Effect of treatment with 1 IU/ml neuraminidase (Neu), 50 mM $NaClO_3$ and Neu plus $NaClO_3$ in porcine oocytes matured for 0, 28 and 44 h on dissolution time of ZPs caused by 0.1% protease. Values are expressed as the mean ± SEM. The total number of oocytes examined was 81 to 102 for each IVM culture period. [a,b] Values with different superscripts are significantly different within each treatment group ($P<0.05$).

in IVM oocytes compared with those of oocytes freshly isolated from their follicles (Fig. 3). However, the increase in sialic acid was completely abolished in oocytes treated with neuraminidase, and the increases in the number of sperm bound to ZPs and the percentage of AR in sperm bound to ZPs with oocyte maturation were suppressed in the desialylated oocytes, resulting in a significant reduction in penetration and polyspermy rates. These findings indicate that the sialylation of ZP glycoproteins during oocyte maturation is associated with sperm-ZP interactions. A similar phenomenon is observed in bovine oocytes, and the number of sperm bound to ZPs and the rate of penetration were remarkably decreased in oocytes treated with neuraminidase compared with untreated oocytes [19]. In our preliminary study, SSA (recognizing the α-2,6-linked sialic acid) and MAL II (maackia amurensis lectin II; recognizing the α-2,3-linked sialic acid residues) lectins added to IVF medium strongly diminished the number of sperm bound to ZPs in matured oocytes (30.9 ± 1.4 and 35.6 ± 2.0, respectively) compared with the control (63.7 ± 1.7; unpublished data). In contrast, Velásquez *et al.* [19] reported that the α-2,3-linked, but not α-2,6-linked, sialic acids residues in bovine ZP glycoproteins were necessary for the binding between gametes. Thus, it seems that there is a difference between the two species in the sialylated oligosaccharide form associated with sperm-ZP interactions.

Although the addition of $NaClO_3$ during 28 and 44 h of IVM to block the sulfation of ZP glycoproteins efficiently decreased the number of sperm bound to ZPs and the rate of AR-inducing sperm to levels comparable with those in the neuraminidase-treated groups, the blocking of sulfation had no inhibitory effect on the progressive increase in the sperm penetration rate in contrast with the findings in the desialylated oocytes and strongly decreased the incidence of polyspermy compared with the control. From the different findings between the desialylated and sulfation-blocked

oocytes, it is considered that ZP sulfation during oocyte maturation influences sperm-ZP interactions in a sialylation-independent manner. The amount of sulfate is high in the basic structures of *N*-linked oligosaccharides in the porcine ZP [27], and the sulfated glycans of ZP glycoproteins play important roles in the binding of boar sperm to eggs and penetration by the sperm [28]. Töpfer-Petersen and Henschen [29] also reported that the polysulfated groups on ZP glycoproteins bind to the boar proacrosin, a zymogen of a serine protease present in the acrosomal vesicle. The binding of proacrosin and β-acrosin to ZP glycoproteins involves strong ionic bonds between basic residues on the surface of proacrosin/acrosin and polysulfate groups on ZP glycoproteins in pigs [30, 31] and mice [32]. Porcine ZP glycoproteins, fucoidan, dextran sulfate and polyvinyl sulfate potentiate the conversion of proacrosin to β-acrosin [20], and the interactions between mouse ZP2 glycoprotein and proacrosin are important for retention of AR sperm on the egg surface during fertilization [32]. In addition, the binding of polysulfide of ZP glycoproteins to the polysulfate-binding domain (PSBD) of proacrosin stimulates the rate of proacrosin activation in boar [33] and mouse [34] sperm, and this interaction mediates post-AR binding of spermatozoa to the ZP long enough for penetration to begin in pigs [35, 36]. Therefore, in the present study, it is likely that the sulfation block of ZP glycoproteins had a specific influence on the secondary binding of AR sperm to the ZP during the initial stages of fertilization, thus resulting in a decrease in polyspermic fertilization. We also clearly demonstrated that the suppression of AR functionality induced by sperm-ZP interactions through the antihyaluronidase action of ellagic acid effectively prevented polyspermy with no effect on sperm penetration during porcine IVF [37]. However, further studies are needed to identify the precise mechanism in sperm-ZP interactions relating to polyspermic fertilization because the interactions between the sperm and the ZP are mediated by a complex involving several sperm plasma membrane proteins and several carbohydrates contained in the ZP [5–8] and the modifications of ZP glycans responsible for sperm AR induction are established by glycosylation, sulfation, sialylation and fucosylation during oocyte growth and maturation [38]. As reported by Velásquez *et al*. [19] and Katsumata *et al*. [39], neuraminidase released from cortical granules during IVF would participate in blockage of polyspermy by removing sialic acid from the ZP in bovine oocytes. In contrast, the time required for cortical granule exocytosis is greatly delayed after insemination in porcine oocytes, and the exact mechanism used by porcine oocytes to block polyspermy is still unclear [40].

To our knowledge, the present study is the first to prove that ZP acidification in accordance with the sialylation and sulfation of ZP glycoproteins during oocyte maturation contributes to the porcine ZPs acquiring the capacity to accept sperm. According to a very recent report [12], the increase in the amount of terminal GlcNAc residues in ZP3 glycoproteins through new *N*-glycosylation for periods in excess of 20–24 h after meiotic maturation played an important role in porcine sperm-ZP interactions. We have confirmed that treatment with neuraminidase and $NaClO_3$ does not inhibit the increase in terminal GlcNAc residues on the ZP by detecting lectin blotting with S-WGA (unpublished data). Moreover, the desialylation and blocking of sulfation did not protect against the proteolytic modification of the ZP matrix before fertilization (Fig. 5). However, in the present study, it is still unclear whether cumulus cells are concerned with the sialylation and sulfation of ZP glycoproteins during porcine oocyte maturation. To address this issue, further experiments will be needed to examine the effect of cumulus cells on the acidification of the ZP.

In summary, the following findings were made in the present study. 1) Porcine ZP glycoproteins undergo acidification in accordance with sialylation and sulfation in the final maturation phase of oocytes prior to fertilization. 2) The progressive increases in the number of sperm bound to ZPs and the percentage of AR in sperm bound to ZPs with IVM for 28 and 44 h were suppressed in oocytes treated with neuraminidase, resulting in a significant reduction in the sperm penetration and polyspermy rates. 3) Blockage of sulfation markedly reduced the incidence of polyspermy with no inhibitory effect on penetration, despite the fact that the number of sperm bound to ZPs and the rate of AR-inducing sperm were decreased to the same level as in desialylated oocytes. 4) The lower incidences of sperm penetration and polyspermy elicited by treatment with neuraminidase and $NaClO_3$ were not due to protective proteolytic modifications of the ZP matrix before fertilization. It is concluded that ZP acidification caused by the sialylation and sulfation of ZP glycoproteins during porcine oocyte maturation is indispensable for the acquisition of sperm-ZP interactions, responsible for sperm penetration, binding to the ZP and induction of the AR in sperm bound to the ZP.

Acknowledgments

This research was supported by a Grant-in-Aid for Scientific Research (C, 20580312) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank the staff of the Okinawa Prefectural Livestock Research Center for providing semen and the Meat Inspection Office of the City of Oozato for supplying ovaries.

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