Studies on involvement of the zona pellucida modifications during meiotic maturation of porcine oocytes in the sperm-egg interactions

（ブタ卵母細胞の減数分裂時における透明帯変化の
精子－卵子間相互作用への関与に関する研究）

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List of Abbreviations

AR   Acrosome reaction

ART   Assisted reproductive technology(ies)

BSA   Bovine serum albumin

CG   Cortical granule

COCs   Cumulus-oocyte complexes

Con A   Concancavalin A

2D   Two dimensional

DAPI   4'6-diamidino-2-phenylin-dole-2HCl

DBA   Dolichos biflorus agglutinin

DOs   Denuded oocytes

D-PBS   Dulbecco's phosphate-buffered saline

EDTA   Ethylenediaminetetracetic acid

FSH   Follicle stimulating hormone

g   Gram(s)

g   Gravities (Relative centrifugal force)

Galtase   β1,4-galactosyltransferase

GlcNAc	*N*-acetylglucosamine
GLM	Generalized linear model
GSH	Glutathione
GVBD	Germinal vesicle breakdown
h	hour(s)
HABP	Hyaluronan binding proteins
Hepes	N-2-Hydroxyethylpiperazine- N'-2-ethanesulphonic acid
H-TL-PVA	Hepes-buffered Tyrode's medium containing 0.01% (w/v) polyvinyl alcohol
IU	International unit
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
kg	Kilogram(s)
LH	Luteinizing hormone
M	Molarity/molar (moles per litre of solution)
MAL II	Maackia amurensis lectin II
M-II	Metaphase II (Second anaphase)
min	Minute(s)

mo	Month
MPN	Male pronucleus
mTBM	Modified Tris-buffered medium
NaClO$_3$	Sodium chlorate
Neu	Neuraminidase
PI	Propidium iodide
PNA	Peanut agglutinin
PSBD	Polysulfate-binding domain
PVA	Polyvinyl alcohol
PVDF	Polyvinylidene fluoride
PZM	Porcine zygote medium
RCA	Ricinus communis agglutinin
SBA	Soybean agglutinin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second(s)
SSA	Sambucus sieboldiana agglutinin
S-WGA	Succinylated wheat germ agglutinin
TCM 199	Tissue culture medium 199

TM   Tunicamycin

UEA   Ulex europaeus agglutinin

WGA   Wheat germ agglutinin

ZP   Zona pellucida

Chapter 1

General Introduction

The *in vitro* fertilization (IVF) is becoming an integral part of assisted reproductive technologies (ART) in the area of veterinary and human reproductive research.   Although the techniques for IVF have proceeded very rapidly during the past decade, polyspermic penetration still remains a persistent obstacle to porcine IVF systems.   Despite of that the embryo production by IVF had been developed successfully in many other species, their developmental potential is very low in pigs, thus the developmental rate to the blastocyst stage following maturation and fertilization *in vitro* (Abeydeera and Day, 1997; Wang *et al*., 1997; Kano *et al*., 1998; Marchal *et al*., 2003) is lower than *in vivo* (Beckmann and Day, 1993; Petters and Wells, 1993; Dobrinsky *et al*., 1996).   The poor developmental competence might be caused by the lack of cytoplasmic maturation in *in vitro* matured porcine oocytes, even though they undergo normal nuclear maturation (Ka *et al*., 1997; Yamauchi and Nagai, 1999; Marchal *et al*., 2001; 2003).   Porcine maturational competence appears to be more related to immatured cumulus-oocyte complexes (COCs) characteristics than to the type of cumulus behavior during culture.   Although the nuclear maturation could not be mediated by cumulus characteristics, the cytoplasmic maturation could be mediated by cumulus features (Alvarez *et al*., 2009).   Moreover, the addition of growth hormone to maturation medium improved nuclear maturation in equine and porcine, but had no

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effect on porcine cytoplasmic maturation responsible for developmental competence (Marchal *et al*., 2003). The nuclear and cytoplasmic maturation of porcine oocytes were mediated by the oocyte's ability to accumulate intracellular glutathione (GSH) during maturation and by extracellular steroid hormones and cumulus cells (Liu *et al*., 2002). Tatemoto *et al*. (2001) also reported that male pronucleus (MPN) formation and blastocyst formation are improved by supplementation of ascorbic acid 2-*O*-α-glucoside during *in vitro* maturation (IVM) culture of denuded porcine oocytes (DOs).

Recently, it would be known that oocyte maturation is mediated by not only nuclear and cytoplasmic maturation but also zona pellucida (ZP) maturation. The ZP, a transparent envelope surrounding the plasma membrane of mammalian oocyte, is a highly glycosylated extracellular matrix. The porcine ZP is composed of three glycoprotein families, ZP1 (ZPA; 92 kDa), ZP3α (ZPB; 55 kDa) and ZP3β (ZPC; 55 kDa) (Hedrick and Wardrip, 1986; 1987). ZP1 is split into two smaller molecules, ZP2 (69 kDa) and ZP4 (23 kDa), under reducing conditions (Hasegawa *et al*., 1994). The ZP3 families have been shown to comprise approximately 60% of the total glycoprotein content (Hedrick and Wardrip, 1987; Yurewicz *et al*., 1987). Porcine ZP3α seems to be responsible for the primary binding of boar sperm to ZP, since preincubation of sperm with solubilized and purified ZP3α inhibits the subsequent sperm attachment to ZP (Berger *et al*., 1989). Yurewicz *et al*. (1993) and Gupta *et al*. (1996) also confirmed that the ZP3α interaction closely linked with the sperm binding to the porcine ZP. Different carbohydrates on ZP3, such as galactose in α-linkage, *N*-acetylglucosamine (GlcNAc) in β-linkage, were suggested as the complementary sperm receptors, mediating the primary binding between the spermatozoon and the ZP

(Shalgi and Raz, 1997).

The ability of sperm to bind ZP has been detected in ZP glycoproteins, and it is generally accepted that this activity is ascribed to the carbohydrate moieties in ZP glycoproteins (Benoff, 1997; Dell *et al*., 1999; Dean, 2004; Shur *et al*., 2004; Wassarman, 2005). ZP glycoproteins are considered to have multiple sperm-binding sites, because it has been shown in mice that ZPC glycoprotein binds to the plasma membrane of acrosome-intact sperm and induces the acrosome reaction (AR), while ZPA glycoprotein binds to acrosome-reacted sperm (Bleil and Wassarman, 1986).

During fertilization, sperm initially bind to the oocyte, undergo the AR, penetrate into ZP, and fuse with oolemma to form a zygote (Hoodbhoy and Talbot, 1994; Yanagimachi, 1994; Green, 1997; Wassarman *et al*., 2001; Sun, 2003). It is generally accepted that the specific interaction between sperm and ZP is a carbohydrate mediated event in different species including human (Chapman and Barratt, 1996; Benoff, 1997; Ozgur *et al*., 1998; Dell *et al*., 1999; Primakoff and Myles, 2002; Talbot *et al*., 2003). Due to a critical involvement of carbohydrate in the sperm-ZP interactions, a detailed description of the carbohydrate composition in ZP is necessary.

The sperm surface hyaluronan binding protein (HABP1) present in rat, mice, bull and human sperm plasma membrane is associated with the sperm-ZP interactions (Ranganathan *et al*., 1994) and interacts with the clustered mannose residues of the ZP glycoproteins (Ghosh and Datta, 2003). Mannose residues in ZP have been proposed to play an important role in human sperm-oocyte interactions (Mori *et al*., 1989; Tulsiani *et al*., 1990; Tesarki *et al*., 1991; Benoff *et al*., 1993a-c; Miranda *et al*., 1997; Maegawa *et al*., 2002). In bovine oocytes, the nonreducing terminal α-mannosyl residues of a high-mannose-type *N*-linked chain of ZP glycoproteins possess the

sperm-binding activity (Amari *et al*., 2001). Similarly, mouse sperm bind to the nonreducing terminal α-galactosyl residue (Bleil and Wassarman, 1988) or β-*N*-acetylglucosaminyl residue (Miller *et al*., 1992), mannose residue (Cornwall *et al*., 1991), and α-fucosyl residue (Johnston *et al*., 1998) of the *O*-linked carbohydrate chains of ZPC. Fucose residues of ZP glycoproteins are involved in the sperm-oocyte interactions in different species from invertebrate to human (Ahuja, 1982; Tesarik *et al*., 1993; Miranda *et al*., 1997). The terminal galactose residues in ZP glycoproteins have been implicated in the sperm-oocyte binding in many different mammalian species (Benoff, 1997; Shalgi and Raz, 1997; Tulsiani, 2000). Terminal β-linked GlcNAc residues are required for human sperm binding (Mori *et al*., 1989; Miranda *et al*., 1997; 2000), and the bull sperm binding to ZP is mediated by α-2,3 linked sialic acid of ZP glycoproteins (Velásquez *et al*., 2007).

Moreover, it is reported that ZP modifications, such as glycosylation, sialylation, sulfation, glucosaminylation, fucosylation and galactosylation of glycoproteins, have been implicated in various events during fertilization. However, to our knowledge, there has been no report directly demonstrating the ZP modifications during IVM.

Therefore, the present study was undertaken with the following objectives:

1) to evaluate the involvement of *N*-glycosylation during meiotic maturation in sperm-ZP interactions by using tunicamycin, an inhibitor for *N*-glycosylation of glycoproteins (Chapters 2 and 3);

2) to determine essential roles of sialylation of ZP glycoproteins in sperm-ZP interactions (Chapter 4);

3) to elucidate the effect of blocking the sulfation of ZP glycoproteins during

meiotic maturation on sperm-ZP interactions (Chapter 5).

Chapter 2

N-glycosylation of zona glycoproteins during meiotic maturation of porcine cumulus-oocyte complexes is involved in sperm-zona pellucida interactions

2.1 Abstract

The objective was to determine whether *N*-glycosylation of ZP glycoproteins occurred during meiotic maturation of porcine oocytes, and whether this *N*-glycosylation had influence upon fertilization.   In the first of four experiments, the sperm penetrability to ZP, sperm binding ability and ZP solubility with the advance of oocyte maturation following various IVM culture periods were examined.   In the second experiment, carbohydrate residues in the ZP of *in vitro* matured porcine oocytes were blocked with various lectins and the influence of such blocking on sperm-ZP interactions was studied.   The third experiment used a lectin-binding assay to determine whether the number of GlcNAc residues in ZP was changed by *N*-glycosylation during IVM of porcine oocytes.   The last experiment determined the effects of tunicamycin, a specific *N*-glycosylation inhibitor, for various intervals during IVM, on sperm-ZP interactions in porcine oocytes.   The primary findings demonstrated that: 1) *N*-glycosylation of GlcNAc residues in porcine ZP occurred during the first 24 h of IVM; and 2) such glycosylation was indispensible for sperm-ZP interactions, e.g., increases in the number of sperm bound to ZP, acrosome-reacted sperm, sperm penetration rate, and level of polyspermy ($P<0.05$).   However, blocking *N*-glycosylation by tunicamycin treatment during IVM did not adversely influence the progression of oocytes to meiotic metaphase II (M-II) and MPN formation, indicating that this glycosylation was involved only in the initial stages of fertilization.   We inferred that the increase in terminal GlcNAc residues in ZP glycoprotein through new *N*-glycosylation during

the first 24 h of meiotic maturation played a critical role in porcine ZP acquiring the capacity to accept sperm.

2.2 Introduction

During fertilization, sperm initially bind to the oocyte ZP, undergo the AR, penetrate the ZP, and fuse with the oolemma to form a zygote (Wassarman *et al*., 2001). Sperm-ZP interactions are carbohydrate-mediated events in various species, including humans (Chapman and Barratt, 1996; Benoff, 1997; Ozgur *et al*., 1998; Dell *et al*., 1999; Primakoff and Myles, 2002; Talbot *et al*., 2003). In rodents, the initial sperm-ZP recognition is mediated by the binding of sperm surface β1,4-galactosyltransferase (GalTase) to terminal GlcNAc residues in ZP3 (Bleil and Wassarman, 1980; Shur, 1991; Miller *et al*., 1992). In hamsters, GlcNAc residues on ZP glycoproteins specifically influenced primary binding of sperm to ZP (Zitta *et al*., 2004). The participation of terminal GlcNAc residues in the ZP of human oocytes in sperm-ZP binding and AR has also been reported (Brandelli *et al*., 1994; Miranda *et al*., 1997; 2000). Biochemical changes during oocyte maturation, such as *N*-glycosylation of ZP glycoproteins, were implicated in sperm-ZP binding in several species (Rath *et al*., 2005). However, there are apparently no reports regarding whether *N*-glycosylation of ZP glycoproteins occur during *in* IVM of porcine oocytes and, if so, whether such alterations are crucial for sperm-ZP interactions.

Therefore, the present study was conducted to understand the role of *N*-glycosylation of zona glycoproteins during IVM in sperm-ZP interactions of porcine oocytes using tunicamycin, which specifically inhibits glycosylation of proteins *N*-glycosylated at asparagine residues (Waechter and Lennarz, 1976; Struck and Lennarz, 1977; Waechter and Harford, 1977) by blocking the enzymatic transfer of GlcNAc-1-phosphate from UDP-GlcNAc to dolichol phosphate (Tkacz and Lampen, 1975; Lehle and Tanner, 1976). Porcine COCs were cultured in IVM medium, in the absence or presence of tunicamycin, and then inseminated to examine the effects of *N*-glycosylation of ZP glycoproteins during IVM on sperm penetration, ZP hardness, sperm binding to ZP, and induction of the AR in ZP-bound sperm.

2.3 Materials and Methods

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

2.3.1 Washing and cleaning

The laboratory instruments (plastic ware, glassware and metallic equipment, etc.) were washed thoroughly and rinsed vigorously 30-50 times with tap water and one time with pure water. After washing, glassware and instruments kept in upside-down arrangement were dried in an oven at 30ºC for 1 day. The laboratory and all exposed surfaces (floor, bench, microscope, etc.) were regularly cleaned with 70% alcohol. Additionally, in order to minimize the contamination, the room and all exposed surfaces were irradiated by cleaning especially prior to oocyte or embryo handling.

2.3.2 Sterilization

Oven-dried instruments (e.g. beaker, test tube, membrane filter, stirrer bars, spatula, etc.) were covered with aluminium foil and sterilized by dry heat at 117°C for 2 h. The media were sterilized by filtration through 0.22 μm membrane filters in clean bench. Glass micropipettes for handling oocytes and embryos were prepared from sterilized capillary tubes. Capillary tubes were pulled over a gas burner to obtain a desired internal diameter of 150-500 μm. The resulting micropipettes were filed by a glasscutter and the sharp tips were fire-polished, and sterilized by dry heat at 117°C for 2 h.

2.3.3 Culture media

The culture medium used for oocyte maturation was tissue culture medium 199 (TCM 199, HEPES-buffered), supplemented with 0.57 mM cysteine, 0.04 unit/ml ovine FSH, 0.02 unit/ml ovine LH, and 10% (v/v) porcine follicular fluid (Table 1). 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.　The basic medium used for IVF was the same as modified Tris-buffered medium (mTBM) (Abeydeera and Day, 1997).　This medium, supplemented with 2 mM Caffeine-sodium benzoate and 0.1% (w/v) bovine serum albumin (BSA), was used as the IVF medium.　Similarly, mTBM supplemented with 4 mM caffeine-sodium benzoate and 0.4% (w/v) BSA was used as sperm pre-incubation medium. The embryo culture medium was a porcine zygote medium (PZM 5) containing 0.3% (w/v) BSA, 100 unit/ml penicillin G, and 50 μg/ml streptomycin (Suzuki *et al*., 2007).

Table 1. Composition of medium 199 for *in vitro* maturation medium of porcine oocytes.

Components	Concentration (mM)	mg/100 ml
Medium 199[1]		1,500.00
NaHCO$_3$	26.18	220.00
Penicillin G	100 IU/ml	6.25
Streptomycin	50 µg/ml	5.00
Cysteine[2]	0.60	7.27
FSH[2]	0.04 units/ml	4 units
LH[2]	0.02 units/ml	2 units
Porcine follicular fluid[2]	10.0%	10.00 ml

The solution was stored at 4°C and used within 2 weeks.

[1] Medium 199 HEPES Modification Powder (M-2520; Sigma) with L-glutamine and 25 mM HEPES and without sodium bicarbonate.

[2] It was supplemented just before used.

2.3.4   Oocyte collection and *in vitro* maturation

Porcine ovaries were collected from prepubertal gilts (85-110 kg, mean age 6 mo) at a local abattoir and transported to the laboratory.   Within 2 h after slaughter, follicular contents were recovered by excising the visible small antral follicles (2-6 mm in diameter) on the ovarian surface using a razor blade, and by scraping the inner surface of the follicle walls with a disposal surgical blade.   COCs were viewed under a stereomicroscope; those with four or five layers of unexpanded cumulus cells and a homogenous ooplasm, were selected and washed three times with HEPES-buffered Tyrode's medium containing 0.01% (w/v) polyvinyl alcohol (H-TL-PVA, Table 2).   After washing in the IVM medium, groups of 15-20 COCs were placed in 100-µl droplets of IVM medium, covered with light weight mineral oil, and incubated for 44 h at 39°C in an atmosphere of 5% CO_2 in air.

Table 2. Composition of H-TL-PVA as oocyte-handling medium.

Components	Concentration (mM)	mg/100 ml
NaCl	114.00	666.33
KCl	3.20	23.86
NaH_2PO_4	0.34	5.30
$MgCl_2 \cdot 6H_2O$	0.50	10.17
$CaCl_2 \cdot 2H_2O$	2.00	29.40
$NaHCO_3$	2.00	16.80
HEPES	10.00	238.30
Na-pyruvate	0.20	2.20
Na-lactate[1]	10.00	186.8 μl
Penicillin G	100 IU/ml	6.50
Streptomycin	50 μg/ml	5.00
PVA[2]	0.01%	10.00

The solution was adjusted pH to 7.4 and filtered (0.22 μm) with vacuum filter system. It was stored at 4°C until use.

[1] 60% syrup
[2] Polyvinyl alcohol

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2.3.5 *In vitro* fertilization

After maturation culture, cumulus-granulosa cells were removed from the COCs by passage through a narrow-bore pipette in H-TL-PVA containing 0.1% (w/v) hyaluronidase. The cumulus-free oocytes were rinsed three times with IVF medium (Table 3) and then transferred to 50-µl droplets of IVF medium, which had been previously covered with warm mineral oil. The droplets, containing 15-20 oocytes, were incubated at 39°C in an atmosphere of 5% CO_2 in air for 1 h, at which time sperm were added.

The sperm preparation consisted of thawing (39°C) (three 100-µl pellets of frozen sperm, each containing ~5 × 10^7 sperm) ejaculated from an Okinawan native Agu boar. The pooled sperm sample was then centrifuged twice at 600 × g for 4 min in Dulbecco's phosphate buffered saline (PBS) supplemented with 0.1% (w/v) polyvinyl alcohol (PVA; pH 7.2). After the washing procedure, sperm were re-suspended (concentration, 4 × 10^8 sperm/ml) in sperm pre-incubation medium, and incubated for 90 min at 39°C in an atmosphere of 5% CO_2 in air to induce capacitation. After pre-incubation, 50 µl of diluted sperm suspension with IVF medium was added to each droplet containing oocytes (final sperm concentration, 1 × 10^6 sperm/ml), and the gametes were co-incubated for 7 h under the conditions indicated above. At the end of co-incubation, accessory sperm were removed by gentle vortexing. Oocytes with sperm attached to the ZP were washed three times and cultured in 50-µl droplets of PZM 5 (Table 4) for 3 h at 39°C in an atmosphere of 5% CO_2 in air to ensure pronucleus formation.

Table 3.　Composition of modified Tris-buffered medium (mTBM) for IVF of frozen-thawed boar sperm.

Components	Concentration (mM)	mg/100 ml
NaCl	113.1	661.10
KCl	3.0	22.40
$CaCl_2$ 2 H_2O	7.5	110.20
Tris	20.0	242.30
D-Glucose	11.0	198.20
Na-pyruvate[1]	5.0	55.00
Caffeine sodium benzoate	2.0	77.68
PVA	0.1%	100.00
BSA[1]	0.1%	100.00

The solution was stored at 4°C and used within 2 weeks.
[1] They were supplemented just before used.

Table 4. Composition of porcine zygote medium (PZM 5) for *in vitro* development of porcine embryos.

Components	Concentration (mM)	mg/100 ml
NaCl	108.00	631.15
KCl	10.00	74.55
KH_2PO_4	0.35	4.76
$MgSO_4 \cdot 7H_2O$	0.40	9.86
$NaHCO_3$	25.00	210.03
Na-pyruvate	0.20	2.20
L-Lactic acid hemicalcium salt	2.00	43.64
L-Glutamine	2.00	29.22
Hypotaurine[1]	5.00	54.55
BME amino acids (ml/100 ml)	20.00	2.00
MEM non-essential amino acids (ml/100 ml)	10.00	1.00
BSA[1]	3 mg/ml	300.00
Penicillin G	100 IU/ml	6.25
Streptomycin	50 μg/ml	5.00
Phenol Red	5 μg/ml	0.50

After preparing the solution, pH was adjusted to 7.4 and stored at 4°C until use. It was used within 2 weeks.

[1] They were supplemented just before used.

2.3.6 Sperm-ZP binding

After cumulus-free oocytes were co-incubated with the pre-incubated sperm for 2 h, a portion of 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 at room temperature for 40 min with 2% formaldehyde.   Oocytes were mounted on a glass slide and then treated with 10 µg/ml *bis*-benzimide Hoechst 33342 for 10 min.   Oocytes were washed in H-TL-PVA, mounted, and sperm tightly bound to the ZP were counted under a fluorescence microscope (Nikon, Tokyo, Japan).

2.3.7 Assessment of *in vitro* nuclear maturation and fertilization

After IVM culture or 10 h of *in vitro* insemination, groups of 30-40 oocytes were mounted, fixed, stained with aceto-lacmoid, and examined under a phase-contrast microscope (400 × magnification) to assess oocyte maturation at M-II stage and the fertilization parameters. The following were evidence that sperm penetrated the oocytes at the M-II stage: two polar bodies, one or more swollen sperm head(s), and/or a MPN and a corresponding sperm tail(s).

2.3.8 Detection of GlcNAc residues by lectin-blotting

Intact ZP was isolated from oocytes and washed as previously described (Kurasawa *et al.*, 1989).   Briefly, ZPs were ruptured and removed mechanically with a narrow micropipette (about 60 µm in diameter) in PBS containing 1 mg/ml ethylenediaminetetracetic 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.   Ten ZPs were subjected to SDS-PAGE on a 7.5% polyacrylamide gel under reducing conditions and transferred to polyvinylidene fluoride (PVDF) membranes (Hybond-P; GE Healthcare, Buckinghamshire, UK).   Following electrophoresis, membranes were blocked for 90 min 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).   Membranes were treated with 0.25 µg/ml and 5 µg/ml of biotin-labeled WGA and S-WGA lectins, respectively, in TBS-T for 40 min.   They were incubated for 50 min at room temperature with 0.1% (v/v) streptavidin-horseradish peroxidase conjugate (GE Healthcare) in TBS-T-gelatin.   Membranes were washed three times with TBS-T at room temperature.   Bound peroxidase was then detected using ECL Western blotting detection kits and ECL-mini camera (GE Healthcare), according to the manufacturer's instructions.   The amount of lectin-blotting intensity was quantified using UN-SCAN-IT gel (Silk Scientific, Orem, UT, USA).   The value of lectin-blotting intensity in oocytes freshly isolated from their follicles was taken to be 1.0, with data reported as relative intensities.

To identify each band of ZP glycoproteins in gel mobility, intact ZPs were biotinylated with water soluble succinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin II; Pierce Chemical Co., IL, USA) as described previously (Moos *et al*., 1994), and two biotinylated ZPs were subjected to electrophoresis as mentioned above, except for the membrane incubation with lectins.

2.3.9   Assessment of ZP solubility

After washing three times in H-TL-PVA, 15-20 cumulus-free oocytes were transferred into a 50-µl drop of H-TL-PVA containing 0.1% (w/v) protease, and continuously observed for dissolution of ZP at room temperature using an inverted microscope (magnification, × 200). For each oocyte, the time required for dissolution was recorded as the ZP dissolution time.

2.3.10   Evaluation of sperm acrosomal status

To examine the induction of AR in sperm bound to ZP, pre-incubated sperm were co-incubated with cumulus-free oocytes in IVF medium for 2 h, since sperm penetration into the ZPs was observed from 2 h after the start of insemination under our experimental conditions. After rinsing several times with PBS with a wide-bore pipette to remove loosely attached sperm, sperm-oocyte complexes were immediately stained with 2 µg/ml Alexa-labeled peanut

agglutinin (PNA; Invitrogen, Burlingame, CA, USA), 1 μg/ml 4'6-diamidino-2-phenylin-dole-2HCl (DAPI), and 10 μg/ml propidium iodide (PI) in PBS at 39°C for 20 min. The complexes were then fixed with 4% formaldehyde for 15 min at room temperature and mounted on glass slides.

2.3.11   Experimental design

In Experiment 1, to elucidate whether the sperm penetrability to ZP, sperm binding ability and ZP solubility could be changed with the advance of oocyte maturation, following IVM culture for 0, 6, 12, 20, 24, 28, 36, and 44 h in IVM medium, COCs were freed from their cumulus cells. Some oocytes were subjected to the ZP solubility assay, and other oocytes were co-incubated with pre-incubated sperm to examine the fertilization parameters and the number of sperm bound to ZP.

In Experiment 2, to evaluate which types of carbohydrate residues were involved in the sperm binding to ZP and sperm penetration, *in vitro* matured oocytes were inseminated in IVF medium supplemented with various types of 1 μg/ml lectin (DBA, Con A, WGA, RCA, PNA, UEA, SBA, and S-WGA; all lectins were purchased from Vector Laboratory, Burlingame, CA, USA), and the number of sperm bound to ZP and the incidence of sperm penetration were observed.

In Experiment 3, to investigate whether the amount of GlcNAc residues in ZP glycoproteins could be changed by *N*-glycosylation during oocyte maturation, a lectin-blotting assay was performed for ZPs of oocytes freshly isolated from their follicles and *in vitro* matured oocytes cultured for 44 h in IVM medium, with or without 10 μg/ml of tunicamycin.

Experiment 4 was designed to elucidate the effect of tunicamycin treatment (10 μg/ml) for various intervals (0, 8, 16, 20, 24, 32, 38, and 44 h) after pre-cultivation in the control medium (44, 36, 28, 24, 20, 12, 6, and 0 h, respectively) during a total of 44 h of IVM culture on subsequent sperm-ZP interactions in porcine oocytes. At the end of IVM, some oocytes were used to assess ZP dissolution by 0.1% protease, and others were inseminated with pre-incubated sperm to examine nuclear status, fertilization parameters, sperm binding to ZP, and AR induction in ZP-bound sperm.

2.3.12   Statistical analysis

All statistical analyses were performed using the Statistical Analysis System R software package (http://www.R-project.org/).   To evaluate differences between groups, percentage data (non-parametric data) were analyzed using a generalized linear model (GLM, in accordance with a binomial distribution) and ANOVA procedures followed by the Tukey test for non-parametric multiple comparisons (Ryan, 1960).   Analyses of other data (parametric data) were carried out using the Shapiro-Wilk normality test and the GLM (in accordance with Gaussian distribution) and ANOVA procedures, followed by the Tukey-Kramer test.   A probability of $P<0.05$ was considered statistically significant.   The effect of the replicate was excluded from the analysis, since none of the parameters were influenced by the replicate.

2.4   Results

Experiment 1:   Effects of IVM culture periods on the fertilization parameters, ZP solubility and sperm binding to ZP

The sperm penetration rates were progressively increased with the advance of oocyte maturation (Table 5).   Moreover, the rates of polyspermy and MPN formation, and the mean number of sperm/oocyte also increased in matured oocytes compared with immatured oocytes. As shown in Fig. 1, the dissolution times of ZP caused by 0.1% protease action were prominently reduced as the meiotic maturation was progressively advanced, and were 253.7 ± 12.6 and 125.1 ± 2.0 sec in freshly isolated and matured oocytes, respectively.   In addition, the number of sperm bound to ZP also gradually enhanced in a maturation time-dependent manner (Fig. 2).   The significantly low number of sperm bound to ZP was found in oocytes freshly isolated from their follicles (10.1 ± 1.9) compared with that of matured oocytes (67.3 ± 6.6, $P<0.05$).   All these alterations according to the different maturation times implied that ZP modifications occurred during oocyte maturation.

Table 5.　Effect of different IVM culture periods on the fertilization parameters in porcine oocytes.

IVM culture period (h)	No. of oocytes examined	Oocytes (%; mean ± SEM)			No. of sperm per penetrated oocyte (mean ± SEM)
		Penetrated	Polyspermic [1]	Male pronucleus [1]	
0	120	16.7 ± 3.4 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.0 ± 0.0 [a]
6	141	20.6 ± 3.4 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.0 ± 0.0 [a]
12	147	34.7 ± 3.9 [b]	0.0 ± 0.0 [a]	2.0 ± 1.9 [a]	1.0 ± 0.0 [a]
20	144	37.5 ± 4.0 [b]	5.6 ± 3.1 [a]	1.9 ± 1.8 [a]	1.1 ± 0.1 [a]
24	129	49.6 ± 4.4 [b]	1.6 ± 1.6 [a]	6.3 ± 3.0 [a]	1.0 ± 0.0 [a]
28	148	49.3 ± 4.1 [b]	37.0 ± 5.7 [b]	50.7 ± 5.9 [b]	1.5 ± 0.1 [b]
36	147	68.0 ± 3.9 [c]	65.0 ± 4.8 [c]	76.0 ± 4.3 [c]	2.3 ± 0.1 [c]
44	147	72.8 ± 3.7 [c]	67.3 ± 4.5 [c]	72.9 ± 4.3 [c]	2.7 ± 0.2 [d]

[1] Percentage of oocytes that were penetrated.
[a-d] Values with different superscripts within the same column are significantly different.
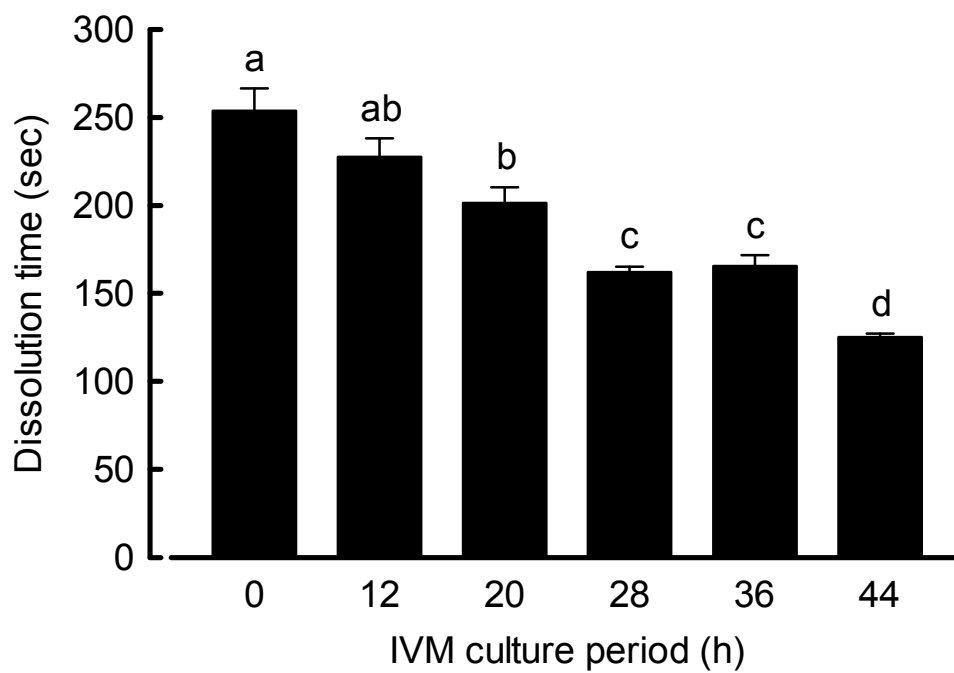
Figure 1.   Changes in the dissolution time of ZP caused by 0.1% protease action in porcine oocytes matured different culture periods.   Values are expressed as the mean ± SEM.   Total number of oocytes in each treatment group ranged from 85-96.   [a-d] Values with different superscripts are significantly different (P<0.05).
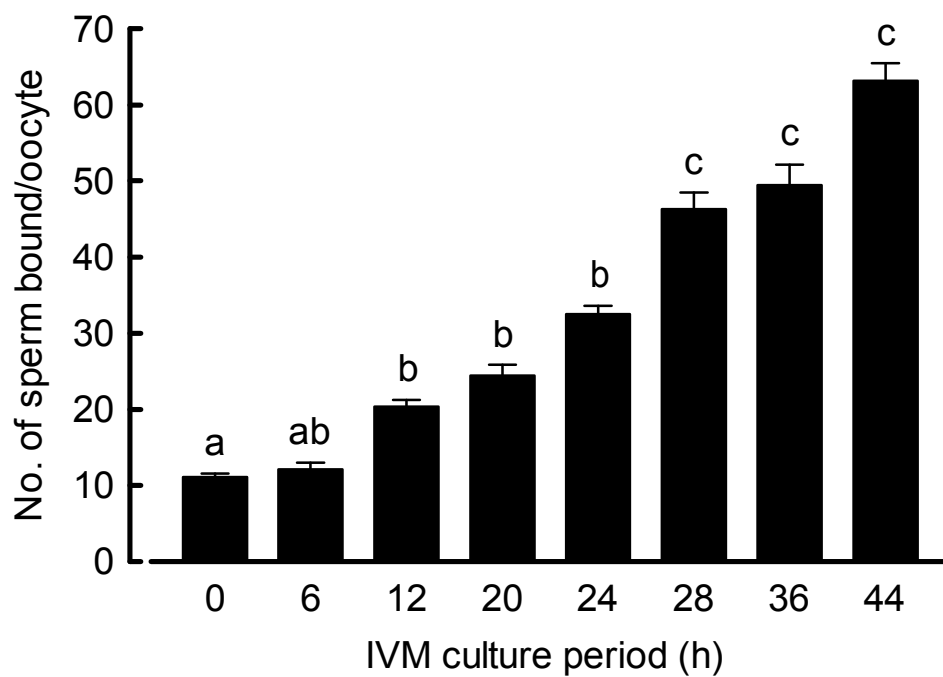
Figure 2. The number of sperm bound to porcine ZP in oocytes matured different culture periods. Values are expressed as the mean ± SEM. Total number of oocytes in each treatment group ranged from 41-45. [a-c] Values with different superscripts are significantly different (P<0.05).

Experiment 2:   Effects of various types of lectin added to IVF medium on sperm

binding to ZP and fertilization parameters of porcine oocytes

The effects of various types of lectin added to IVF medium on sperm binding to ZP and fertilization parameters were shown in Tables 6 and 7, respectively.   Among these various types of lectin, Con A, WGA and S-WGA diminished sperm binding to ZP (46.5 ± 1.6, 42.4 ± 1.7 and 48.4 ± 2.7 sperm, respectively, Table 6).   Moreover, these lectins (Con A, WGA and S-WGA) also had strong inhibitory effect on the sperm penetration rates (21, 13 and 8%, respectively) compared with control (in the absence of lectin; 89%, Table 7).   However, no difference was found in the incidence of polyspermic fertilization in all lectin treatment groups. Although WGA and S-WGA significantly inhibited the MPN formation (35 and 31%, respectively, $P<0.05$), other lectins did not have this inhibitory effect when compared with that in the absence of lectin (80%).   The mean number of sperm/oocyte decreased from 2.2 sperm (control) to 1.3, 1.4 and 1.7 sperm by treatment with Con A, WGA and S-WGA, respectively. From these findings, it was clear that the GlcNAc residues in porcine ZP glycoproteins played a key role in the sperm-ZP binding and the sperm penetration.

Table 6. Effects of various types of lectin added to IVF medium on sperm binding to ZP in porcine oocytes.

Types of lectin (1 μg/ml)	Specific affinity to carbohydrate residues	No. of oocytes examined	No. of sperm bound per oocyte (mean ± SEM)
Control		42	62.3 ± 1.7 [a]
DBA	GalNAc α	46	55.2 ± 1.6 [ab]
Con A	Man α, Glc α, GlcNAc α	45	46.5 ± 1.6 [c]
WGA	GlcNAc, Sialic acid	45	42.4 ± 1.7 [c]
RCA	Gal, GalNAc	40	56.4 ± 2.0 [ab]
PNA	Gal β1-3GalNAc	41	55.0 ± 1.8 [ab]
UEA	Fuc α	41	58.4 ± 1.7 [a]
SBA	GalNAc	44	59.3 ± 2.3 [a]
S-WGA	GlcNAc	40	48.4 ± 2.7 [bc]

[a-c] Values with different superscripts within the same column are significantly different (P<0.05).

Table 7. Effects of various types of lectin added to IVF medium on the fertilization parameters in porcine oocytes.

Types of lectin (1 µg/ml)	No. of oocytes examined	Oocytes (%; mean ± SEM)			No. of sperm per penetrated oocyte (mean ± SEM)
		Penetrated	Polyspermic [1]	Male pronucleus [1]	
Control	137	89.1 ± 2.7 [a]	56.6 ± 4.5	79.5 ± 3.7 [a]	2.2 ± 0.1 [a]
DBA	137	82.5 ± 3.3 [a]	46.9 ± 4.7	81.4 ± 3.7 [a]	1.7 ± 0.0 [abc]
Con A	123	21.1 ± 3.7 [c]	26.9 ± 8.7	57.7 ± 9.7 [ab]	1.4 ± 0.1 [cd]
WGA	133	12.8 ± 2.9 [cd]	29.4 ± 11.1	35.3 ± 11.6 [b]	1.3 ± 0.1 [d]
RCA	126	63.5 ± 4.3 [b]	43.8 ± 5.6	78.8 ± 4.6 [a]	1.7 ± 0.1 [bcd]
PNA	134	81.3 ± 3.4 [a]	58.7 ± 4.7	78.9 ± 3.9 [a]	2.1 ± 0.1 [ab]
UEA	130	76.2 ± 3.7 [ab]	57.6 ± 5.0	80.8 ± 4.0 [a]	2.1 ± 0.1 [ab]
SBA	116	62.9 ± 4.5 [b]	54.8 ± 5.8	78.1 ± 4.8 [a]	2.0 ± 0.1 [ab]
S-WGA	161	8.1 ± 2.2 [d]	46.2 ± 13.8	30.8 ± 12.8 [b]	1.7 ± 0.3 [abcd]

[1] Percentage of oocytes that were penetrated.
[a-d] Values with different superscripts within the same column are significantly different (P<0.05).

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Experiment 3: Detection of the amount of GlcNAc residues in ZP glycoproteins during oocyte maturation by the lectin-blotting assay

The relative intensities of terminal GlcNAc residues were specifically detected in ZP3 glycoprotein by lectin-blotting with WGA and S-WGA (Fig. 3), and these relative intensities labeling with WGA and S-WGA increased to 1.32 ± 0.07 and 1.28 ± 0.06, respectively, in oocytes cultured for 44 h in the control culture (Fig. 4) compared with that of freshly isolated oocytes (1.00 ± 0.07).   In contrast, increases in the relative intensities of GlcNAc labeled with WGA and S-WGA were significantly reduced in oocytes treated with 10 μg/ml tunicamycin during IVM culture (1.14 ± 0.03 and 1.05 ± 0.03, respectively).
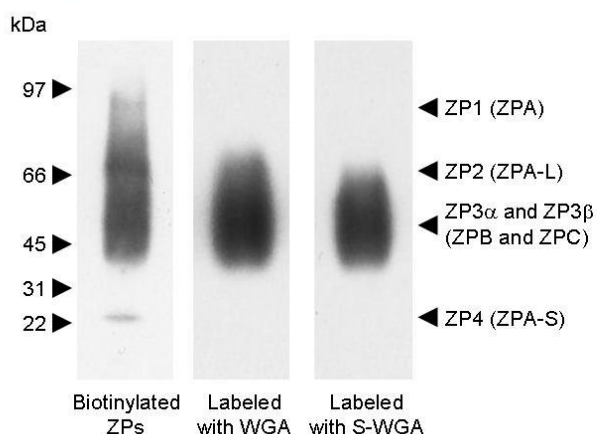
Figure 3.   Binding affinities of lectins to glycoprotein composition of porcine ZP.   Ten ZPs were fractionated on a 7.5% SDS-PAGE under reducing conditions.   Proteins were transferred to PVDF membranes and labeled with biotinylated WGA and S-WGA.   In biotinylated ZPs, two ZPs were biotinylated by the water soluble NHS-LC Biotin II before electrophoresis to identify each ZP glycoprotein band.
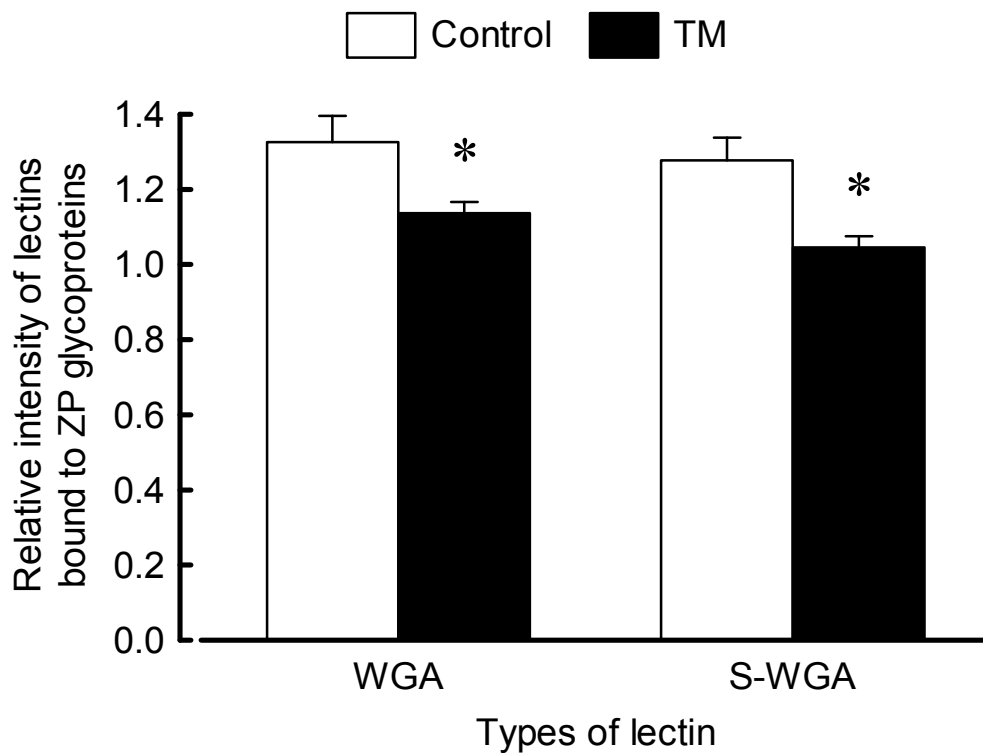
Figure 4. Effect of treatment with 10 μg/ml tunicamycin (TM) during 44-h IVM culture on the relative intensities of ZP3 glycoproteins labeled with WGA and S-WGA. Values are expressed as the mean ± SEM. The value of ZP3 glycoprotein in oocytes freshly isolated from their follicles is taken to be 1.0. * Values are significantly different compared with those in the absence of treatment with TM (control) (P<0.05).

Experiment 4: **Effects of tunicamycin treatment during IVM of COCs on nuclear maturation and sperm-ZP interactions**

When porcine COCs were cultured for 44 h in the absence or presence of tunicamycin (0-20 μg/ml), the treatment with tunicamycin at all concentrations had no effect on oocyte maturation (Fig. 5). However, treatment with tunicamycin during IVM of COCs significantly blocked sperm penetration in a dose-dependent manner, but these rates were not significantly different from that in the presence of 10 μg/ml tunicamycin (Fig. 6). These findings indicate that the optimal concentration of tunicamycin was 10 μg/ml to block the glycosylation of ZP glycoproteins during oocyte maturation.
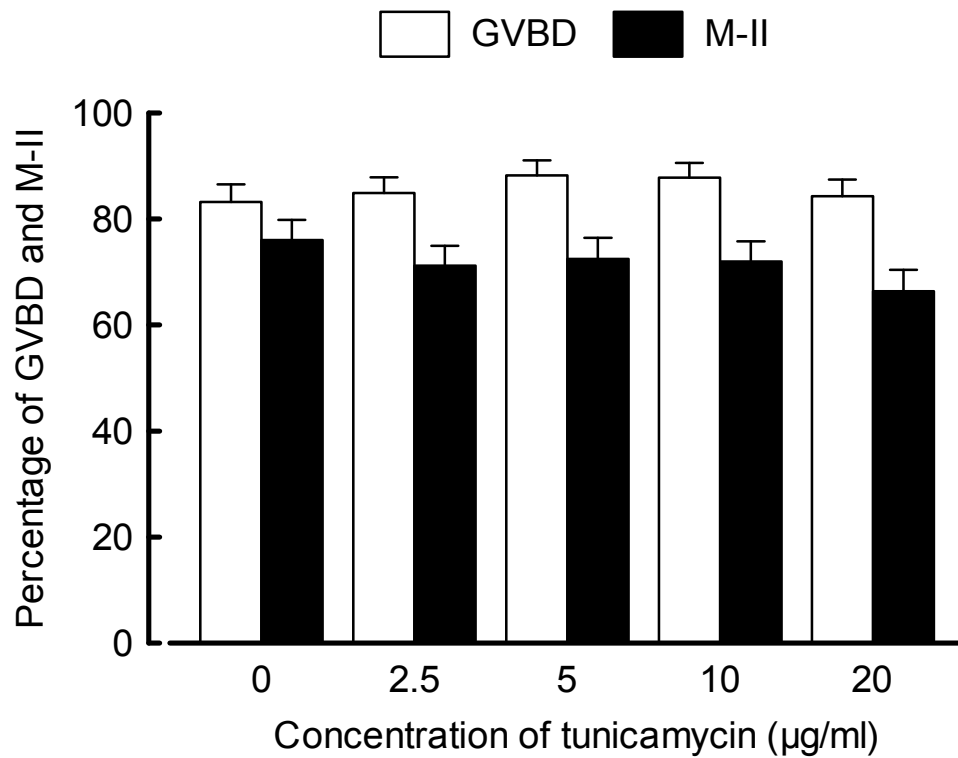
Figure 5. Effect of treatment with tunicamycin during IVM culture on meiotic division of porcine oocytes. COCs were treated with or without tunicamycin during maturation. After 44 h of IVM, cumulus-free oocytes were mounted on the glass slide to examine the nuclear maturation. Values are expressed as the mean ± SEM. The numbers of oocyte examined are about 130 in each group. GVBD = germinal vesicle breakdown, M-II = metaphase II.
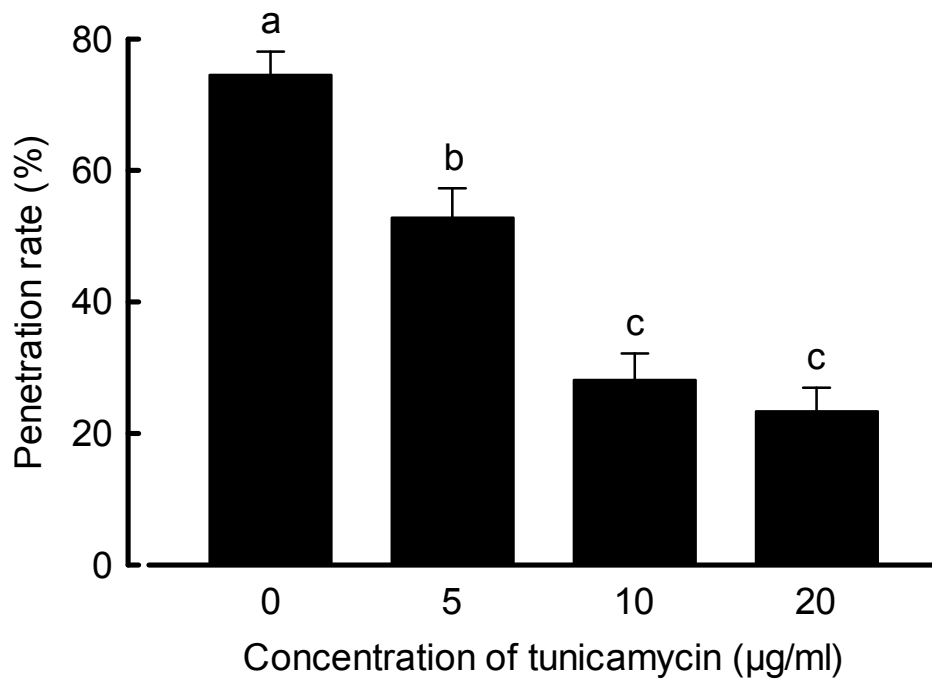
Figure 6. Effect of treatment with tunicamycin during IVM culture of porcine COCs on the sperm penetration rates. After treatment with or without tunicamycin during 44 h of IVM, cumulus-free oocytes were co-incubated with pre-incubated sperm in IVF medium. Following 10 h of IVF, oocytes were mounted, fixed, stained, and examined for fertilization parameters. Values are expressed as the mean ± SEM. Total number of oocytes examined was about 150 for each group. [a-c] Values with different superscripts are significantly different (P<0.05).

To determine the time interval before new glycosylation was sufficient for fertilization in porcine oocytes, COCs were cultured for 44 h and 10 μg/ml tunicamycin was added to the culture beginning at specific times during this 44-h cultivation. The porcine IVM oocytes in tunicamycin for up to 44 h did not adversely influence meiotic progression, but a significant reduction in the sperm penetration rate was observed in oocytes treated with tunicamycin for 32 h (Table 8). The treatment with tunicamycin for 24 h after the onset of normal IVM culture for 20 h was needed to reach at the same level of the penetration rate compared with that in the absence of treatment with tunicamycin through 44-h IVM culture periods in COCs. Although treatment with tunicamycin through 44-h IVM culture periods did not inhibit the MPN formation in COCs, the incidences of polyspermic penetration were increased by the short treatment time of tunicamycin in parallel to the progressive increases of the penetration rates.

Treatment with tunicamycin for up to 44 h had no effect on the dissolution time of ZP caused by 0.1% protease action (Fig. 7). In contrast, treatment with tunicamycin during IVM culture significantly decreased in the number of sperm bound to ZP in COCs (63-10) (Fig. 8, $P<0.05$). However, the number of sperm bound to ZP was progressively increased as treatment with tunicamycin was delayed after the onset of IVM in inhibitor-free medium, and these values of oocytes treated with tunicamycin for 8 h reached at the same levels compared with those in the absence of treatment with tunicamycin through 44-h IVM culture periods. Interestingly, the treatment with tunicamycin for 44, 38, 32 and 24 h resulted in a significant decrease of the percentages of acrosome-reacted sperm (16.7 ± 0.7, 17.7 ± 0.8, 18.9 ± 0.8 and 23.2 ± 0.9%, respectively) compared with that (28.5 ± 0.9%) in the absence of treatment with tunicamycin through 44-h IVM culture periods ($P<0.05$), suggesting that new glycosylation of ZP during the first 24 h of IVM culture was needed for the acquisition of inducible AR in ZP-bound sperm (Fig. 9).

All results from this study suggest that N-glycosylation for periods in excess of 20-24 h after the start of IVM was essential for a significant increase of the sperm penetration rate, the polyspermic fertilization rate (Table 8), the number of sperm bound to ZP (Fig. 8), and the number of acrosome-reacted sperm (Fig. 9). However, the proportion of penetrated oocytes exhibiting MPN was not influenced by the duration of culture in tunicamycin (Table 8).

Table 8. Effect of treatment with 10 μg/ml of tunicamycin (TM) during IVM of porcine COCs on the fertilization parameters.

Treatment time (h) [1]	No. of oocytes examined	Oocytes (%; mean ± SEM)			No. of sperm per penetrated oocyte (mean ± SEM)
		Penetrated [2]	Polyspermic [3]	Male pronucleus [3]	
0	143	71.8 ± 4.0 [a]	68.5 ± 4.9 [a]	85.4 ± 3.7	2.9 ± 0.2 [a]
8	139	68.7 ± 4.3 [a]	54.4 ± 5.6 [ab]	78.5 ± 4.6	2.5 ± 0.1 [ab]
16	153	58.4 ± 4.4 [a]	54.8 ± 5.8 [ab]	87.7 ± 3.9	2.5 ± 0.1 [ab]
20	117	56.9 ± 5.3 [a]	36.7 ± 6.9 [b]	87.8 ± 4.7	2.3 ± 0.1 [b]
24	130	58.8 ± 4.6 [a]	59.7 ± 6.0 [ab]	88.1 ± 4.0	2.2 ± 0.1 [b]
32	151	32.6 ± 4.1 [b]	40.5 ± 7.6 [b]	71.4 ± 7.0	1.6 ± 0.1 [c]
38	140	29.2 ± 4.3 [b]	42.4 ± 8.6 [ab]	78.8 ± 7.1	2.3 ± 0.1 [b]
44	130	29.7 ± 4.6 [b]	53.3 ± 9.1 [ab]	83.3 ± 6.8	2.0 ± 0.2 [bc]

[1] Each treatment time indicates the duration of TM treatment following pre-cultivation (44, 36, 28, 24, 20, 12, 6, or 0 h) in the control medium during a total of 44-h IVM culture.

[2] Percentage within the number of oocytes examined.

[3] Percentage of oocytes that were penetrated.

[a-c] Values with different superscripts within the same column are significantly different (P<0.05).
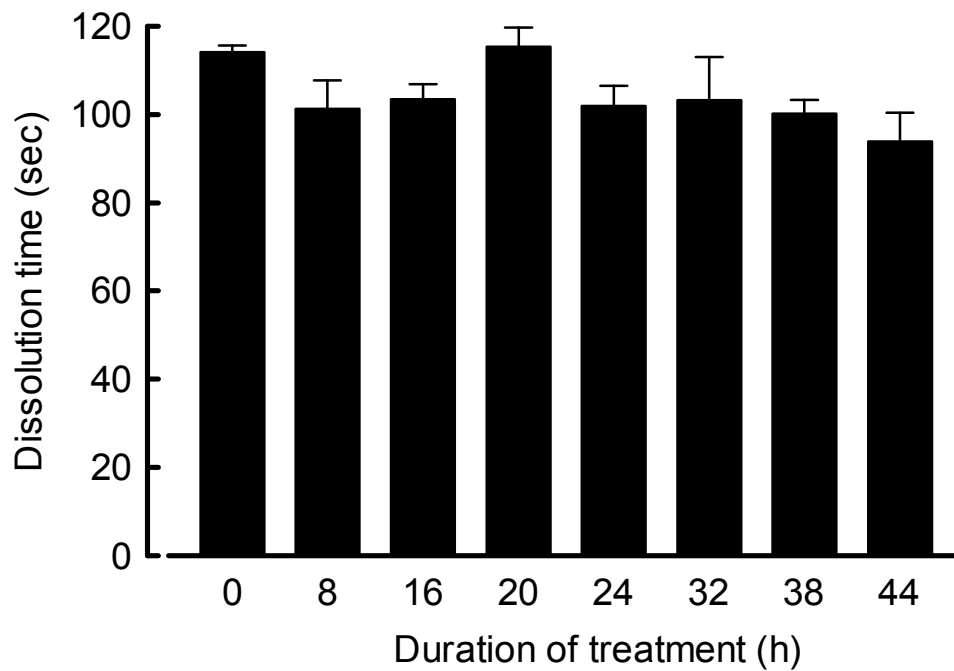
Figure 7. Effect of treatment with 10 μg/ml tunicamycin (TM) during IVM of porcine COCs on dissolution time of ZP caused by 0.1% protease. Each treatment time indicates the duration of TM treatment following pre-cultivation (44, 36, 28, 24, 20, 12, 6, or 0 h) in the control medium, during a total of 44-h IVM culture.   Values are expressed as the mean ± SEM.   Total number of oocytes in each treatment group ranged from 77-103.
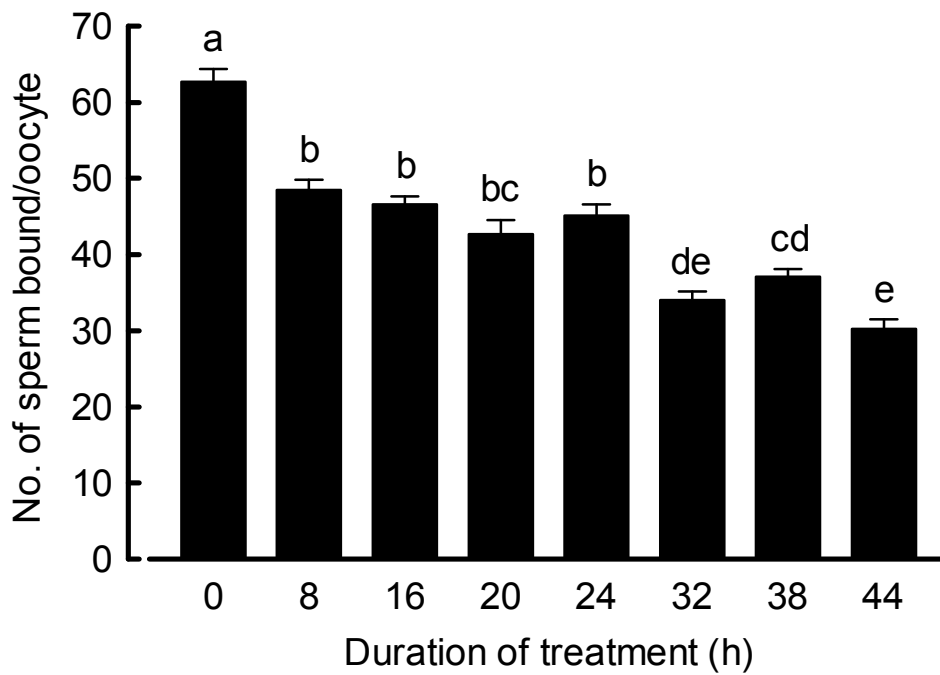
Figure 8. Effect of treatment with 10 μg/ml tunicamycin (TM) during IVM of porcine COCs on the sperm binding to ZP. Each treatment time indicates the duration of TM treatment following pre-cultivation (44, 36, 28, 24, 20, 12, 6, or 0 h) in the control medium, during a total of 44-h IVM culture. After treatment with TM during IVM culture, cumulus-free oocytes were co-incubated with pre-incubated sperm in IVF medium for 2 h. Values are expressed as the mean ± SEM. The total numbers of oocytes examined were 50-58 for determination of the number of sperm binding to ZP in each group. [a-e] Values without a common superscript differed (P<0.05).
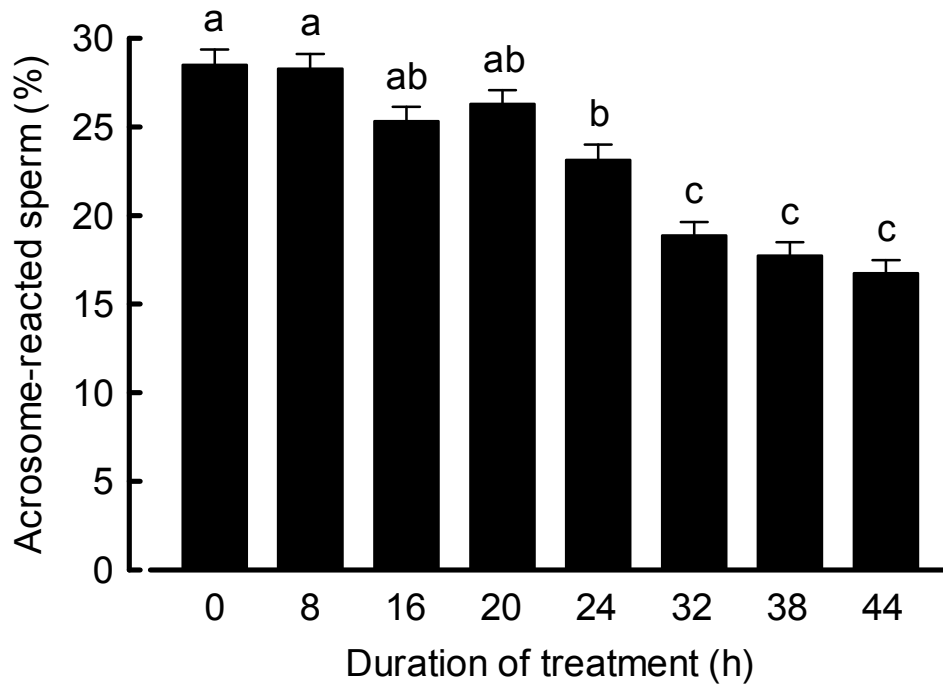
Figure 9. Effect of treatment with 10 μg/ml tunicamycin (TM) during IVM of porcine COCs on the AR induction in ZP-bound sperm. Each treatment time indicates the duration of TM treatment following pre-cultivation (44, 36, 28, 24, 20, 12, 6, or 0 h) in the control medium, during a total of 44-h IVM culture. After treatment with TM during IVM culture, cumulus-free oocytes were co-incubated with pre-incubated sperm in IVF medium for 2 h. Values are expressed as the mean ± SEM. The total numbers of living sperm examined were 2391-3129 in each treatment group. [a-c] Values without a common superscript differed (P<0.05).

2.5   Discussion

In the present study, masking GlcNAc residues in the zonae of porcine oocytes treated with lectins (Con A, WGA, and S-WGA) strongly reduced the number of sperm bound to ZP and the sperm penetration rate (Tables 6 and 7) at the same level of immatured oocytes (Table 5). Apparently, GlcNAc residues in ZP were important for the initial steps in fertilization of the porcine oocytes.   Similar observations on the involvement of zona GlcNAc residues in the fertilization of hamster and human oocytes have been reported (Mori *et al.*, 1989; Miranda *et al.*, 1997; 2000; Zitta *et al.*, 2004).   Moreover, the number of sperm bound to ZP and the sperm penetration rate in immatured oocytes at the germinal vesicle stage were markedly lower than those of matured oocytes (Fig. 2 and Table 5) according to the findings reported by Campos *et al.* (2001).

As reported by Rath *et al.* (2005), ZP glycoproteins underwent biochemical changes, such as *N*-glycosylation, during final maturation of porcine oocytes.   In the present study, a significant increase in terminal GlcNAc residues, detected by lectin-blotting with WGA and S-WGA, occurred in oocytes cultured in the control IVM medium.   This increase of terminal GlcNAc residues was significantly reduced by treatment with tunicamycin, a specific *N*-glycosylation inhibitor.

When porcine oocytes were treated with tunicamycin for 32 h after the onset of IVM in inhibitor-free medium for 12 h significantly lower rates of sperm penetration were found than that of the control group, but there was no effect on MPN formation (Table 8).   However, the treatment with tunicamycin for 24 h after the onset of IVM in inhibitor-free medium for 20 h resulted in a significant increase in the number of sperm bound to ZP and the same penetration rate as those in the control group (Fig. 8 and Table 8).   Similarly ZP glycosylation during the first 24 h of culture was needed to reach the levels of AR induction in ZP-bound sperm compared to those in the control group (Fig. 9).   Under our culture condition, germinal vesicle break down (GVBD) in COCs occurred at about 20-24 h after the onset of IVM culture regardless of treatment with tunicamycin (data not shown).   Therefore, these results suggest that *N*-glycosylation for periods in excess of 20-24 h after the beginning of IVM, played an essential role in sperm-ZP interactions, resulting in significant increases of the sperm penetration

rate, the polyspermic fertilization rate (Table 8), the number of sperm bound to ZP (Fig. 8), and the number of acrosome-reacted sperm (Fig. 9), although meiotic progression and MPN formation were unaffected by treatment with tunicamycin during the entire IVM culture periods.

Additionally, the present study examined whether blocking further *N*-glycosylation by treatment with tunicamycin had an effect on proteolytic modification of the ZP matrix. The hardening of porcine ZP takes place in accordance with a substantial decrease in the amounts of ZP1 and ZP2 glycoproteins (Hatanaka *et al*., 1992). However, tunicamycin, as indicated by zona dissolution time in protease, did not alter the zona hardening properties of porcine COCs (Fig. 7). These results indicate that a reduction in the rate of sperm penetration by treatment with tunicamycin does not result from any effect on physiological functions related to ZP hardening. Therefore, we inferred that *N*-glycosylation of GlcNAc residues in the ZP was required for sperm-ZP interactions, but not for *in vitro* maturation and pronucleus formation in the porcine COCs. To our knowledge, this is the first clear demonstration of the involvement of *N*-glycosylation of ZP glycoproteins in the sperm-ZP interactions in porcine COCs. Similarly, *N*-linked carbohydrates chains of ZP3α play a major role in the sperm binding to ZP (Yonezawa *et al*., 1995). Conversely, the *O*-linked carbohydrate chain of mouse ZP3 is involved in mediating the sperm binding to ZP (Berger *et al*., 1989).

In the present study, although the increase in the relative intensity of the GlcNAc residue labeled with S-WGA was completely blocked in matured oocytes treated with tunicamycin (1.05 ± 0.03), the intensity of labeling with WGA was slightly increased (1.14 ± 0.03, Fig. 4). This difference was attributed to the fact that S-WGA has a specific affinity only to GlcNAc residues, but WGA could bind to sialic acid residues in addition to GlcNAc residues (Debray *et al*., 1981). Perhaps sialylation may be concomitant with glycosylation of ZP glycoproteins during maturation of porcine oocytes. Gunaratne (2007) mentioned that the induction of AR in sperm bound to ZP could be mediated by not only *N*-glycosylation, but also sialylation and sulfation of ZP glycoproteins. In Chapter 4 and 5, therefore, we investigated whether sialylation and sulfation occur in concert with *N*-glycosylation during maturation of porcine oocytes.

Based on the present results, we concluded that: 1) *N*-glycosylation of GlcNAc residues in ZP glycoproteins was indispensible for sperm-ZP interactions; and 2) such

N-glycosylation occurred during the first 24 h of *in vitro* maturation of porcine COCs.

Chapter 3

Effect of cumulus cells during meiotic maturation on *N*-glycosylation of zona glycoproteins responsible for sperm-zona pellucida interactions of porcine oocytes

3.1 Abstract

The present study was conducted to examine whether cumulus cells were related to *N*-glycosylation of ZP glycoproteins during meiotic maturation of porcine oocytes. After mechanical removal of cumulus cells from COCs, cumulus-denuded oocytes (DOs) were cultured for a total of 44 h in IVM culture in the absence or presence of tunicamycin, a specific *N*-glycosylation inhibitor, for various intervals during IVM. The results determined that the first 36 h of *N*-glycosylation of ZP glycoproteins during meiotic maturation of DOs was crucial in sperm penetration, and sperm binding to ZP, while the *N*-glycosylation of GlcNAc residues in ZP glycoprotein during the first 24 h of meiotic maturation played a critical role in sperm-ZP interactions of porcine COCs (Chapter 2). The induction of AR in sperm-bound to ZP of porcine DOs was mediated by the first 24 h of *N*-glycosylation of ZP glycoproteins during oocyte maturation. However, the inhibition of *N*-glycosylation by tunicamycin treatment during IVM of DOs did not significantly influence on the meiotic maturation, MPN formation and ZP dissolution time as well in COCs (Chapter 2).

Therefore, these results suggest that cumulus cells are partly involved in ZP glycosylation during oocyte maturation, because the longer culture period in the absence of tunicamycin after the onset of IVM culture periods is needed to obtain the sperm penetration at the same levels of untreated oocytes in DOs rather than COCs.

3.2 Introduction

The *N*-glycosylation of GlcNAc residues in ZP glycoproteins during oocyte maturation was indispensible for sperm-ZP interactions of porcine COCs (Chapter 2). In agreement with this, biochemical changes during oocyte maturation, such as *N*-glycosylation of ZP glycoproteins were implicated in sperm-ZP binding in several species (Rath *et al*., 2005). Moreover, it is suggested that the sperm receptor activity depends on the carbohydrate components of ZP in mammals. In mouse, the sperm receptor activity is mediated by *O*-linked carbohydrate chains bearing terminal α-galactose residues (Florman and Wassarman, 1985; Bleil and Wassarman, 1988). Similarly, Yurewicz *et al*. (1991) show that *O*-linked carbohydrate chains isolated from porcine ZP3 inhibited sperm-egg binding. In contrast, the *N*-linked carbohydrate chains of ZP3α play a major role in mediating the sperm-ZP binding in pig (Noguchi *et al*., 1992; Yonezawa *et al*., 1995).

In general, the relationship between the cumulus cells and oocytes is important not only in the process of nuclear maturation but also in the cytoplasmic maturation closely associated with the developmental competences of oocytes after fertilization (Moor *et al*., 1990; Ka *et al*., 1997). Several studies have indicated that DOs can undergo meiotic maturation in mice (Binor and Wolf, 1979), rats (Magnusson, 1980), sheep (Staigmiller and Moor, 1984), and cattle (Chian *et al*., 1994; Tatemoto *et al*., 1994) *in vitro*. However, there are apparently no reports regarding whether the cumulus cells during maturation was correlated with the *N*-glycosylation of ZP glycoproteins responsible for sperm-ZP interactions.

Therefore, the present study was conducted to evaluate the effects of cumulus cells during meiotic maturation on *N*-glycosylation of ZP glycoproteins responsible for sperm-ZP interactions of porcine DOs by using tunicamycin, which has been shown to specifically inhibit the glycosylation of proteins *N*-glycosylated at asparagine residues (Waechter and Lennarz 1976; Struck and Lennarz 1977; Waechter and Harford 1977). Porcine DOs were cultured in IVM medium in the absence or presence of tunicamycin for various intervals a total of 44 h. After maturation, oocytes were inseminated with capacitated spermatozoa to examine the effects of *N*-glycosylation of ZP glycoproteins during IVM on sperm penetration, the number of sperm bound to ZP, and induction of

AR in ZP-bound sperm.   At the end of IVM culture, oocytes were assessed to investigate ZP hardness by 0.1% protease action.

3.3   Materials and Methods

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

3.3.1   Oocyte collection and *in vitro* maturation

Oocytes were collected by using the methods described in Chapter 2.   Only COCs with uniform ooplasm and a compact cumulus cell mass were selected to use in the experiments.   Oocytes were freed from cumulus cells by repeated passage through a narrow-bore pipette to obtain DOs.   After washing in IVM medium, groups of 15-20 DOs were placed into 100-μl droplets of IVM medium covered with light weight mineral oil and incubated for 44 h at 39°C in an atmosphere of 5% CO_2 in air.

3.3.2   *In vitro* fertilization

IVF was carried out by use of methods described in Chapter 2.

3.3.3   Sperm-ZP binding

At 2 h after sperm insemination, the number of sperm tightly bound to the ZP was counted under a fluorescence microscope as described in Chapter 2.

3.3.4   Assessment of fertilization

The nuclear status after IVM and fertilization parameters after IVF were examined as described in Chapter 2.

3.3.5 Assessment of ZP solubility

After washing three times in H-TL-PVA, 15-20 DOs were transferred into a 50-μl drop of H-TL-PVA containing 0.1% (w/v) protease, and continuously observed for dissolution of ZP at room temperature using an inverted microscope at magnification: × 200. The time required for dissolution in a given oocyte was recorded as the ZP dissolution time.

3.3.6 Evaluation of sperm acrosomal status

The induction of AR in sperm bound to ZP was assayed by use of methods described in Chapter 2.

3.3.7 Experimental design

Experiments were designed to elucidate the effect of treatment with 10 μg/ml of tunicamycin during IVM on sperm-ZP interactions in DOs. In all treatment groups, DOs were treated with tunicamycin for various intervals (0, 8, 16, 20, 24, 32, 38 and 44 h) after pre-cultivation in the control medium (44, 36, 28, 24, 20, 12, 6, and 0 h, respectively) during a total of 44 h of IVM culture.

Experiment 1: At the end of IVM culture, DOs were inseminated with pre-incubated sperm in IVF medium to examine the fertilization parameters.

Experiment 2: At the end of IVM culture, DOs were assessed for ZP hardness by 0.1% protease action.

Experiment 3: At the end of IVM culture, DOs were inseminated with pre-incubated sperm in IVF medium to evaluate the sperm binding to ZP and induction of AR in ZP-bound sperm.

3.3.8 Statistical analysis

Statistical analysis was performed as described in Chapter 2.

3.4   Results

When porcine DOs were cultured for 44 h in the absence or presence of tunicamycin (0-20 μg/ml), the treatment with tunicamycin during IVM culture significantly blocked sperm penetration in a dose-dependent manner (Fig. 10). However, there is no significant different in sperm penetration rate between 10 μg/ml and 20 μg/ml of tunicamycin concentration. These results imply that the optimal concentration of tunicamycin was 10 μg/ml to block the glycosylation during oocyte maturation.

A significant reduction in the sperm penetration rate was observed in DOs treated with tunicamycin for 16 h after the onset of normal IVM culture for 28 h through 44-h IVM culture periods ($P<0.05$). However, the treatment of tunicamycin for 8 h after the onset of IVM in inhibitor-free medium for 36 h was obtained the same level of the penetration rate compared with that in the absence of treatment with tunicamycin through 44-h IVM culture period (Table 9). Although treatment with tunicamycin during IVM culture did not influence the MPN formation, the incidences of polyspermic penetration were decreased only in DOs treated with tunicamycin for 38 h. The new glycosylation of ZP glycoproteins during the first 36 h of culture was needed to reach at the same level of the penetration rate compared with that in the absence of treatment with tunicamycin through 44-h IVM culture periods in DOs.

The treatment with tunicamycin for up to 44 h in porcine DOs did not adversely influence on the zona dissolution time in 0.1% protease (Fig. 11). In contrast, treatment with tunicamycin for 44 h during IVM culture significantly decreased in the number of sperm bound to ZP in DOs (27 ± 1.8 sperm) compared with that of DOs treated without tunicamycin (61 ± 2.4 sperm, $P<0.05$), and the number of sperm bound to ZP was progressively increased as treatment time of tunicamycin was decreased after the onset of IVM in inhibitor-free medium during IVM culture (Fig. 12). Interestingly, treatment of tunicamycin for 44, 38, 32 and 24 h after the onset of IVM in inhibitor-free medium through 44-h IVM culture periods resulted in a significant decrease of the percentages of AR-inducing sperm (15.6 ± 0.7, 16.4 ± 0.7, 17.3 ± 0.7 and $22.7 \pm 0.9\%$, respectively) compared with that ($27.8 \pm 0.9\%$) in the absence of

44

treatment with tunicamycin (P<0.05; Fig. 13). The proportion of AR-inducing sperm in DOs treated with tunicamycin for 20 h after the onset of IVM in inhibitor-free medium for 24 h during IVM culture (25.2 ± 0.8%) was comparable to that of the control group (27.8 ± 0.9%), suggesting that new glycosylation of ZP during the first 24 h of IVM culture was needed for the acquisition of inducible AR in ZP-bound sperm.
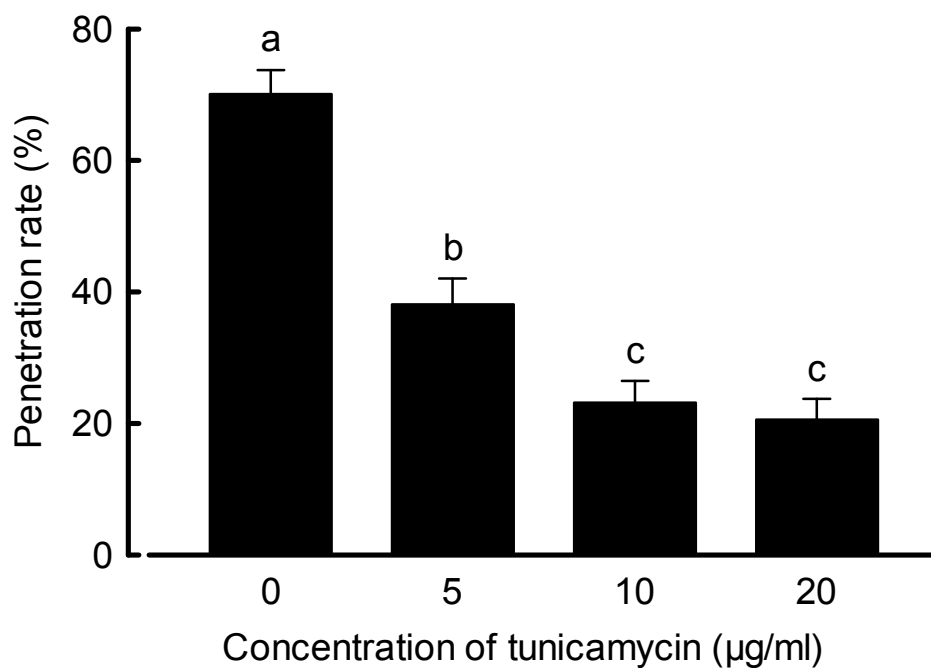
Figure 10.　Effect of treatment with tunicamycin (TM) during IVM culture of porcine DOs on the sperm penetration rates.　After treatment with or without TM during 44 h of IVM, oocytes were co-incubated with pre-incubated sperm in IVF medium. Following 10 h of IVF, oocytes were mounted, fixed, stained, and examined for fertilization parameters.　Values are expressed as the mean $\pm$ SEM.　Total number of oocytes examined was about 150 for each group.　[a-c] Values with different superscripts are significantly different ($P<0.05$).

Table 9. Effect of treatment with 10 µg/ml of tunicamycin (TM) during IVM of porcine DOs on the fertilization parameters.

Treatment time (h) [1]	No. of oocytes examined	Oocytes (%; mean ± SEM)			No. of sperm per penetrated oocyte (mean ± SEM)
		Penetrated [2]	Polyspermic [3]	Male pronucleus [3]	
0	122	72.9 ± 4.0 [a]	43.8 ± 5.3 [a]	61.8 ± 5.2	1.5 ± 0.1 [ab]
8	148	62.2 ± 4.0 [a]	55.4 ± 5.2 [a]	72.8 ± 4.6	1.9 ± 0.1 [a]
16	126	46.8 ± 4.5 [b]	52.5 ± 6.5 [a]	66.1 ± 6.2	1.8 ± 0.1 [ab]
20	138	40.6 ± 4.2 [b]	46.4 ± 6.7 [a]	60.7 ± 6.5	1.6 ± 0.1 [ab]
24	135	45.2 ± 4.3 [b]	54.1 ± 6.4 [a]	67.2 ± 6.0	1.8 ± 0.1 [a]
32	120	34.2 ± 4.3 [bc]	60.9 ± 7.6 [a]	58.5 ± 7.7	1.8 ± 0.1 [a]
38	126	34.1 ± 4.2 [bc]	14.0 ± 5.3 [b]	58.1 ± 7.5	1.1 ± 0.1 [c]
44	131	23.7 ± 3.7 [c]	29.0 ± 8.2 [ab]	58.4 ± 8.9	1.4 ± 0.1 [bc]

[1] Each treatment time indicates the duration of TM treatment following pre-cultivation (44, 36, 28, 24, 20, 12, 6, or 0 h) in the control medium during a total 44-h IVM culture.

[2] Percentage within the number of oocytes examined.

[3] Percentage of oocytes that were penetrated.

[a-c] Values with different superscripts within the same column are significantly different (P<0.05).
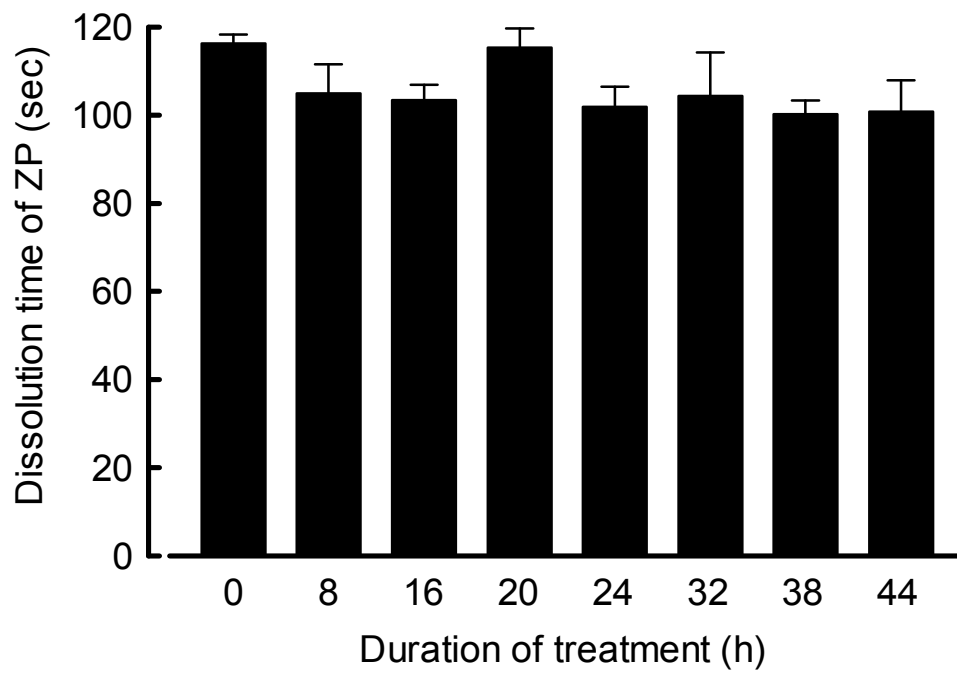
Figure 11. Effect of treatment with 10 μg/ml tunicamycin (TM) during IVM of porcine DOs on dissolution time of ZP caused by 0.1% protease. Each treatment time indicates the duration of TM treatment following pre-cultivation (44, 36, 28, 24, 20, 12, 6, or 0 h) in the control medium, during a total of 44-h IVM culture. Values are expressed as the mean ± SEM. Total number of oocytes in each treatment group ranged from 85-102.
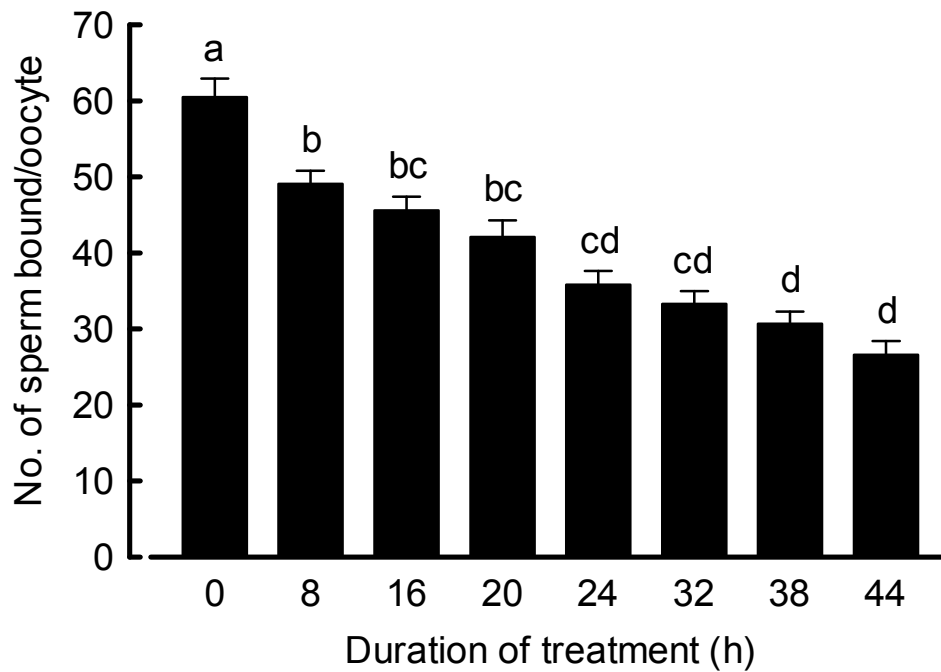
Figure 12. Effect of treatment with 10 μg/ml tunicamycin (TM) during IVM of porcine DOs on the sperm binding to ZP. Each treatment time indicates the duration of TM treatment following pre-cultivation (44, 36, 28, 24, 20, 12, 6, or 0 h) in the control medium, during a total of 44-h IVM culture. After treatment with TM during IVM culture, oocytes were co-incubated with pre-incubated sperm in IVF medium for 2 h. Values are expressed as the mean ± SEM. The total numbers of oocytes examined were 43-55 for determination of the number of sperm binding to ZP in each group. [a-d] Values without a common superscript differed (P<0.05).
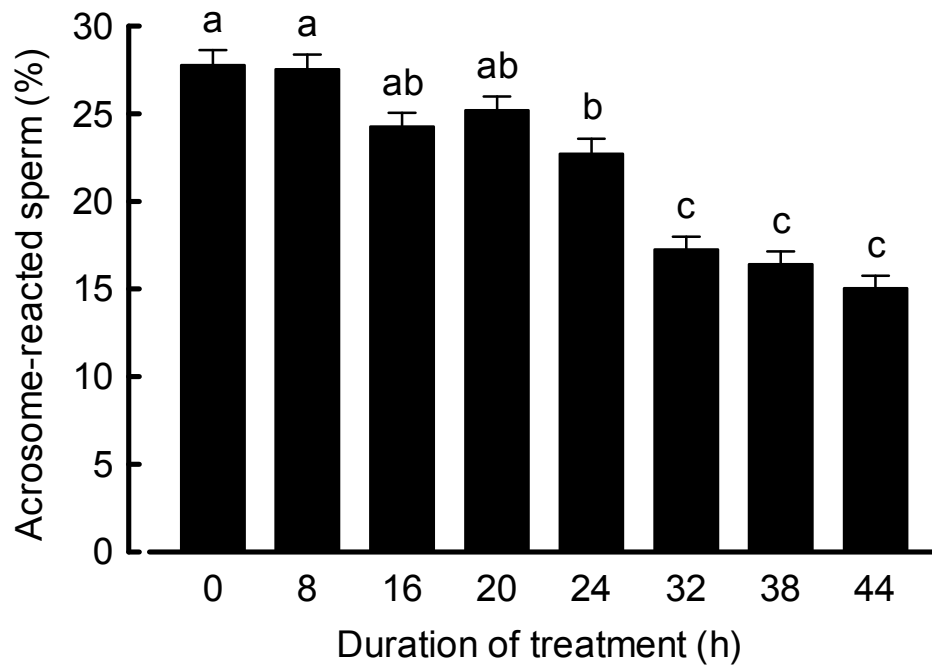
Figure 13. Effect of treatment with 10 μg/ml tunicamycin (TM) during IVM of porcine DOs on the AR induction in ZP-bound sperm. Each treatment time indicates the duration of TM treatment following pre-cultivation (44, 36, 28, 24, 20, 12, 6, or 0 h) in the control medium, during a total of 44-h IVM culture. After treatment with TM during IVM culture, oocytes were co-incubated with pre-incubated sperm in IVF medium for 2 h. Values are expressed as the mean ± SEM. The total numbers of living sperm examined were 2552-2914 in each treatment group. [a-c] Values without a common superscript differed (P<0.05).

3.5 Discussion

The present findings showed that the incidences of penetration (Table 9) and the number of sperm bound to ZPs (Fig. 12) significantly decreased in DOs treated with tunicamycin for 44 h during maturation.   These findings indicated that N-glycosylation of ZP glycoproteins during meiotic maturation played important roles in sperm penetration and sperm binding to ZP of porcine DOs.   Similar findings reported that terminal GlcNAc residues are required for sperm binding to ZP in humans (Mori *et al.*, 1989; Miranda *et al.*, 1997; 2000) and that GlcNAc residues in hamster ZP glycoproteins specifically affect the primary binding of sperm to ZP (Zitta *et al.*, 2004). Moreover, N-glycosylation of GlcNAc residues in ZP glycoproteins was indispensible for sperm-ZP interactions of porcine COCs (Chapter 2).

In pig, mechanical removal of the cumulus cells resulted in premature cortical reaction (Galeati *et al.*, 1991), supporting the hypothesis that the cumulus oophorus during oocyte maturation participates in the prevention of spontaneous zona hardening. However, ZP dissolution time due to protease action in porcine DOs cultured with tunicamycin over a time period of 44 h did not differ from that of oocytes cultured in the control group (Fig. 11), indicating that a reduction in the rate of sperm penetration by treatment with tunicamycin from the beginning of IVM was not exerted by ZP hardening.   In addition, the new glycosylation of ZP glycoproteins during the first 36 h of culture is indispensable for acquirement of the capacity of oocytes to accept spermatozoa in DOs (Table 9), while the first 24 h of culture is critical to the new glycosylation of ZP glycoproteins in COCs (Table 8 in Chapter 2).   In general, the presence of cumulus cells during oocyte maturation is fundamental for porcine oocytes to maintain the penetrability by sperm (Galeati *et al.*, 1991).

The treatment with tunicamycin for 44 h during maturation significantly diminished the induction of AR in ZP-bound sperm, and new glycosylation of ZP during the first 24 h of IVM culture was needed for the acquisition of inducible AR in ZP-bound sperm (Fig. 13).   This result suggests that the induction of AR in sperm-bound to ZP was mediated by N-glycosylation of ZP glycoproteins occurred during oocytes maturation, independent of cumulus cells.   On the other hand, hyaluronic acid, one of types of glycosaminoglycans, secreted from the expanded

cumulus cells might be a candidate molecule for ZP modifications during oocyte maturation to improve fertilization processes, since hyaluronic acid has been proposed to bind to sperm plasma membrane PH-20 responsible for the induction of AR (Ball *et al*., 1982).   Further experiments will be necessary to determine whether cumulus cells during oocyte maturation facilitate the ZP modifications implicated in the AR induction in ZP-bound sperm.

Therefore, the present results suggest that cumulus cells are partly involved in ZP glycosylation during oocyte maturation, because the longer culture period in the absence of tunicamycin after the onset of IVM culture periods is needed to obtain the sperm penetration at the same levels of untreated oocytes in DOs rather than COCs.

Chapter 4

Sialylation of zona glycoproteins during meiotic maturation is involved in sperm-zona pellucida interactions of porcine oocytes

4.1   Abstract

The porcine ZP undergoes biochemical, structural and functional alterations in the final maturation phase prior to fertilization.   In the present study, four experiments were conducted to elucidate whether the sialylation of ZP glycoproteins during oocyte maturation had influences upon sperm-ZP interactions.   In the first experiment, sialic acid residues in the ZP of *in vitro* matured porcine oocytes were masked with Sambucus sieboldiana (SSA) and Maackia amurensis lectin II (MAL II) lectins and the influence of such lectin-masking on sperm binding and sperm penetration was examined.   In the second experiment, lectin-blotting assay and two-dimensional (2D) gel electrophoresis were used to determine the sialylation of ZP glycoproteins and ZP acidification associated with sialylation during oocyte maturation, respectively.   The third experiment was carried out to investigate the role of sialylation of ZP glycoproteins during oocyte maturation in sperm-ZP interactions by using neuraminidase.   The last one examined the effect of cumulus cells during oocyte maturation on the sialylation of ZP glycoproteins responsible for sperm-ZP interactions.   The results described that the lower number of sperm bound to ZP and the sperm penetration rate were observed in oocytes blocked with SSA and MAL II lectins.   The lectin-blotting assay and 2D gel electrophoresis clearly demonstrated that ZP acidification occurred in accordance with the sialylation 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 via treatment with neuraminidase after 28, 32 and 44 h of culture as a consequence of significant reductions in the number of sperm bound to ZP and AR induction in ZP-bound sperm in COCs ($P<0.05$).   Similarly, the sperm penetration, polyspermy,

the number of sperm bound to ZP and induction of AR in ZP-bound sperm were significantly reduced by ZP desialylation via treatment with neuraminidase after 24, 28 and 44 h of culture in DOs ($P<0.05$).   In contrast, sialylation was not associated with a protective proteolytic modification of the ZP matrix before fertilization.   These findings suggest that ZP acidification elicited by the sialylation of ZP glycoproteins during oocyte maturation contributed to the porcine ZP acquiring the capacity to accept sperm.   Moreover, the cumulus cells during oocyte maturation were not essential in the sialylation of ZP glycoproteins responsible for sperm-ZP interactions, and the time course of this sialylation in ZP glycoproteins was correlated with the induction of GVBD during oocyte maturation.

4.2 Introduction

The early events of mammalian fertilization are implicated in the initial binding of acrosome-intact sperm to ZP glycoproteins. After the sperm-ZP binding induces AR, the acrosome-reacted sperm binds transiently to ZP before penetrating the zona matrix. Presumably, the sperm penetration through ZP is facilitated by hydrolytic enzymes released from the sperm acrosome. Following penetration into perivitelline space, the sperm fuses with oolemma and activates the egg, triggering the ZP block to polyspermy. Although these cellular events are well described in many species, their underlying molecular mechanisms are less well understood (Yanagimachi, 1988). Moreover, the interactions between sperm and ZP are mediated by multiple complexes involving several sperm plasma membrane proteins and several carbohydrates contained in ZP glycoproteins (Storey, 1995; Shur, 1998; Thaler and Cardullo, 2002; Rodeheffer and Shur, 2004). The GalTase on the sperm surface at least partly mediates gamete adhesion by binding to its appropriate carbohydrate substrate in ZP (Shur, 1986). The sperm surface HABP1 present in rat, mice, bull and human sperm plasma membrane is also responsible for the sperm-ZP interactions (Ranganathan *et al*., 1994). On the other hand, as the aspect of ZP, several carbohydrates including fucose, GlcNAc, mannose and galactose in ZP glycoproteins, participate in the sperm-ZP interactions in rat, hamster, and/or mouse (Shalgi *et al*., 1986; Boldt *et al*., 1989; Ponce *et al*., 1994). Moreover, we reported that the increase in the amount of terminal 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 (Chapter 2). This new *N*-glycosylation is responsible for significant increases in the sperm penetration rate, the polyspermic fertilization rate, the number of sperm bound to ZP and the percentage 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 matured oocytes compared with immatured oocytes as indicated by an acidic shift of pI units in 2D gel electrophoresis elicited by increases in the sialic acid residues of ZP glycoproteins (Rath *et al*., 2005; 2006; von Witzendorff *et al*., 2009). Porcine ZP3 glycoproteins are highly

heterogeneous mainly as a result of the differences in the amount of sulfated *N*-acetyllactosamine and sialic acid in the acidic chains (Nakano *et al*., 1990) and have been shown to be required for the binding of sperm to the intact ZP leading to completion of AR (Yoshizawa *et al*., 1994). It is reported that the sialic acid of ZP glycoproteins are physiologically involved in regulating the binding of sperm to ZPs in porcine (Lo Leggio *et al*., 1994) and bovine (Velásquez *et al*., 2007) oocytes. Gunaratne (2007) further mentioned that the induction of AR in sperm bound to ZP could be mediated by not only *N*-glycosylation, but also sialylation and sulfation of ZP glycoproteins. Contrary to these reports, Liu *et al*. (1997) suggest that neither the sulfate nor sialic acid present on mouse ZP3 oligosaccharides is directly involved in sperm receptor or AR-inducing activity.

Interestingly, the mammalian oocyte and cumulus cells are metabolically coupled through gap junctions, that provide a unique means of entry into the ooplasm for several metabolites (Moor *et al*., 1980; Larsen and Wert, 1988). The attachment of cumulus cells to the oocytes during IVM has a critical role in nuclear maturation, fertilization and subsequent embryo development. The attachment of cumulus cells to the oocytes during IVF has a positive influence on the embryonic development but not on fertilization (Wongsrikeao *et al*., 2005). However, cumulus-free oocytes are widely used for pig IVF in many laboratories, and acceptable rates of penetration and monospermy have been reported (Abeydeera and Day, 1997; Funahashi and Nagai, 2001; Marchal *et al*., 2002). Moreover, there are apparently no reports regarding whether the cumulus cells during oocyte maturation are necessary for the sialylation of ZP glycoproteins responsible for sperm-ZP interactions.

Therefore, in the present study, experiments were undertaken 1) to examine whether sialic acid residues in the ZP of *in vitro* matured porcine oocytes have influence upon sperm binding to ZP and sperm penetration by using SSA and MAL II lectins, 2) to investigate whether porcine ZP glycoproteins are acidified in parallel to sialylation during oocyte maturation, 3) to evaluate the effects of desialylation of ZP glycoproteins of both COCs and DOs during IVM on fertilization responses including sperm penetration, polyspermy, sperm binding to ZP, induction of AR in ZP-bound sperm and ZP hardness, and 4) to examine the effect of cumulus cells during oocyte maturation on the sialylation of ZP glycoproteins involved in sperm-ZP interactions.

4.3   Materials and Methods

4.3.1   Collection of oocytes and *in vitro* maturation

Oocytes were collected by using the methods described in Chapter 2.   Only COCs with uniform ooplasm and a compact cumulus cell mass were selected to use the experiments. Some oocytes were freed from cumulus cells by repeated passage through a narrow-bore pipette to obtain DOs.   COCs and DOs were washed three times with H-TL-PVA.   After washing in basic IVM medium mentioned previously, groups of 15-20 COCs or DOs were transferred into 100-µl droplets of the basic IVM medium for 0, 6, 12, 20, 24, 28, 36 and 44 h.   Since GVBD and oocyte maturation at M-II stage were achieved at 28 and 44 h of IVM in COCs and at 24 and 44 h of IVM in DOs, respectively, under our culture conditions.   Oocytes cultured in IVM medium for 0, 16, 28, 32 and 44 h for COCs and 0, 12, 24, 28 and 44 h for DOs of IVM were employed for various experiments to determine sperm-ZP interactions associated with meiotic progression.

4.3.2   Removal of sialic acid residues

After 0, 16, 28, 32 and 44 h of maturation culture, COCs were sucked through a narrow-bore pipette to remove their cumulus cells in H-TL-PVA containing 0.1% (w/v) hyaluronidase.   After 0, 12, 24, 28 and 44 h of IVM culture for DOs, both types of oocytes derived from COCs and DOs were washed three times with PZM 5 and then placed into 20-µl droplets of PZM 5 containing neuraminidase (sialidase) from *Clostridium perfringens* (1 IU/ml). The droplets containing oocytes were kept in an incubator for 1 h before insemination.   After 1 h of incubation, the oocytes were washed three times in IVF medium and transferred into 50-µl droplets of IVF medium until spermatozoa were added for fertilization.

4.3.3 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*. (1989). Briefly, ZPs were ruptured and removed mechanically with a narrow micropipette (about 60 μm in diameter) in PBS containing 1 mg/ml 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 (Moos *et al*., 1994, Tatemoto *et al*., 2005). 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, UK). 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.

4.3.4 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 in Chapter 2. After blocking for 2 h in TBS-T-gelatin, the membranes were treated with 2.5 μg/ml of biotin-labeled SSA (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.

4.3.5 *In vitro* fertilization

IVF was carried out by use of methods described in Chapter 2.

4.3.6 Sperm-ZP binding

At 2 h after sperm insemination, the number of sperm tightly bound to the ZP was counted under a fluorescence microscope as described in Chapter 2.

4.3.7 Assessment of *in vitro* nuclear maturation and fertilization

The nuclear status after IVM and fertilization parameters after IVF were examined as described in Chapter 2.

4.3.8 Assessment of ZP solubility

In vitro matured cumulus-free oocytes were treated for 1 h in PZM 5 medium with or

without neuraminidase and then washed three times in H-TL-PVA.   The dissolution times of ZP caused by 0.1% (w/v) protease action were observed by use of methods described in Chapter 2.

4.3.9   Evaluation of sperm acrosomal status

The induction of AR in sperm bound to ZP was assayed by use of methods described in Chapter 2.

4.3.10   Experimental design

In Experiment 1, the involvement of sialic acid residues in ZP of matured oocytes in sperm binding to ZP and sperm penetration was examined.   *In vitro* matured COCs were inseminated in IVF medium supplemented with 1 μg/ml of SSA and MAL II, recognizing α-2, 6-linked and α-2, 3-linked sialic acid, respectively, and the number of sperm bound to ZP and the fertilization parameters were observed.

Experiment 2 examined whether porcine ZP glycoproteins were acidified in parallel to sialylation during oocyte maturation.   The lectin-blotting assay and 2D gel electrophoresis were performed for ZPs of oocytes freshly isolated from their follicles and *in vitro* matured COCs and DOs treated with or without neuraminidase.

Experiment 3 was designed to evaluate the effects of the sialylation of ZP glycoproteins during oocyte maturation on subsequent sperm-ZP interactions of porcine oocytes by using neuraminidase.   After IVM culture for 0, 6, 12, 20, 24, 28, 36, and 44 h in IVM medium, COCs were freed from their cumulus cells.   Both types of oocytes derived from COCs and DOs were mounted on the glass slide to observe the nuclear status.   The other oocytes treated with or without neuraminidase were inseminated with pre-incubated 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 during IVM were co-incubated with pre-incubated sperm in IVF medium containing 1 IU/ml neuraminidase with attention paid to the blocking of sialylation during the

IVF procedure.

Experiment 4 investigated the effects of cumulus cells during oocyte maturation on the sialylation of ZP glycoproteins subsequent in sperm-ZP interactions of porcine oocytes.

4.3.11 Statistical analysis

Statistical analysis was performed as described in Chapter 2.

4.4 Results

Experiment 1: Effects of SSA and MAL II lectins added to IVF medium on sperm binding to ZP and fertilization parameters of porcine oocytes

The number of sperm bound to ZP were significantly lower in the oocytes inseminated in the presence of SSA and MAL II (31 and 36 sperm, respectively) than that in the absence of lectin (control; 64 sperm, $P<0.05$) (Table 10). In addition, the sperm penetration rates were significantly declined in the oocytes inseminated in the presence of SSA and MAL II (56 and 45%, respectively) compared with that in the absence of lectin (control; 71%, $P<0.05$) (Table 11). Although SSA had no effect on polyspermy rate, MAL II significantly decreased the rate of polyspermic fertilization (27%) compared with that in the absence of lectin (70%, $P<0.05$). The proportion of MPN formation was significantly decreased in SSA and MAL II treated oocytes (36, 42%, respectively, $P<0.05$). The mean number of sperm per penetrated oocytes was reduced by treatment with SSA, and MAL II (1.8 and 1.3 sperm respectively), when compared with that of control (2.4 sperm).

Table 10. Effects of SSA and MAL II lectins added to IVF medium on sperm binding to ZP in porcine oocytes.

Types of lectin (1 µg/ml)	Specific affinity to carbohydrate residues	Sperm binding to ZP	
		No. of oocytes examined	No. of sperm bound per oocyte (mean ± SEM)
Control		30	63.7 ± 1.7 [a]
SSA	α-2,6-linked sialic acid	30	30.9 ± 1.4 [b]
MAL II	α-2,3-linked sialic acid	30	35.6 ± 2.0 [b]

[a,b] Values with different superscripts within the same column are significantly different ($P<0.05$).

Table 11. Effects of SSA and MAL II lectins added to IVF medium on the fertilization parameters of porcine oocytes.

Types of lectin (1 µg/ml)	No. of oocytes examined	Oocytes (%; mean ± SEM)			No. of sperm per penetrated oocyte (mean ± SEM)
		Penetrated	Polyspermic [1]	Male pronucleus [1]	
Control	145	71.0 ± 3.7 [a]	69.2 ± 4.5 [a]	76.7 ± 4.2 [a]	2.4 ± 0.2
SSA	120	55.8 ± 4.5 [b]	47.8 ± 6.1 [ab]	35.8 ± 5.9 [b]	1.8 ± 0.1
MAL II	139	44.6 ± 4.2 [b]	27.4 ± 5.7 [b]	41.9 ± 6.3 [b]	1.3 ± 0.1

[1] Percentage of oocytes that were penetrated.
[a,b] Values with different superscripts within the same column are significantly different ($P<0.05$).

Experiment 2.1:   Detection of sialylation of ZP glycoproteins during oocyte maturation by the lectin-blotting assay

The relative intensity of sialic acid residues in ZP glycoproteins detected by lectin-blotting with SSA 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).   However, this increase was completely repressed in oocytes treated with neuraminidase (0.8 ± 0.1; Figs. 14 and 15).
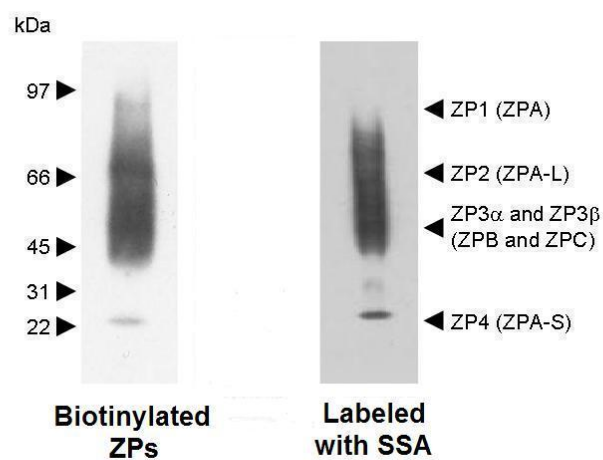
Figure 14.   Binding affinities of lectin to glycoprotein composition of porcine ZP.   Ten ZPs were fractionated on a 7.5% SDS-PAGE under reducing conditions.   Proteins were transferred to PVDF membranes and labeled with biotinylated SSA.   In biotinylated ZPs, two ZPs were biotinylated by the water soluble NHS-LC Biotin II before electrophoresis to identify each ZP glycoprotein band.
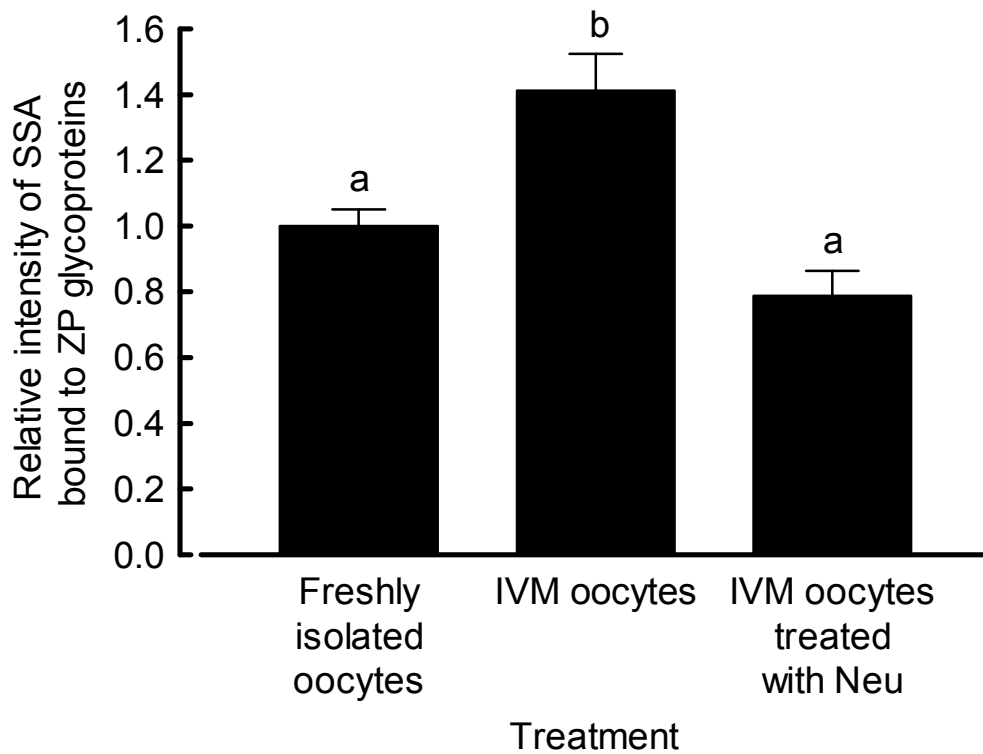
Figure 15. Changes in the affinity of SSA lectin for glycoproteins of porcine ZPs collected from IVM oocytes. After 44 h of IVM, oocytes were treated with 1 IU/ml of neuraminidase (Neu) before collection of ZPs. Values are expressed as the mean ± SEM. The values for ZP glycoproteins of freshly isolated oocytes are taken to be 1.0. [a,b] Values with different superscripts are significantly different ($P<0.05$).

Experiment 2.2:   Acidification according to sialylation of ZP glycoproteins during oocyte maturation

Two-dimensional (2D) 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. 16-19).   Accordingly, at a pI of 5.5-7.0, the intensity of the ZP1 + ZP2 and ZP3 bands in COCs cultured for 44 h in IVM medium declined dramatically to 0.2 ± 0.1 and 0.1 ± 0.1, respectively, compared with those in oocytes freshly isolated from follicles (1.0 ± 0.1 and 1.0 ± 0.1, respectively, P<0.05; Fig. 17). However, the mobility shift caused by the sialylation 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 neuraminidase (Figs. 16C and 17).   The same phenomenon was also observed in DOs (Figs. 18 and 19), but the level of the mobility shift elicited by sialylation during IVM in DOs was weaker than that of COCs under the condition without neuraminidase treatment.
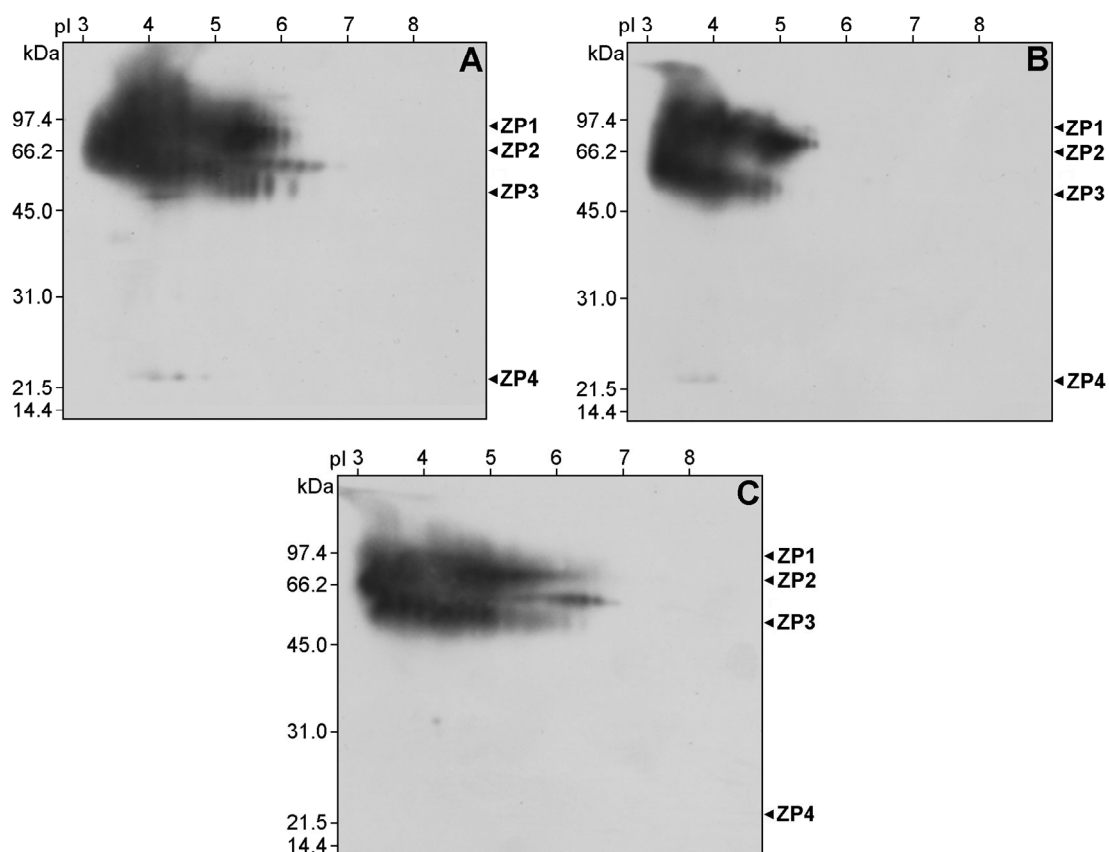
Figure 16. Two-dimensional (2D) gel electrophoresis of ZP glycoproteins of porcine COCs analyzed by biotinylation and ECL in oocytes freshly isolated from follicles (A) and after IVM for 44 h (B).   After the IVM, some oocytes were treated with 1 IU/ml of neuraminidase to remove sialic acid residues in ZP glycoproteins before the collection of ZPs (C).   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.

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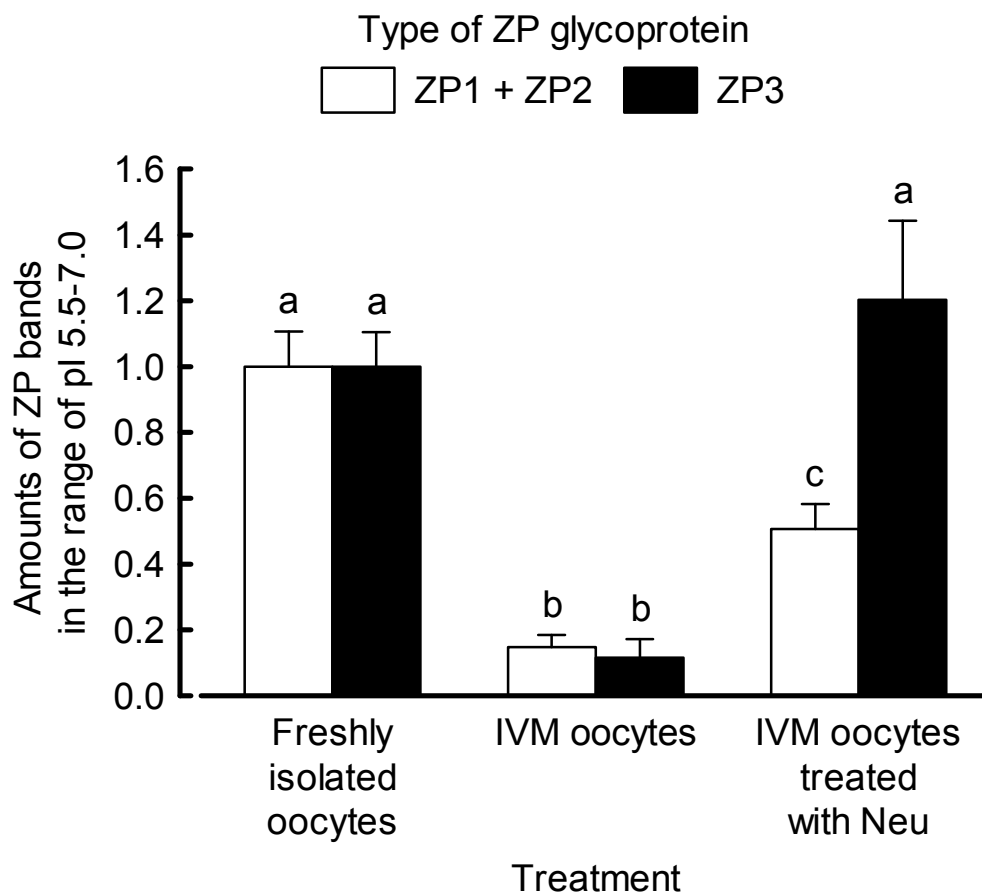
Figure 17.   Changes in amounts of ZP1 + ZP2 and ZP3 glycoproteins (in the range of pI 5.5-7.0) of porcine COCs subjected to 2D gel electrophoresis under reducing conditions.   After 44 h of IVM, some oocytes were treated with 1 IU/ml of neuraminidase (Neu) before collection of ZPs. Values are expressed 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).
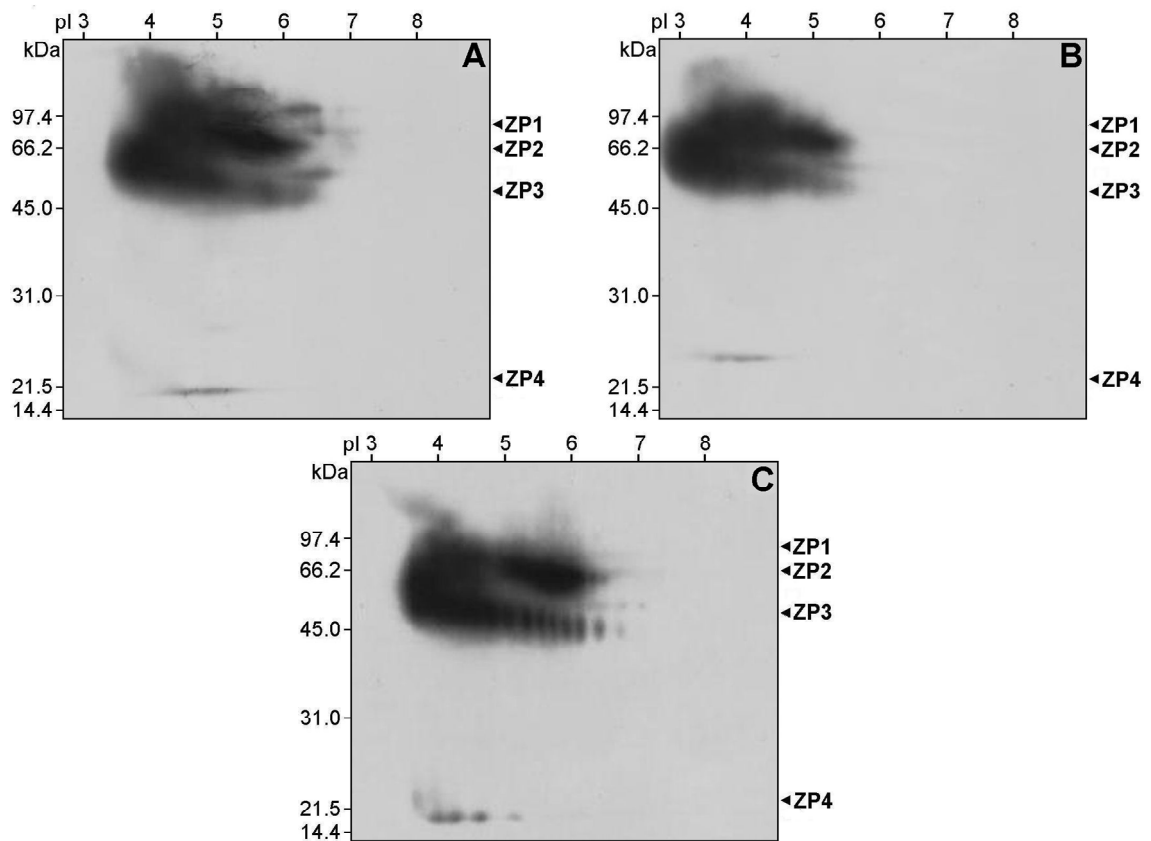
Figure 18. Two-dimensional (2D) gel electrophoresis of ZP glycoproteins of porcine DOs analyzed by biotinylation and ECL in oocytes freshly isolated from follicles (A) and after IVM for 44 h (B).   After the IVM, some oocytes were treated with 1 IU/ml of neuraminidase to remove sialic acid residues in ZP glycoproteins before the collection of ZPs (C).   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.
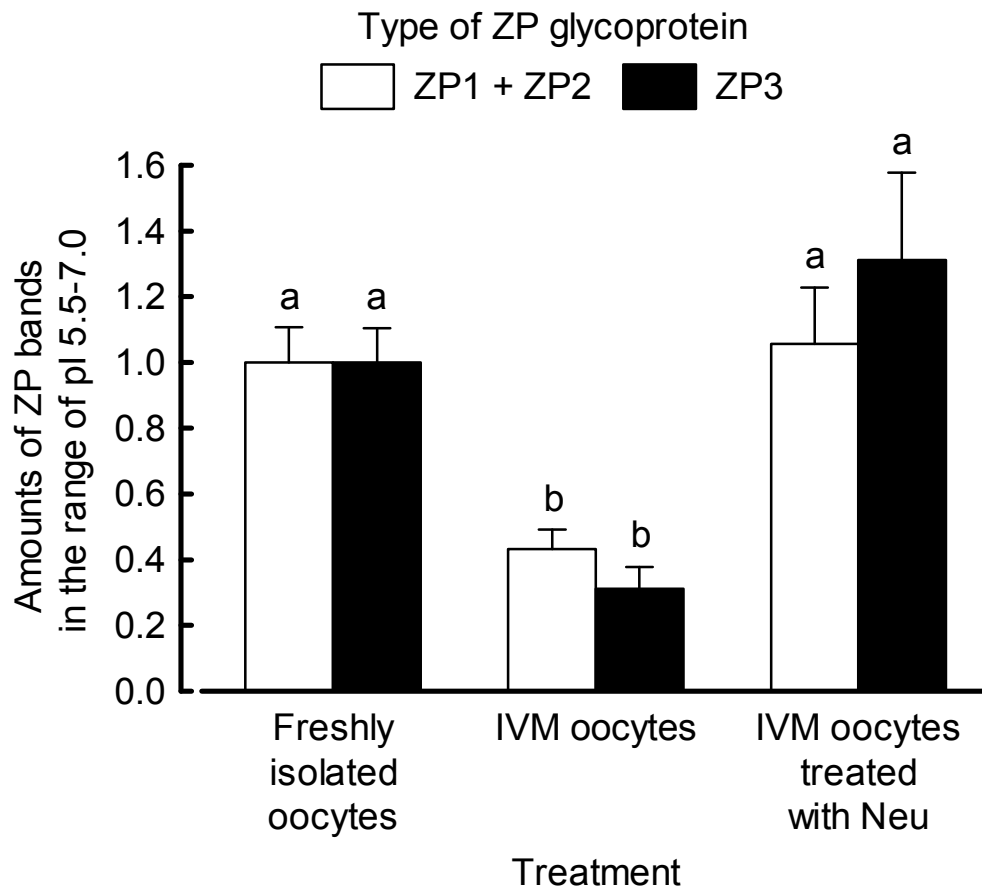
Figure 19. Changes in amounts of ZP1 + ZP2 and ZP3 glycoproteins (in the range of pI 5.5-7.0) of porcine DOs subjected to 2D gel electrophoresis under reducing conditions. After 44 h of IVM, some oocytes were treated with 1 IU/ml of neuraminidase (Neu) before collection of ZPs. Values are expressed as the mean ± SEM. The value for each ZP glycoprotein of freshly isolated oocytes was taken to be 1.0. [a,b] Values with different superscripts within each ZP glycoprotein are significantly different (P<0.05).

Experiment 3:   Effects of sialylation in ZP glycoproteins during oocyte maturation on sperm-ZP interactions and ZP solubility

Porcine COCs and DOs were cultured in IVM medium for various intervals (0, 6, 12, 20, 24, 28, 36 and 44 h).   In COCs, GVBD was found at 20 h following the start of IVM culture.   Up to 28 h of maturation time was required to accomplish GVBD in 74% oocytes, and the proportions of GVBD were not changed at 36, and 44 h of IVM culture periods (73 and 72%, respectively) (Fig. 20).

In DOs, GVBD was observed at 12 h of IVM culture and was accomplished at 24 h of IVM culture period.   The maturation rate at 36 h of maturation time was reached maximum level (73%) comparable to that shown by oocytes cultured for 44 h (72%, Fig. 21).   Therefore, under our experimental condition, almost oocytes were finished GVBD and maturation at 28 and 36-44 h (COCs), and at 24 and 36-44 h (DOs), respectively, after cultivation, and we use the oocytes cultured for 0, 16, 28, 32 and 44 h (COCs) and 0, 12, 24, 28 and 44 h (DOs) in IVM medium as the oocytes inducing GVBD and accomplishing maturation in the following experiments.

The incidences of sperm penetration, polyspermy, MPN formation and mean number of sperm per penetrated oocyte were increased in untreated oocytes with the progress of the IVM culture period in COCs (Table 12) and DOs (Table 13).   The polyspermy and MPN formation were not observed in oocytes at 0 h IVM culture despite of the absence or presence of treatment with neuraminidase.   However, in COCs, the rates of penetration, polyspermy and mean number of sperm per penetrated oocyte were significantly decreased in oocytes treated with neuraminidase after 28, 32 and 44 h of IVM culture compared with those in untreated oocytes.   Similarly, in DOs, the rates of penetration were significantly decreased in oocytes treated with neuraminidase after 24, 28 and 44 h of IVM, and polyspermy were significantly decreased in oocytes treated with neuraminidase after 28 and 44 h of IVM compared with those in untreated oocytes (Table 13).   Moreover, the significant reductions in the number of sperm bound to ZPs (Fig. 22) and the percentage of AR induction in sperm bound to ZPs (Fig. 23) by treatment with neuraminidase were also observed in COCs cultured for 28, 32 and 44 h and DOs cultured

for 24, 28 and 44 h (P<0.05).　However, MPN formation rate (Tables 12 and 13) and the ZP dissolution time in 0.1% protease (Fig. 24) were not altered by treatment with neuraminidase, and the pronase resistance of ZPs decreased with the progress of IVM in all treatment groups (P<0.05).
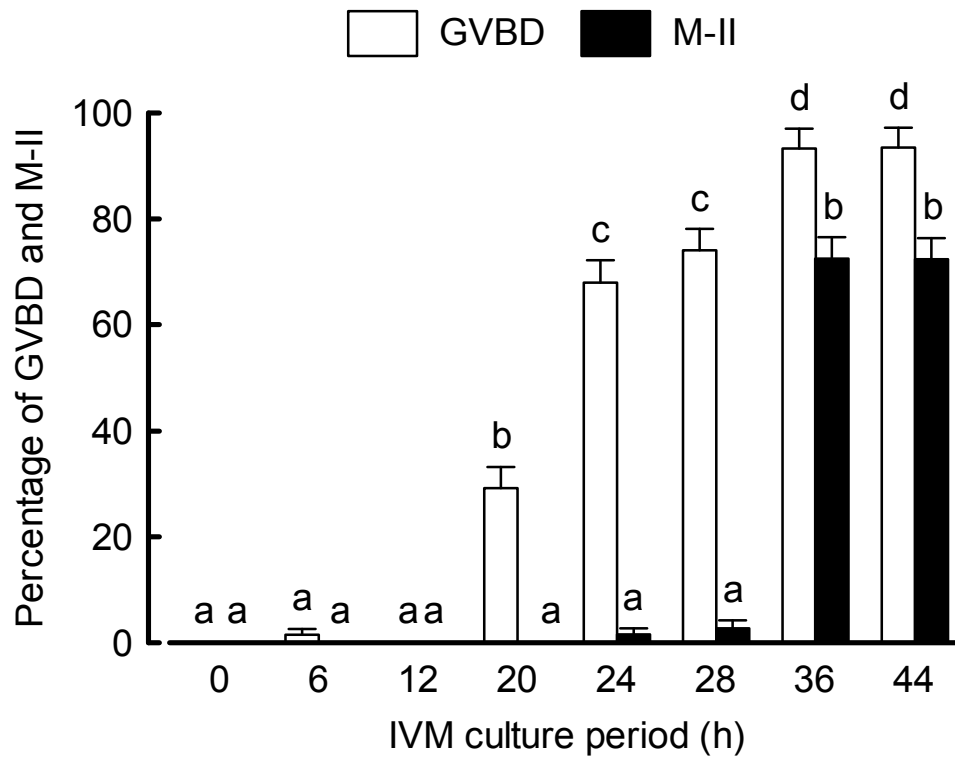
Figure 20. Time sequence of GVBD and maturation of porcine COCs cultured in IVM medium. Values are expressed as the mean ± SEM (n = 112-131 oocytes individually to examine at each time point). [a-d] Values with different superscripts within each stage are significantly different (P<0.05). GVBD = germinal vesicle breakdown, M-II = metaphase II.
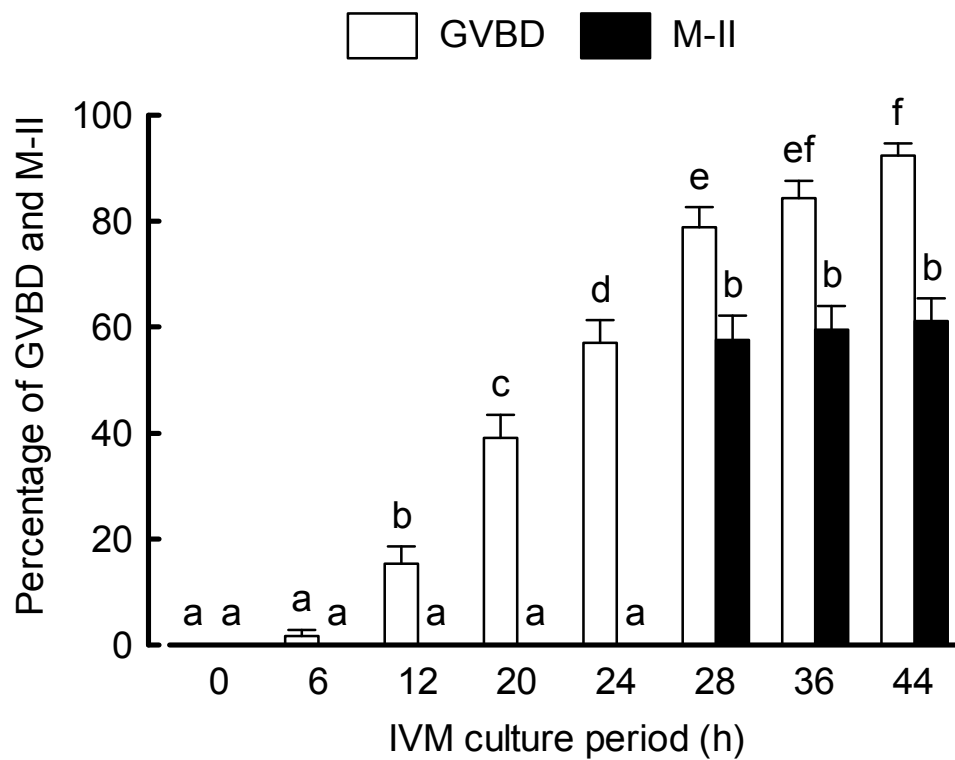
Figure 21. Time sequence of GVBD and maturation of porcine DOs cultured in IVM medium. Values are expressed as the mean ± SEM ($n =$ 102-135 oocytes individually to examine at each time point). [a-f] Values with different superscripts within each stage are significantly different (P<0.05). GVBD = germinal vesicle breakdown, M-II = metaphase II.

Table 12. Effect of treatment with neuraminidase (Neu) in porcine COCs matured for various culture periods 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 [1]	Male pronucleus [1]	
None (control)	0	120	16.7 ± 3.4 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.0 ± 0.0 [a]
	16	130	32.3 ± 4.1 [b]	2.4 ± 2.4 [a]	4.8 ± 3.3 [a]	1.0 ± 0.0 [a]
	28	148	48.7 ± 4.1 [c]	26.4 ± 5.2 [b]	37.5 ± 5.7 [b]	1.4 ± 0.1 [b]
	32	122	59.8 ± 4.4 [cd]	49.3 ± 5.9 [c]	50.7 ± 5.9 [bc]	1.9 ± 0.1 [c]
	44	136	69.9 ± 3.9 [d]	62.1 ± 5.0 [c]	65.3 ± 4.9 [c]	1.8 ± 0.1 [c]
1 IU/ml Neu	0	151	9.3 ± 2.4 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.0 ± 0.0 [a]
	16	130	26.9 ± 3.9 [b]	2.9 ± 2.8 [a]	2.9 ± 2.8 [a]	1.0 ± 0.0 [a]
	28	123	32.5 ± 4.2 [b, *]	5.0 ± 3.5 [a, *]	22.5 ± 6.6 [ab]	1.1 ± 0.0 [a, *]
	32	132	27.3 ± 3.9 [b, *]	5.6 ± 3.8 [a, *]	33.3 ± 7.9 [b]	1.1 ± 0.1 [a, *]
	44	122	50.8 ± 4.5 [c, *]	45.2 ± 6.3 [b, *]	66.0 ± 6.0 [c]	1.5 ± 0.1 [b, *]

[1] Percentage of oocytes that were penetrated.

[a-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).

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Table 13. Effect of treatment with neuraminidase (Neu) in porcine DOs matured for various culture periods 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 [1]	Male pronucleus [1]	
None (control)	0	120	15.8 ± 3.3 [a]	0.0 ± 0.0 [ab]	0.0 ± 0.0 [ab]	1.0 ± 0.0 [a]
	12	118	36.4 ± 4.4 [b]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.0 ± 0.0 [a]
	24	139	50.4 ± 4.2 [bc]	17.1 ± 4.5 [bc]	15.7 ± 4.4 [b]	1.2 ± 0.1 [a]
	28	142	46.5 ± 4.2 [b]	27.3 ± 5.5 [c]	36.4 ± 5.9 [c]	1.4 ± 0.1 [b]
	44	149	61.7 ± 4.0 [c]	59.8 ± 5.1 [d]	48.9 ± 5.2 [c]	2.1 ± 0.1 [c]
1 IU/ml Neu	0	149	9.4 ± 2.4 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.0 ± 0.0 [a]
	12	109	30.3 ± 4.4 [b]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.0 ± 0.0 [a]
	24	139	33.1 ± 4.0 [b, *]	6.5 ± 3.6 [a]	8.7 ± 4.2 [ab]	1.1 ± 0.0 [a]
	28	122	29.5 ± 4.1 [b, *]	5.6 ± 3.8 [a, *]	27.8 ± 7.5 [bc]	1.1 ± 0.0 [a, *]
	44	122	44.3 ± 4.5 [b, *]	35.2 ± 6.5 [b, *]	46.3 ± 6.8 [c]	1.5 ± 0.1 [b]

[1] Percentage of oocytes that were penetrated.

[a-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).

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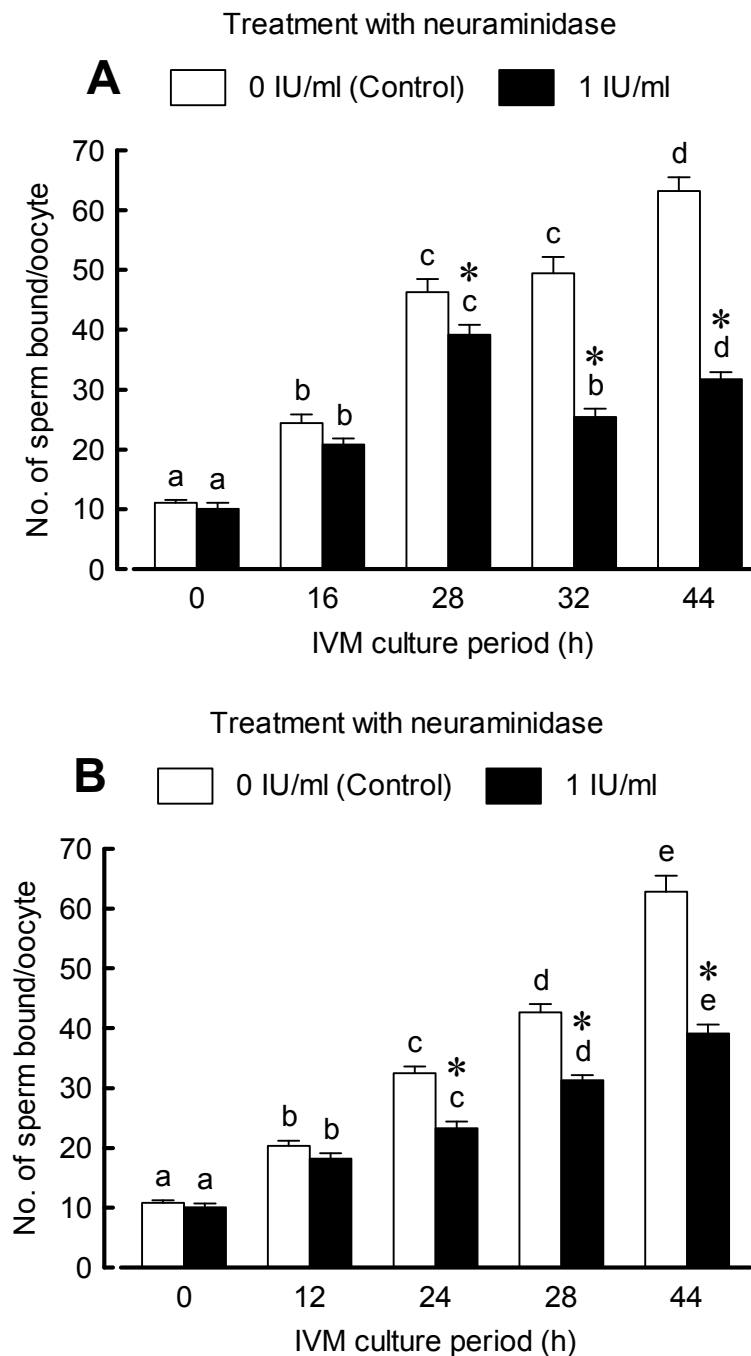
Figure 22. Effect of treatment with 1 IU/ml neuraminidase in porcine COCs (A) and DOs (B) matured for various culture periods on the binding of sperm to ZPs. Treated oocytes were co-incubated with pre-incubated sperm in IVF medium for 2 h. Values are expressed as the mean ± SEM. The total number of oocytes examined was 41-52 for each IVM culture period. [a-e] 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).
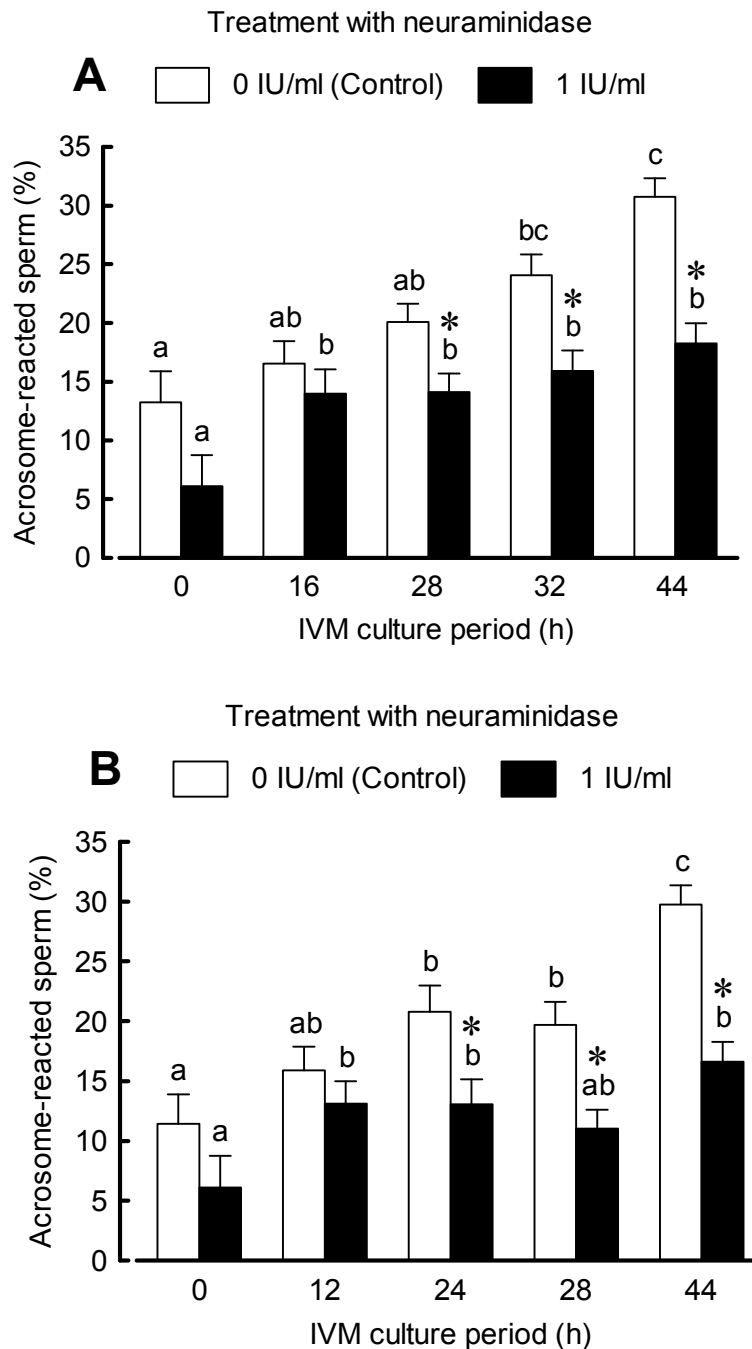
Figure 23. Effect of treatment with 1 IU/ml neuraminidase in porcine COCs (A) and DOs (B) matured for various culture periods on the AR induction in ZP-bound sperm. Treated oocytes were co-incubated with pre-incubated sperm in IVF medium for 2 h. Values are expressed as the mean ± SEM. For each IVM culture period, the total number of live sperm examined was 2931-3225 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).
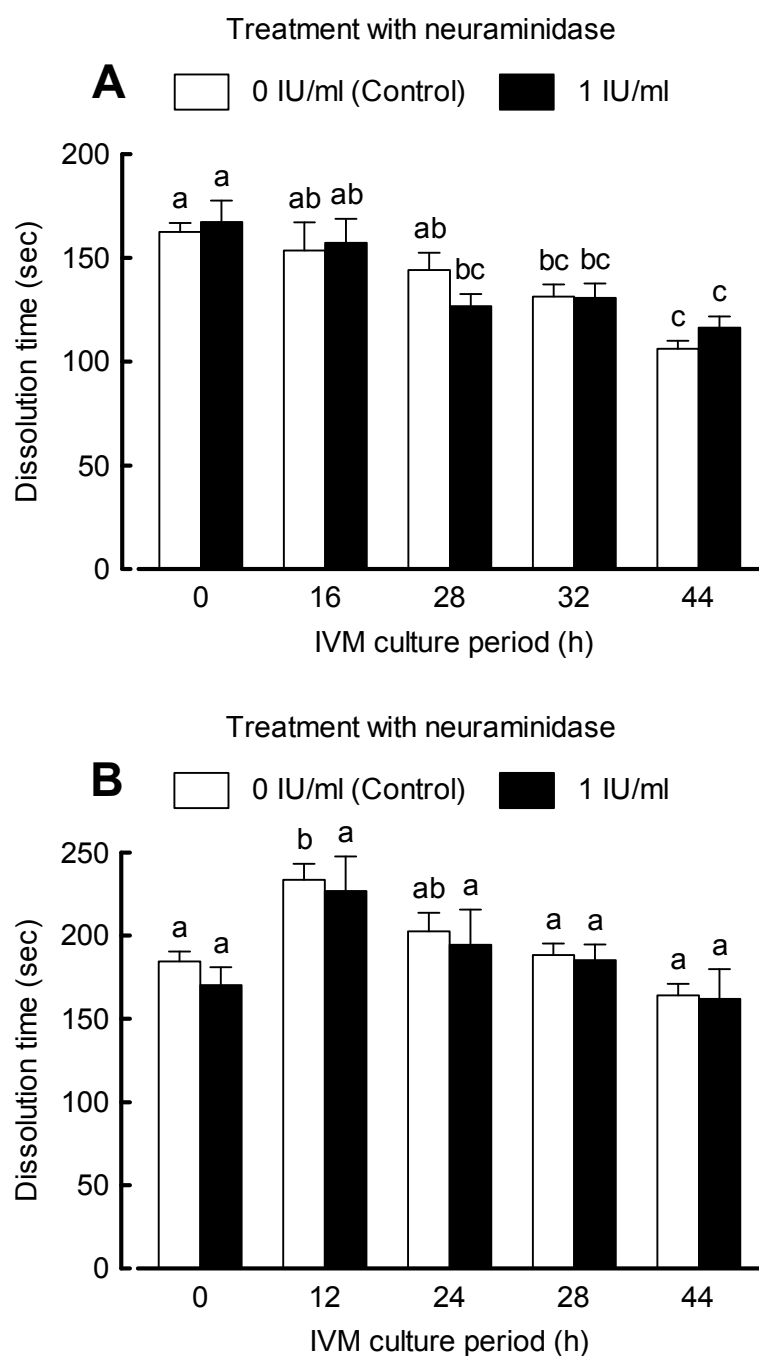
Figure 24. Effect of treatment with 1 IU/ml neuraminidase in porcine COCs (A) and DOs (B) matured for various culture periods on dissolution time of ZPs caused by 0.1% protease. Values are expressed as the mean ± SEM. The total number of oocytes examined was 81-102 for each IVM culture period. [a-c] Values with different superscripts are significantly different within each treatment group (P<0.05).

Experiment 4: **Effects of cumulus cells during oocyte maturation on the sialylation of ZP glycoproteins responsible for sperm-ZP interactions of porcine oocytes**

The sperm penetration rate (Table 14), the number of sperm bound to ZP (Fig. 25) and induction of AR (Fig. 26) were significantly decreased in 44 h of IVM oocytes treated with neuraminidase for 1 h. These interfering effects of treatment with neuraminidase were observed in both types of oocytes derived from COCs and DOs. Although, the increase of degree zona hardening that occurred by oxidation during IVM culture was detected in DOs compared with COCs, neuraminidase treatment did not alter the ZP dissolution time by 0.1% protease action (Fig. 27) in both types of oocytes. These results suggest that the sialylation of ZP glycoproteins during oocyte maturation is not assembly with the cumulus cells.

Table 14. Effect of treatment with neuraminidase (Neu) after the onset of 44-h IVM culture in COCs and DOs on the fertilization parameters.

| Types of oocyte | Treatment | No. of oocytes examined | Oocytes (%; mean ± SEM) | | | No. of sperm per penetrated oocyte (mean ± SEM) |
			Penetrated	Polyspermic [1]	Male pronucleus [1]	
COCs	None (Control)	147	64.6 ± 3.9 [a]	61.1 ± 5.0 [a]	71.6 ± 4.6	2.3 ± 0.2
	1 IU/ml Neu	130	26.9 ± 3.9 [b]	34.3 ± 8.0 [b]	65.7 ± 8.0	1.5 ± 0.2
DOs	None (Control)	145	63.5 ± 4.0 [a]	59.8 ± 5.1 [a]	48.9 ± 5.2	2.1 ± 0.1
	1 IU/ml Neu	122	44.3 ± 4.5 [b]	35.2 ± 6.5 [b]	46.3 ± 6.8	1.5 ± 0.1

[1] Percentage of oocytes that were penetrated.
[a,b] Values with different superscripts in the same column are significantly different within each type of oocyte ($P<0.05$).

Figure 25. Effect of treatment with 1 IU/ml neuraminidase after the onset of 44-h IVM culture in COCs and DOs on the binding of sperm to ZPs. Treated oocytes were co-incubated with pre-incubated sperm in IVF medium for 2 h. Values are expressed as the mean ± SEM. For each group, the total number of oocytes examined was 35-50 for determination of the number of sperm bound to ZPs. [a,b] Values with different superscripts are significantly different within each oocyte group (P<0.05).
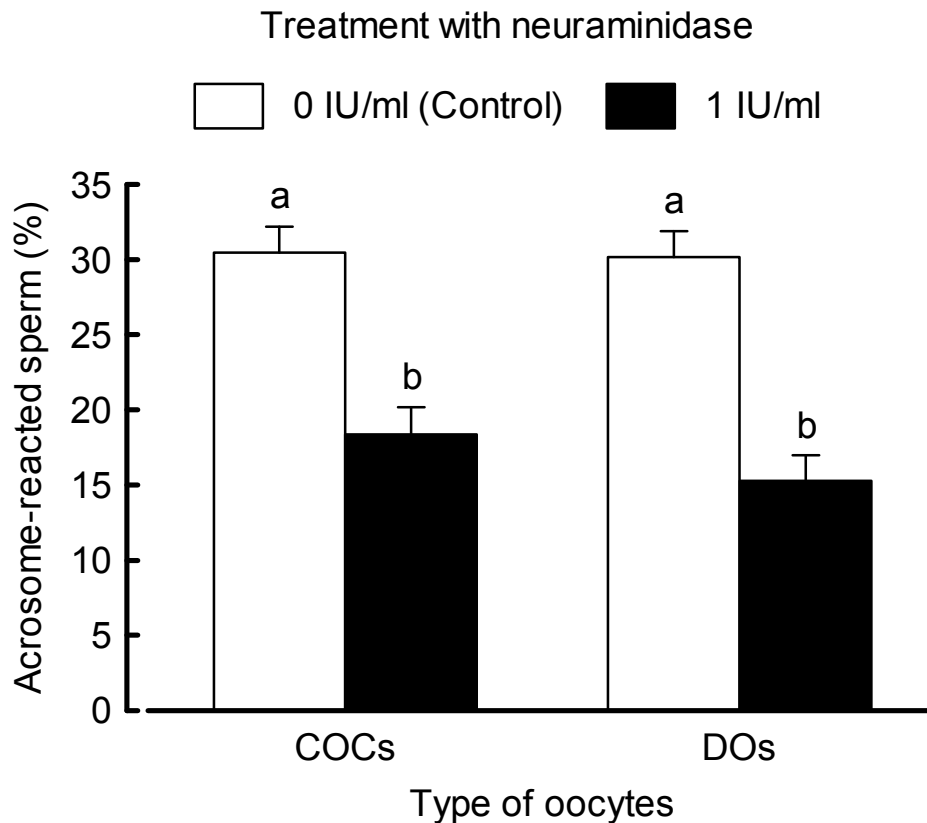
Figure 26. Effect of treatment with 1 IU/ml neuraminidase after the onset of 44-h IVM culture in COCs and DOs on the AR induction in ZP-bound sperm. Treated oocytes were co-incubated with pre-incubated sperm in IVF medium for 2 h. Values are expressed as the mean ± SEM. For each group, the total number of live sperm examined was 1852-2836 for determination of the number of acrosome-reacted sperm. [a,b] Values with different superscripts are significantly different within each oocyte group (P<0.05).
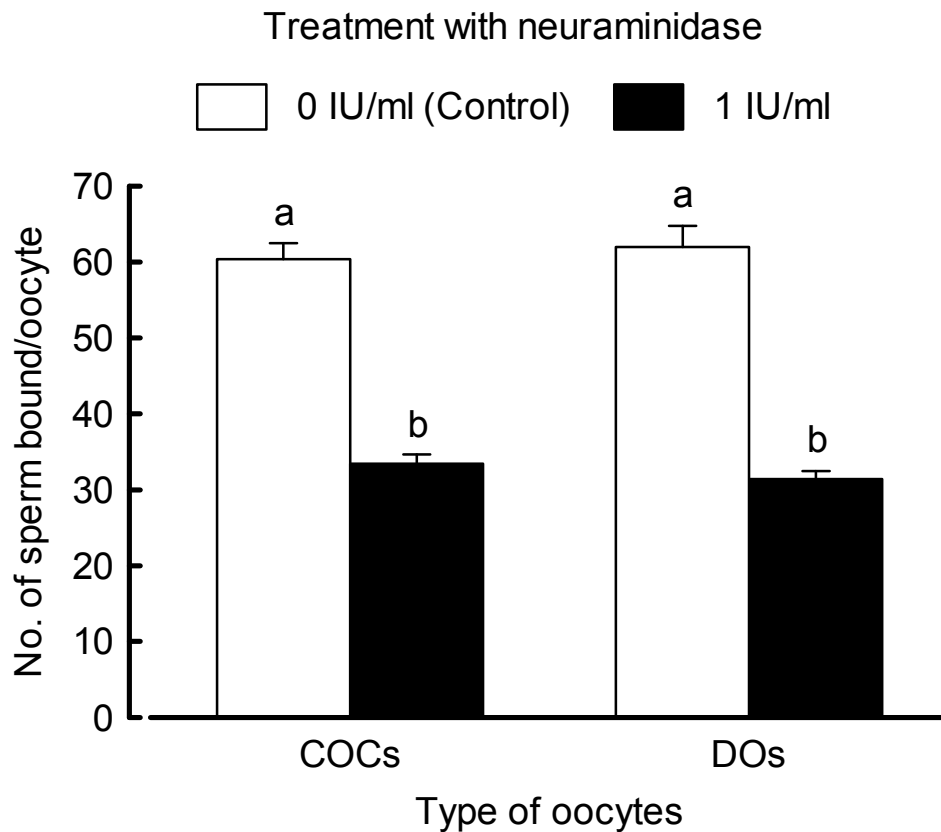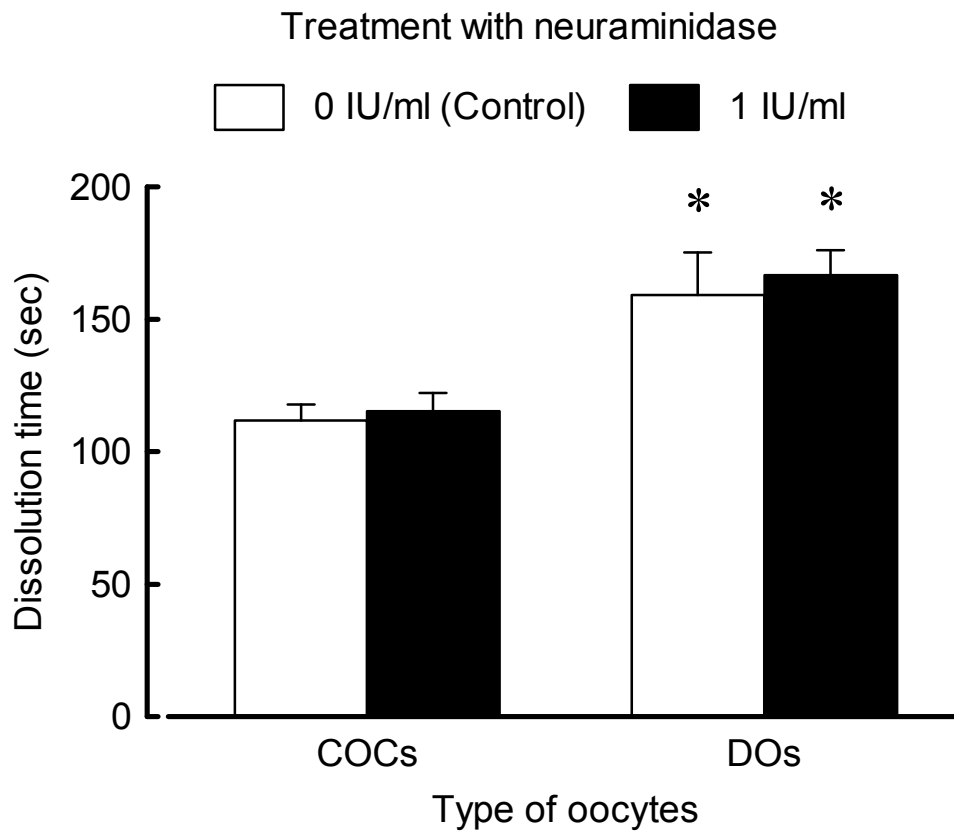
Figure 27. Effect of treatment with 1 IU/ml neuraminidase after the onset of
44-h IVM culture in COCs and DOs on dissolution time of ZPs
caused by 0.1% protease.   Values are expressed as the mean ±
SEM.   The total number of oocytes examined was 85-101 for
each group.   * Values are significantly different between COCs
and DOs in the same treatment (P<0.05).

4.5 Discussion

In the present study, the addition of SSA (recognizing the α-2,6-linked sialic acid) and MAL II (recognizing the α-2,3-linked sialic acid residues) lectins to IVF medium 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; Table 10).   Moreover, these lectins strongly reduced the sperm penetration rates compared to the control group ($P<0.05$; Table 11).   In contrast, Velásquez *et al.* (2007) 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.

The significant increase in sialic acid residues, detected by lectin-blotting with SSA, occurred in oocytes cultured for 44 h in the control IVM medium.   This increase of sialic acid residues was potently removed by treatment with neuraminidase (Fig. 15).   Furthermore, the acidity of ZP glycoproteins significantly increased in COCs and DOs cultured for 44 h compared with immatured oocytes as indicated by 2D gel electrophoresis, which was consistent with the sialylation of ZP glycoproteins during oocyte maturation, because acidification was prevented in the desialylated oocytes (Figs. 16-19).   These findings strongly suggest that porcine ZP glycoproteins undergo the sialic modifications in the final maturation phase of oocytes prior to fertilization, as reported by Rath *et al.* (2005).

The incidences of penetration, polyspermy and mean number of sperm per penetrated oocyte (Tables 12 and 13), the number of sperm bound to ZPs (Fig. 22) and the proportion of AR induction in sperm bound to ZPs (Fig. 23) increased significantly in *in vitro* matured COCs and DOs with the progress of the maturation procedure.   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 COCs after 28, 32 and 44 h, and the desialylated DOs after 24, 28 and 44 h of IVM culture, resulting in a significant reduction in penetration and polyspermy rates. 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 (Velásquez *et al.*, 2007).   Interestingly, under our

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experimental condition, almost COCs and DOs underwent GVBD at 28 and 24 h, respectively (Figs. 20 and 21).   These findings indicate that the sialylation of ZP glycoproteins occurred in accordance with GVBD during oocyte maturation in both COCs and DOs.

Previously, we 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 (Tokeshi *et al.*, 2007).   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 (Storey, 1995; Shur, 1998, Thaler and Cardullo, 2002; Rodeheffer and Shur, 2004) and the modifications of ZP glycans responsible for sperm AR induction are established by glycosylation, sulfation, sialylation and fucosylation during oocyte growth and maturation (Gunaratne, 2007).   As reported by Katsumata *et al.* (1996) and Velásquez *et al.* (2007), neuraminidase released from cortical granules during IVF would participate in blockage of polyspermy by removing sialic acid from the ZP glycoproteins in bovine oocytes.   In contrast, the time required for cortical granule exocytosis is greatly delayed after insemination in porcine oocytes, and the exact mechanism to block polyspermy is still unclear (Wang *et al.*, 1997).

The interfering effects of treatment with neuraminidase on sperm penetration (Table 14), sperm binding to ZP (Fig. 25) and AR induction (Fig. 26) were found in both types of oocytes derived from COCs and DOs after 44 h of maturation culture, and there is no significant difference between COCs and DOs.   These findings indicate that the cumulus cells-oocyte connection during oocyte maturation has no effect on sialylation of ZP glycoproteins relating to sperm-ZP interactions.   It seems that the sialylation of ZP glycoproteins during oocyte maturation would be affected by the oocytes themselves or some factors from ZP.   Although the ZP dissolution time did not alter by neuraminidase treatment in COCs and DOs (Fig. 24), the ZP dissolution time of DOs is longer than that of COCs after 44 h of IVM culture regardless of treatment with neuraminidase (Fig. 27).   The lower incidence of sperm penetration and the lower level of the acidic mobility shift elicited by sialylation during IVM in DOs (Table 13 and Fig. 19) than COCs may be due to the zona hardening caused by oxidation of ZP glycoproteins

during IVM culture.

To our knowledge, the present study is the first to prove that the sialylation of ZP glycoproteins during oocyte maturation contributed to the porcine ZPs acquiring the capacity to accept sperm. 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 (Chapters 2 and 3). We have confirmed that treatment with neuraminidase does not inhibit the increase in terminal GlcNAc residues on the ZP by detecting lectin-blotting with S-WGA (unpublished data). Moreover, the desialylation did not protect against the proteolytic modification of the ZP matrix before fertilization (Fig. 24).

In summary, the following findings were made in the present study. 1) Porcine ZP glycoproteins undergo sialylation without the requirement of cumulus cells-oocyte connection 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 were suppressed in oocytes treated with neuraminidase, resulting in a significant reduction in the sperm penetration and polyspermy rates. 3) The lower incidences of sperm penetration and polyspermy elicited by treatment with neuraminidase were not due to protective proteolytic modifications of the ZP matrix before fertilization. It is concluded that the sialylation of ZP glycoproteins during porcine oocyte maturation is indispensable for the acquisition of sperm-ZP interactions, responsible for sperm penetration, sperm binding to the ZP and induction of the AR in sperm bound to the ZP. In addition, the sialylation of ZP glycoproteins occurred in accordance with GVBD, and the cumulus cells during oocyte maturation would have a very small role in the sialylation of ZP glycoproteins responsible for sperm-ZP interactions.

Chapter 5

Sulfation of zona glycoproteins during meiotic maturation is involved in sperm-zona pellucida interactions of porcine oocytes

5.1 Abstract

Evidence is accumulating that the porcine ZP undergoes biochemical changes during the final phase of maturation prior to fertilization. In this chapter, we conducted to investigate whether the sulfation of ZP glycoproteins during oocyte maturation had influences upon sperm-ZP interactions. Two-dimensional (2D) gel electrophoresis clearly demonstrated that ZP acidification was in good agreement with the sulfation of ZP glycoproteins in oocytes matured for 44 h. The sperm penetration, polyspermy, the number of sperm bound to ZPs and the AR induction in ZP-bound sperm were gradually increased with the progress of the IVM culture period in untreated oocytes ($P<0.05$). The blocking of ZP sulfation by $NaClO_3$ treatment during IVM in COCs and DOs markedly abolished 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. The results support the hypothesis that sulfation in ZP glycoproteins during oocyte maturation is critically important in regulating the fundamental steps of sperm-ZP interactions. Moreover, sulfation of ZP glycoproteins was not associated with a protective proteolytic modification of the ZP matrix before fertilization. Surprisingly, the cumulus cells during maturation were not correlated with the sulfation of ZP glycoproteins incorporated in sperm-ZP interactions, and the time course of this sulfation in ZP glycoproteins was related to the induction of GVBD during oocyte maturation.

5.2 Introduction

All mammalian eggs are surrounded by a relatively thick, insoluble extracellular coat called the ZP (Wassarman, 1988; 1993; Yanagimachi, 1994). During fertilization, the ZP mediates species-selective recognition between the oocyte and spermatozoon (Töpfer-Petersen, 1999). There is evidence that the sperm-ZP interaction is a carbohydrate-mediated event (Storey, 1995; Shur, 1998; Thaler and Cardullo, 2002; Rodeheffer and Shur, 2004) that triggers a signal transduction pathway that results in the fenestration and fusion of the sperm plasma membrane and the outer acrosomal membrane (AR, Yanagimachi, 1994).

We have previously confirmed that *N*-glycosylation for periods in excess of 20-24 h after meiotic maturation played a critical role in sperm-ZP interactions (Chapter 2). 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. Moreover, the sialylation in ZP glycoproteins during oocyte maturation is critical for sperm-ZP interactions (Chapter 4). 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 matured oocytes compared with immatured oocytes as indicated by an acidic shift of pI units in 2D gel electrophoresis elicited by increases in the sulfate of ZP glycoproteins (Rath *et al.*, 2005; 2006; von Witzendorff *et al.*, 2009). Porcine ZP3 glycoproteins are highly heterogeneous mainly due to differences in the amount of sulfated *N*-acetyllactosamine and sialic acid in the acidic chains (Nakano *et al.*, 1990) and have been shown to be required for the binding of sperm to the intact ZP leading to completion of AR (Yoshizawa *et al.*, 1994). It is reported that the binding of sperm to ZPs and the partial activation of proacrosin to form α- and β-acrosin in porcine (Lo Leggio *et al.*, 1994), mouse (Howes and Jones, 2002) and bovine (Velásquez. *et al.*, 2007) oocytes are mediated by the sialic acid and sulfate of ZP glycoproteins. In contrast, neither the sulfate nor sialic acid present on mouse ZP3 oligosaccharides is directly involved in sperm receptor or AR-inducing activity (Liu *et al.*, 1997). In addition, the porcine oocyte maturational competence appear to be more related to immatured COCs characteristics than to the type of cumulus behavior during culture (Alvarez *et al.*, 2009). The role of cumulus cells

during maturation in the ZP modification such as sulfation of ZP glycoproteins is still under debate, and the role of sulfation of ZP glycoproteins during IVM in sperm-ZP interactions of porcine oocytes is still unclear.

Therefore, in the present study, experiments were undertaken by using COCs and DOs 1) to examine whether porcine ZP glycoproteins are acidified in parallel to sulfation during oocyte maturation, 2) to evaluate the effects of sulfation of ZP glycoproteins during IVM on fertilization responses including sperm penetration, polyspermy, sperm binding to ZPs, induction of AR in ZP-bound sperm and ZP hardness, and 3) to examine the effect of cumulus cells during oocyte maturation on the sulfation of ZP glycoproteins involved in sperm-ZP interactions.

5.3 Materials and Methods

5.3.1 Collection of oocytes and *in vitro* maturation

Oocytes were collected by use of methods described in Chapter 2. COCs were cultured in IVM medium for 0, 16, 28, 32 and 44 h, and DOs were cultured in IVM medium for 0, 12, 24, 28 and 44 h. COCs and DOs were employed for various experiments to determine sperm-ZP interactions associated with meiotic progression.

5.3.2 Blocking of sulfation residues

To block the sulfation of ZP glycoproteins, oocytes were cultured in IVM medium supplemented with 50 mM sodium chlorate ($NaClO_3$), which acts as a sulfation inhibitor of carbohydrate.

5.3.3 Assessment of ZP modifications by two-dimensional gel electrophoresis

The 2 D gel electrophoresis was subjected to the methods described in Chapter 4.

5.3.4 *In vitro* fertilization

IVF was carried out by use of methods described in Chapter 2.

5.3.5 Sperm-ZP binding

At 2 h after sperm insemination, the number of sperm tightly bound to the ZP was counted under a fluorescence microscope as described in Chapter 2.

5.3.6 Assessment of *in vitro* nuclear maturation and fertilization

The nuclear status after IVM and fertilization parameters after IVF were examined as described in Chapter 2.

5.3.7 Assessment of ZP solubility

After washing three times in H-TL-PVA, 15-20 cumulus-free oocytes treated with or without $NaClO_3$ were transferred into a 50-μl drop of H-TL-PVA containing 0.1% (w/v) protease to assess the dissolution time.   The dissolution times of ZP caused by 0.1% (w/v) protease action were observed by use of methods described in Chapter 2.

5.3.8 Evaluation of sperm acrosomal status

The induction of AR in sperm bound to ZP was assayed by use of methods described in Chapter 2.

5.3.9 Experimental design

In Experiment 1, the optimal concentration of $NaClO_3$ supplemented in IVM culture

during oocyte maturation was evaluated. The effect of various concentrations (0-75 mM) of NaClO$_3$ supplemented in IVM medium for a total of 44 h of culture periods on meiotic maturation of porcine COCs was assessed.

In Experiment 2, the acidification of ZP glycoproteins during oocyte maturation was examined by 2D gel electrophoresis for ZPs of oocytes freshly isolated from their follicles and COCs and DOs cultured for 44 h in the absence or presence of NaClO$_3$.

In Experiment 3, the effects of the sulfation of ZP glycoproteins during oocyte maturation on subsequent sperm-ZP interactions were examined in porcine COCs and DOs. After 0, 16, 28, 32 and 44 h (COCs), and 0, 12, 24, 28 and 44 h (DOs) of IVM, a large portion of oocytes treated with NaClO$_3$ were inseminated with pre-incubated 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 blocked sulfation during IVM were co-incubated with pre-incubated sperm in IVF medium containing 50 mM NaClO$_3$ with attention paid to the blocking of sulfation during the IVF procedure.

Experiment 4 investigated the effects of cumulus cells during oocyte maturation on the sulfation of ZP glycoproteins resemble for sperm-ZP interactions of porcine oocytes.

5.3.10 Statistical analysis

Statistical analysis was performed as described in Chapter 2.

5.4 Results

Experiment 1: Effect of various concentrations of NaClO$_3$ added to IVM culture on meiotic maturation

When porcine COCs were cultured for 44 h in the absence or presence of NaClO$_3$ (0-75 mM), the treatment with 25 and 50 mM of NaClO$_3$ had no effect on oocyte maturation (Fig. 28)

compared with control.　However, the meiotic maturation rate decreased in oocytes treated with 75 mM of $NaClO_3$ compared to that of control.　There is no significant different among 0, 25 and 50 mM of $NaClO_3$ concentration.　Therefore, the optimal concentration of $NaClO_3$ we used was 50 mM and the next experiment used 50 mM $NaClO_3$ to inhibit the sulfation of ZP glycoproteins during oocyte maturation.
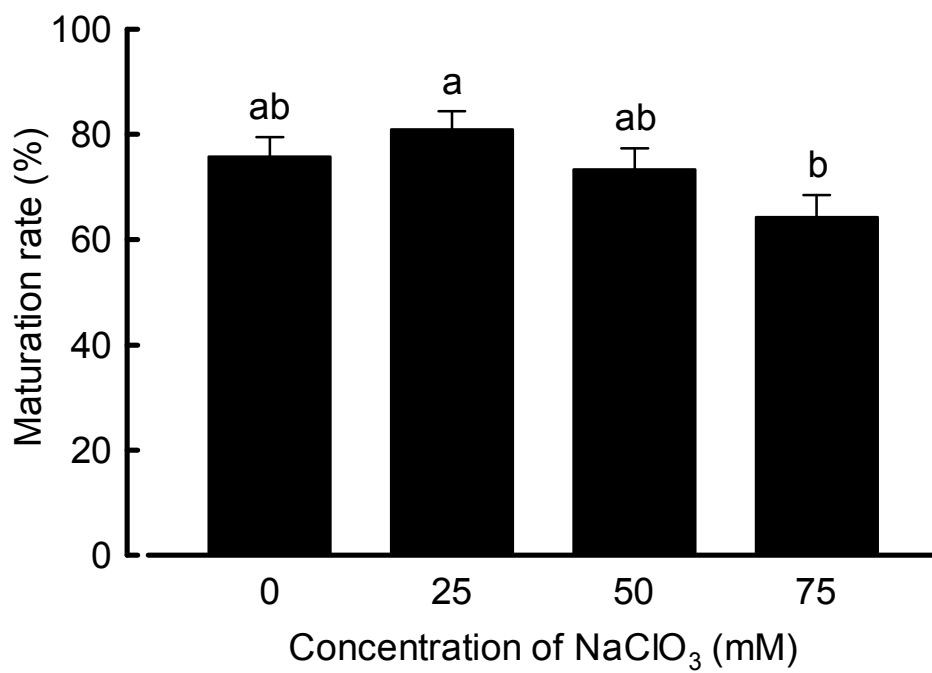
Figure 28. Effect of various concentrations of $NaClO_3$ added to IVM culture on meiotic maturation. Values are expressed as the mean ± SEM. The total number of oocytes examined in each group were about 150. [a,b] Values with different superscripts are significantly different (P<0.05).

Experiment 2: Acidification according to sulfation of ZP glycoproteins during
oocyte maturation

The ZP occurred biochemical changes such as acidification during oocyte maturation process as indicated by shift in mobility of ZP1 + ZP2 and ZP3 glycoproteins and a slight change to the ZP4 glycoprotein in COCs and DOs (Figs. 29 and 32).   The intensity of the ZP1 + ZP2 and ZP3 bands at a pI of 5.5-7.0 in oocytes cultured for 44 h in IVM medium declined dramatically to 0.2 ± 0.1 and 0.1 ± 0.1, respectively, compared with those in oocytes freshly isolated from follicles (1.0 ± 0.1 and 1.0 ± 0.1, respectively, P<0.05; Fig. 30).   However, the blocking of ZP sulfation significantly inhibited the mobility shift in IVM oocytes, and the intensity of the ZP1 + ZP2 and ZP3 bands (0.7 ± 0.1 and 0.6 ± 0.2, respectively) at a pI of 5.5-7.0 was maintained in IVM oocytes treated with $NaClO_3$ (Figs. 29 and 30).   The same result was found in DOs (Figs. 31 and 32), but the intensity of ZP1 + ZP2 and ZP3 bands at a pI of 5.5-7.0 in DOs after 44 h of IVM in the absence of $NaClO_3$ treatment were slightly increased as compared with those in COCs (Figs. 30 and 32).
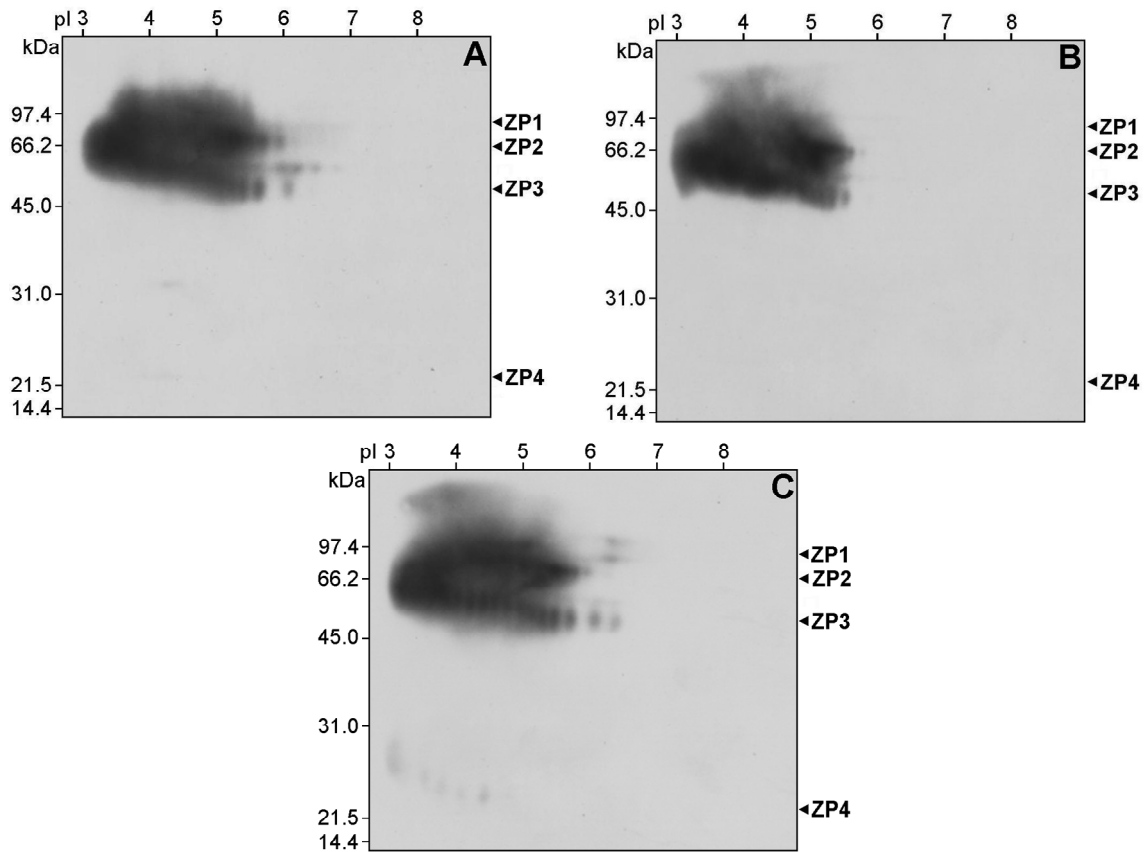
Figure 29. Two-dimensional (2D) gel electrophoresis of porcine ZP glycoproteins of COCs analyzed by biotinylation and ECL in oocytes freshly isolated from follicles (A) and after IVM for 44 h (B).   Oocytes were treated with 50 mM NaClO$_3$ to block ZP sulfation during IVM (C).   Twenty biotinylated 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.
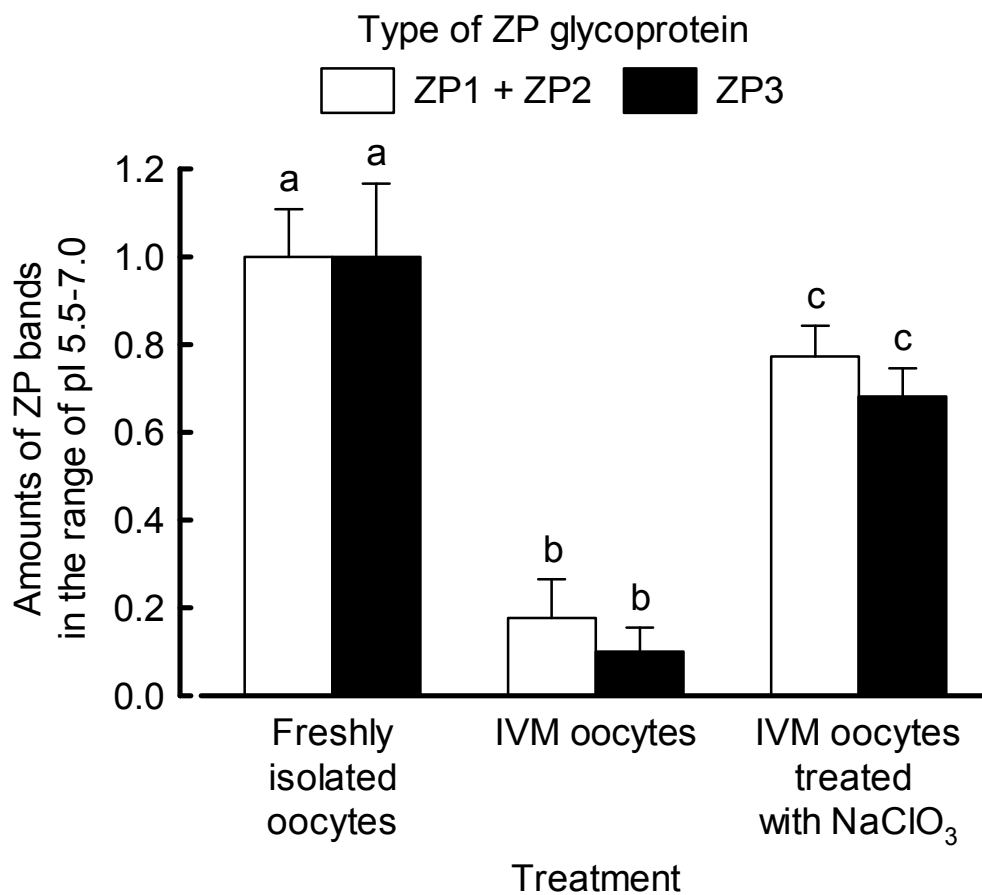
Figure 30. Changes in amounts of ZP1 + ZP2 and ZP3 glycoproteins (in the range of pI 5.5-7.0) of porcine COCs subjected to 2D gel electrophoresis under reducing conditions. Oocytes were treated with 50 mM NaClO$_3$ during IVM. Values are expressed as the mean ± SEM. The value for each ZP glycoprotein 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).

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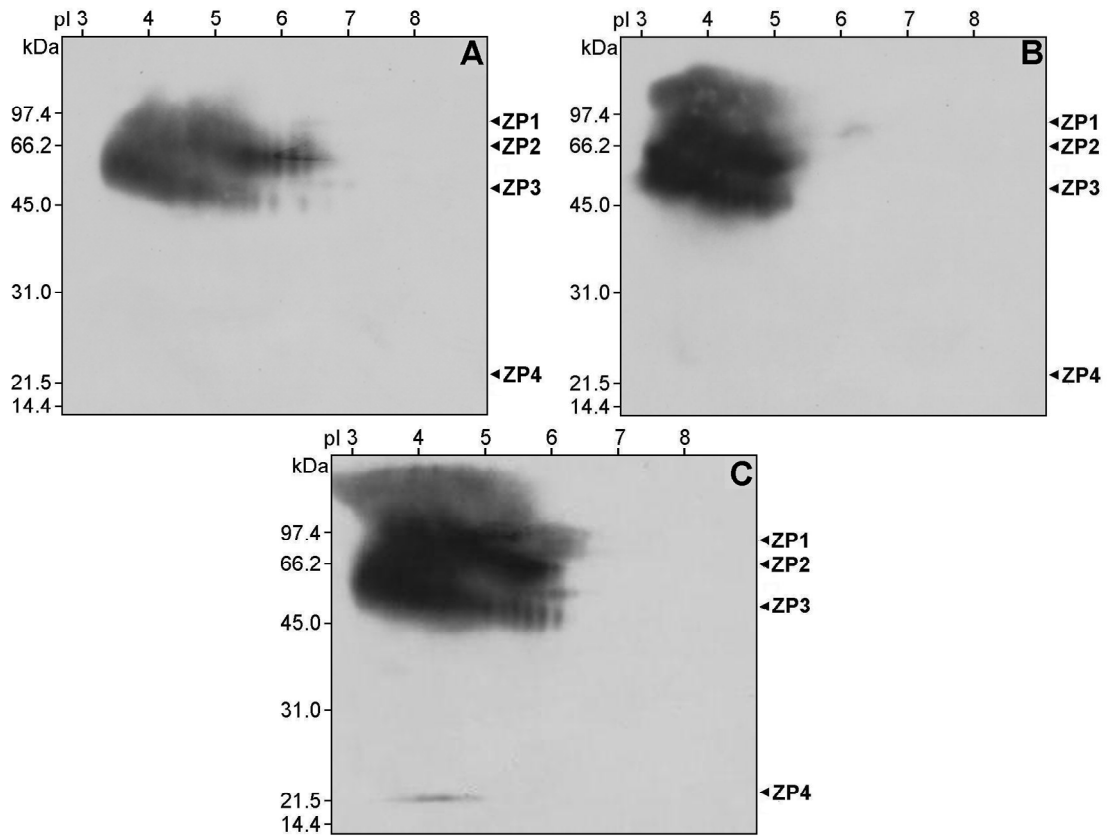
Figure 31. Two-dimensional (2D) gel electrophoresis of porcine ZP glycoproteins of DOs analyzed by biotinylation and ECL in oocytes freshly isolated from follicles (A) and after IVM for 44 h (B).   Oocytes were treated with 50 mM $NaClO_3$ to block ZP sulfation during IVM (C).   Twenty biotinylated ZPs 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.
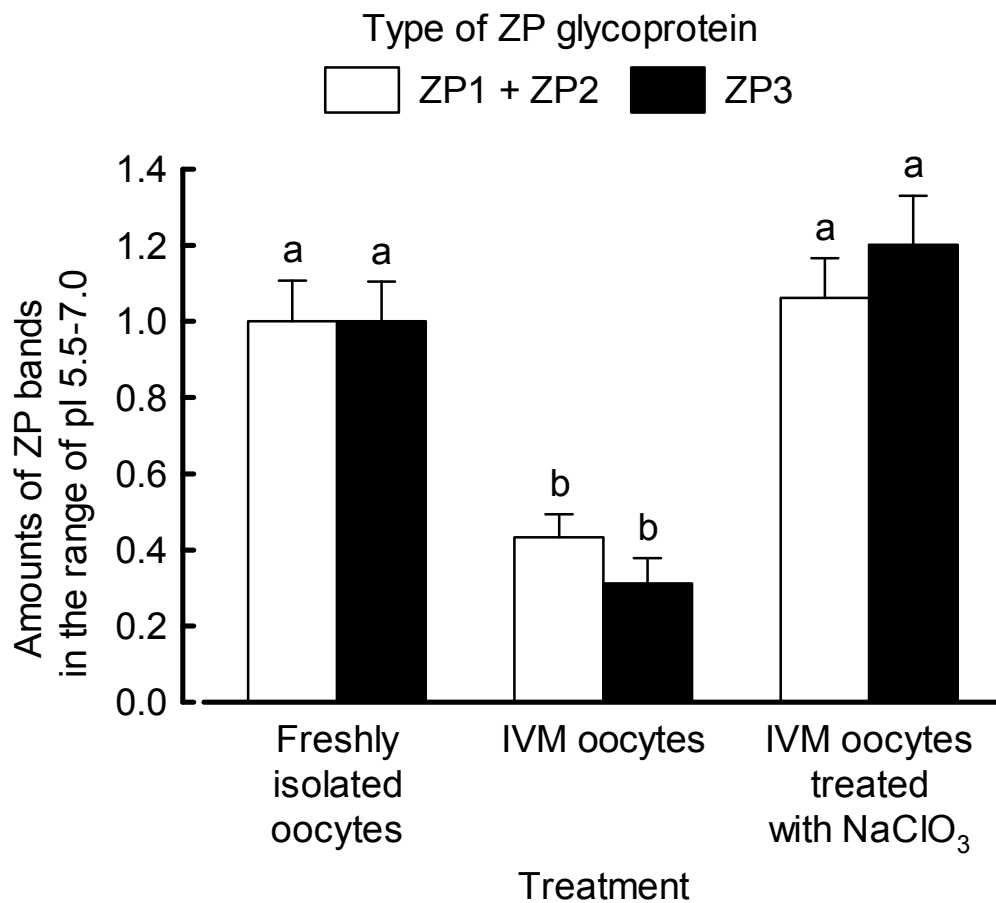
Figure 32. Changes in amounts of ZP1 + ZP2 and ZP3 glycoproteins (in the range of pI 5.5-7.0) of porcine DOs subjected to 2D gel electrophoresis under reducing conditions.   Oocytes were treated with 50 mM $NaClO_3$ during IVM. Values are expressed as the mean ± SEM.   The value for each ZP glycoprotein of freshly isolated oocytes was taken to be 1.0.   [a,b] Values with different superscripts within each ZP glycoprotein are significantly different ($P<0.05$).

Experiment 3: **Effects of sulfation in ZP glycoproteins during oocyte maturation on sperm-ZP interactions and ZP solubility**

The rates of sperm penetration, polyspermy and MPN formation of COCs (Table 15) and of DOs (Table 16) were significantly increased in untreated oocytes in advance of IVM culture period (P<0.05). Similarly, the number of sperm bound to ZPs (Fig. 33) and the proportion of AR-inducing sperm (Fig. 34) were significantly increased in untreated oocytes with the progress of the IVM culture period (P<0.05). The number of sperm bound to ZPs and the percentage of AR in sperm bound to ZPs were reduced significantly in COCs blocked ZP sulfation by $NaClO_3$ after 28, 32 and 44 h of IVM culture compared with those in untreated oocytes (P<0.05; Figs. 33A and 34A). The similar results were observed in DOs blocked ZP sulfation by $NaClO_3$ after 24, 28 and 44 h of IVM culture compared with those in untreated oocytes (P<0.05; Figs. 33B and 34B). On the other hand, the blocking of sulfation during oocyte maturation had no inhibitory effect on the progressive increase in the penetration rate in both types oocytes of COCs and DOs. Nonetheless, the addition of $NaClO_3$ to IVM medium for 28, 32 and 44 h (COCs) and for 24, 28 and 44 h (DOs) to block the sulfation of ZP glycoproteins significantly reduced the incidence of polyspermy compared with the control (P<0.05; Tables 15 and 16). However, the ZP dissolution time in 0.1% protease was not altered by treatment with $NaClO_3$ and significantly decreased with the progress of IVM in all treatment groups in both COCs and DOs (P<0.05; Fig. 35).

Table 15. Effect of treatment with 50 mM $NaClO_3$ in porcine COCs matured for various culture periods 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 [1]	Male pronucleus [1]	
None (control)	0	120	16.7 ± 3.4 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.0 ± 0.0 [a]
	16	130	32.3 ± 4.1 [b]	2.4 ± 2.4 [a]	4.8 ± 3.3 [a]	1.0 ± 0.0 [a]
	28	148	48.7 ± 4.1 [c]	26.4 ± 5.2 [b]	37.5 ± 5.7 [b]	1.4 ± 0.1 [b]
	32	122	59.8 ± 4.4 [cd]	49.3 ± 5.9 [c]	50.7 ± 5.9 [bc]	1.9 ± 0.1 [c]
	44	136	69.9 ± 3.9 [d]	62.1 ± 5.0 [c]	65.3 ± 4.9 [c]	1.8 ± 0.1 [c]
50 mM $NaClO_3$	0	141	18.4 ± 3.3 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.0 ± 0.0 [a]
	16	115	33.9 ± 4.4 [b]	0.0 ± 0.0 [a]	2.6 ± 2.5 [a]	1.0 ± 0.0 [a]
	28	130	38.5 ± 4.3 [b]	10.0 ± 4.2 [a, *]	28.0 ± 6.4 [b]	1.1 ± 0.0 [a, *]
	32	120	58.3 ± 4.5 [c]	20.0 ± 5.9 [b, *]	42.9 ± 5.9 [bc]	1.8 ± 0.2 [b]
	44	127	70.9 ± 4.0 [c]	40.0 ± 5.2 [b, *]	60.0 ± 5.2 [c]	1.6 ± 0.1 [b]

[1] Percentage of oocytes that were penetrated.

[a-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).

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Table 16. Effect of treatment with 50 mM NaClO$_3$ in porcine DOs matured for various culture periods 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 [1]	Male pronucleus [1]	
None (control)	0	120	15.8 ± 3.3 [a]	0.0 ± 0.0 [ab]	0.0 ± 0.0 [ab]	1.0 ± 0.0 [a]
	12	118	36.4 ± 4.4 [b]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.0 ± 0.0 [a]
	24	139	50.4 ± 4.2 [bc]	17.1 ± 4.5 [bc]	15.7 ± 4.4 [b]	1.2 ± 0.1 [a]
	28	142	46.5 ± 4.2 [b]	27.3 ± 5.5 [c]	36.4 ± 5.9 [c]	1.4 ± 0.1 [b]
	44	149	61.7 ± 4.0 [c]	59.8 ± 5.1 [d]	48.9 ± 5.2 [c]	2.1 ± 0.1 [c]
50 mM NaClO$_3$	0	138	18.8 ± 3.3 [a]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.0 ± 0.0 [a]
	12	120	35.8 ± 4.4 [b]	0.0 ± 0.0 [a]	0.0 ± 0.0 [a]	1.0 ± 0.0 [a]
	24	129	48.8 ± 4.4 [bc]	4.8 ± 2.7 [a, *]	6.4 ± 3.1 [a]	1.1 ± 0.1 [a]
	28	127	39.4 ± 4.3 [b]	12.0 ± 4.6 [a, *]	28.0 ± 6.4 [b]	1.1 ± 0.1 [a, *]
	44	127	61.4 ± 4.3 [c]	38.5 ± 5.5 [b, *]	43.6 ± 5.6 [b]	1.5 ± 0.0 [b]

[1] Percentage of oocytes that were penetrated.

[a-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).
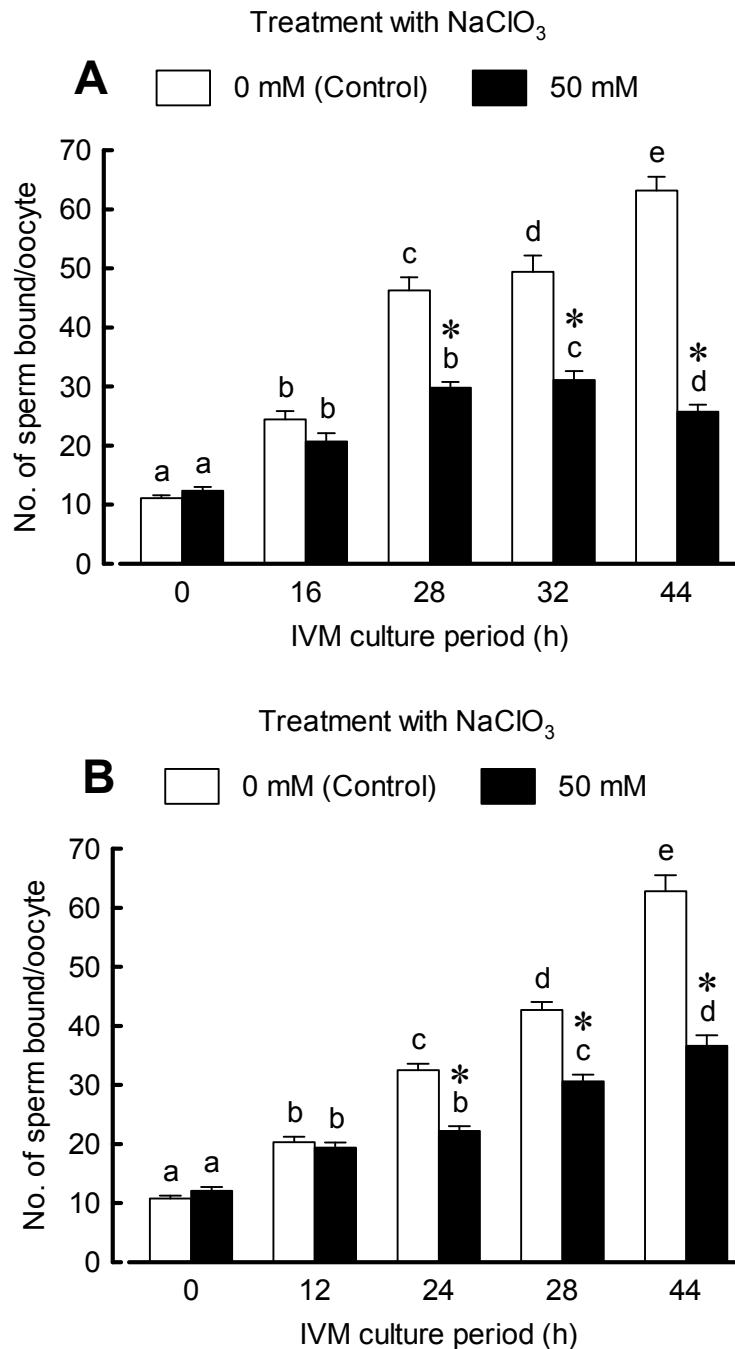
Figure 33. Effect of treatment with 50 mM $NaClO_3$ in porcine COCs (A) and DOs (B) matured for various culture periods on the binding of sperm to ZPs. Treated oocytes were co-incubated with pre-incubated sperm in IVF medium for 2 h. Values are expressed as the mean ± SEM. For each IVM culture period, the total number of oocytes examined was 35-50 for determination of the number of sperm bound to ZPs. [a-e] Values with different superscripts are significantly different within each group at different culture periods ($P<0.05$). * Values are significantly different as compared with the control in the same culture period ($P<0.05$).

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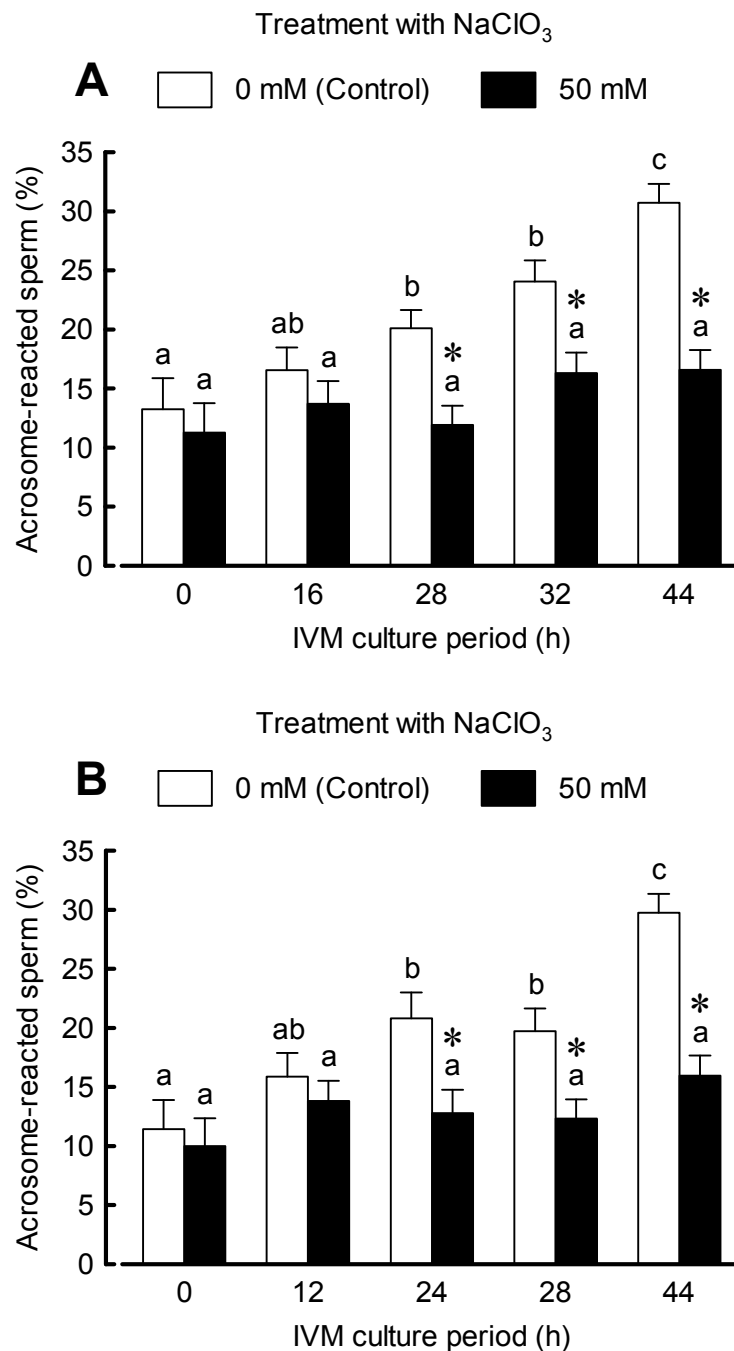
Figure 34. Effect of treatment with 50 mM NaClO$_3$ in porcine COCs (A) and DOs (B) matured for various culture periods on the AR induction in ZP-bound sperm.   Treated oocytes were co-incubated with pre-incubated sperm in IVF medium for 2 h.   Values are expressed as the mean ± SEM.   For each IVM culture period, the total number of live sperm examined was 2852-3384 for determination of the number of acrosome-reacted sperm.   [a-c] Values with different superscripts are significantly different within each group at different culture periods (P<0.05).   * Values are significantly different as compared with the control in the same culture period (P<0.05).

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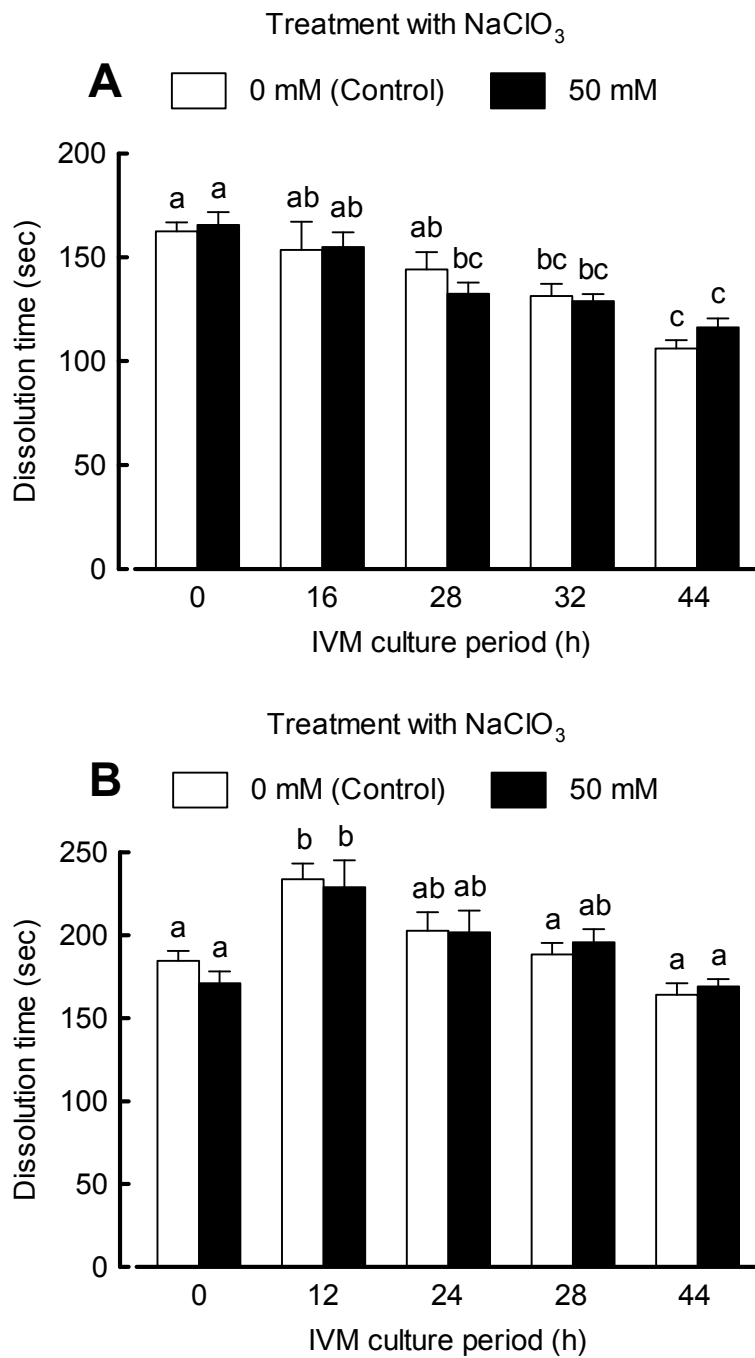
Figure 35. Effect of treatment with 50 mM NaClO$_3$ in porcine COCs (A) and DOs (B) matured for various culture periods on dissolution time of ZPs caused by 0.1% protease. Values are expressed as the mean ± SEM. The total number of oocytes examined was 35-57 for each IVM culture period. [a-c] Values with different superscripts are significantly different within each group at different culture periods (P<0.05).

Experiment 4:   Effects of cumulus cells during oocyte maturation on the sulfation of ZP glycoproteins responsible for sperm-ZP interactions of porcine oocytes

The blocking of ZP sulfation during oocyte maturation for 44 h had no inhibitory effects on the penetration rate, MPN formation and the mean number of sperm per penetrated oocytes in COCs and DOS (Table 17).   Surprisingly, the addition of $NaClO_3$ to IVM medium due to block the sulfation of ZP glycoproteins significantly reduced the incidence of polyspermy in both types of oocytes derived from COCs and DOs compared with the control   ($P<0.05$; Table 17).   The treatment with $NaClO_3$ during 44 h of IVM culture significantly decreased in the number of sperm bound to ZP in COCs (31.9 ± 1.0) and DOs (30.3 ± 0.9) compared with COCs (60.4 ± 2.1) and DOs (62.0 ± 2.8) treated without $NaClO_3$ ($P<0.05$; Fig. 36).   The percentages of acrosome-reacted sperm of COCs and DOs were significantly reduced to 19.7 ± 1.8 and $16.9 \pm 1.7\%$, respectively, by treatment with $NaClO_3$ compared with that of oocytes (30.5 ± 1.7 and $30.2 \pm 1.7\%$, respectively) treated without $NaClO_3$ ($P<0.05$; Fig. 37). However, the ZP dissolution time in 0.1% protease was not altered by treatment with $NaClO_3$ and significantly decreased with the progress of IVM in all treatment groups in both COCs and DOs ($P<0.05$; Fig. 38).   The ZP dissolution time in DOs is longer than that in COCs, indicating that ZP hardening caused by oxidation of ZP glycoproteins during IVM culture. These results suggest that the sulfation of ZP glycoproteins during oocyte maturation is not associated with the cumulus cells.

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Table 17. Effect of treatment with 50 mM NaClO$_3$ during 44-h IVM culture in COCs and DOs on the fertilization parameters.

Types of oocyte	Treatment	No. of oocytes examined	Oocytes (%; mean ± SEM)			No. of sperm per penetrated oocyte (mean ± SEM)
			Penetrated	Polyspermic [1]	Male pronucleus [1]	
COCs	None (Control)	147	64.6 ± 3.9	61.1 ± 5.0 [a]	71.6 ± 4.6	2.3 ± 0.2
	50 mM NaClO$_3$	132	68.2 ± 3.9	34.4 ± 8.0 [b]	60.0 ± 5.2	1.6 ± 0.1
DOs	None (Control)	145	63.5 ± 4.0	59.8 ± 5.1 [a]	48.9 ± 5.2	2.1 ± 0.1
	50 mM NaClO$_3$	127	61.4 ± 4.3	38.5 ± 5.5 [b]	43.6 ± 5.6	1.5 ± 0.0

[1] Percentage of oocytes that were penetrated.
[a,b] Values with different superscripts in the same column are significantly different within each type of oocyte (P<0.05).
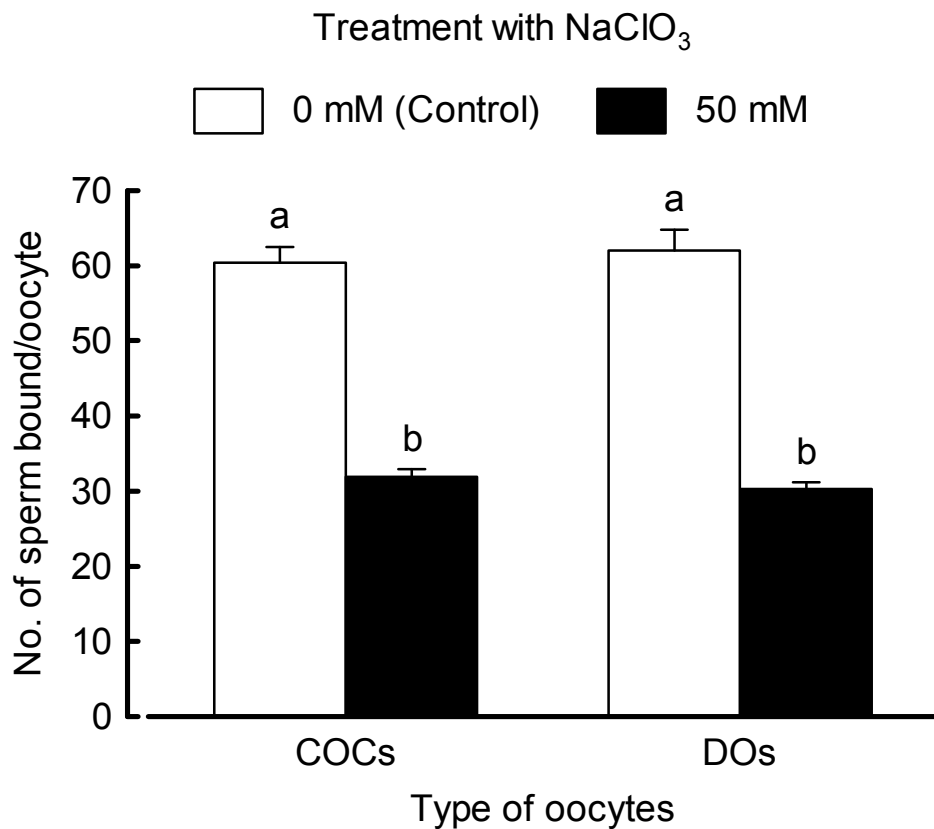
Figure 36. Effect of treatment with 50 mM NaClO$_3$ during 44-h IVM culture in COCs and DOs on the binding of sperm to ZPs.   Treated oocytes were co-incubated with pre-incubated sperm in IVF medium for 2 h. Values are expressed as the mean ± SEM.   For each IVM culture period, the total number of oocytes examined was 30-37 for determination of the number of sperm bound to ZPs.   [a,b] Values with different superscripts are significantly different within each type of oocyte (P<0.05).
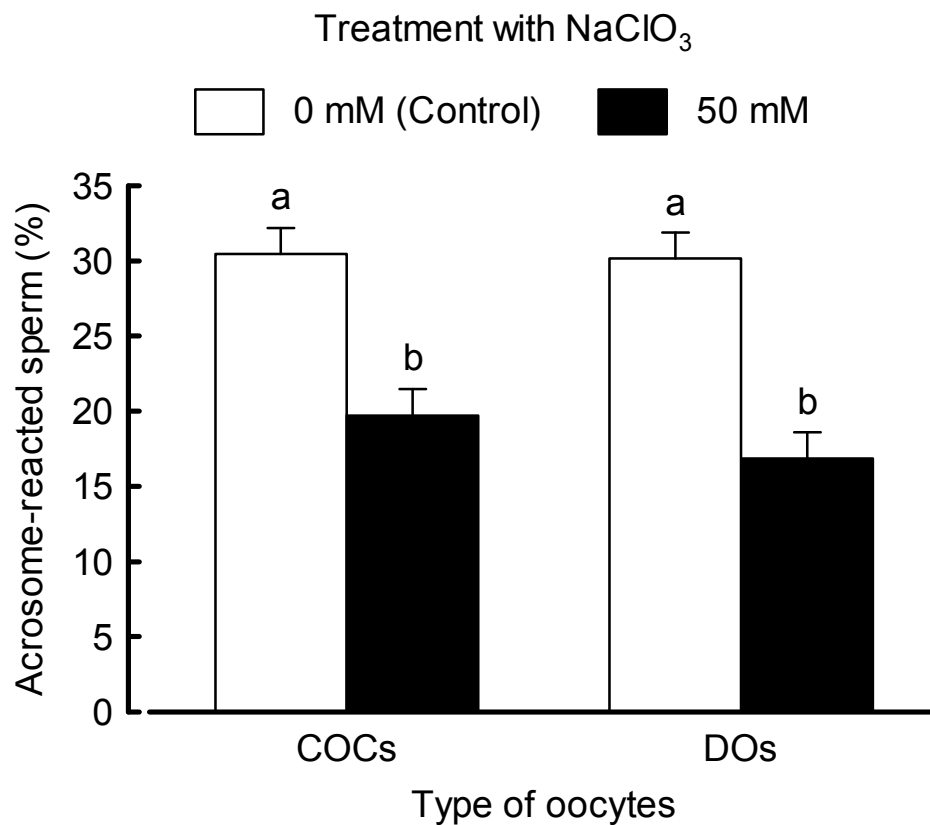
Figure 37. Effect of treatment with 50 mM $NaClO_3$ during 44-h IVM culture in COCs and DOs on the AR induction in ZP-bound sperm. Treated oocytes were co-incubated with pre-incubated sperm in IVF medium for 2 h. Values are expressed as the mean ± SEM. For each IVM culture period, the total number of live sperm examined was 1968-2836 for determination of the number of acrosome-reacted sperm. [a,b] Values with different superscripts are significantly different within each type of oocyte (P<0.05).
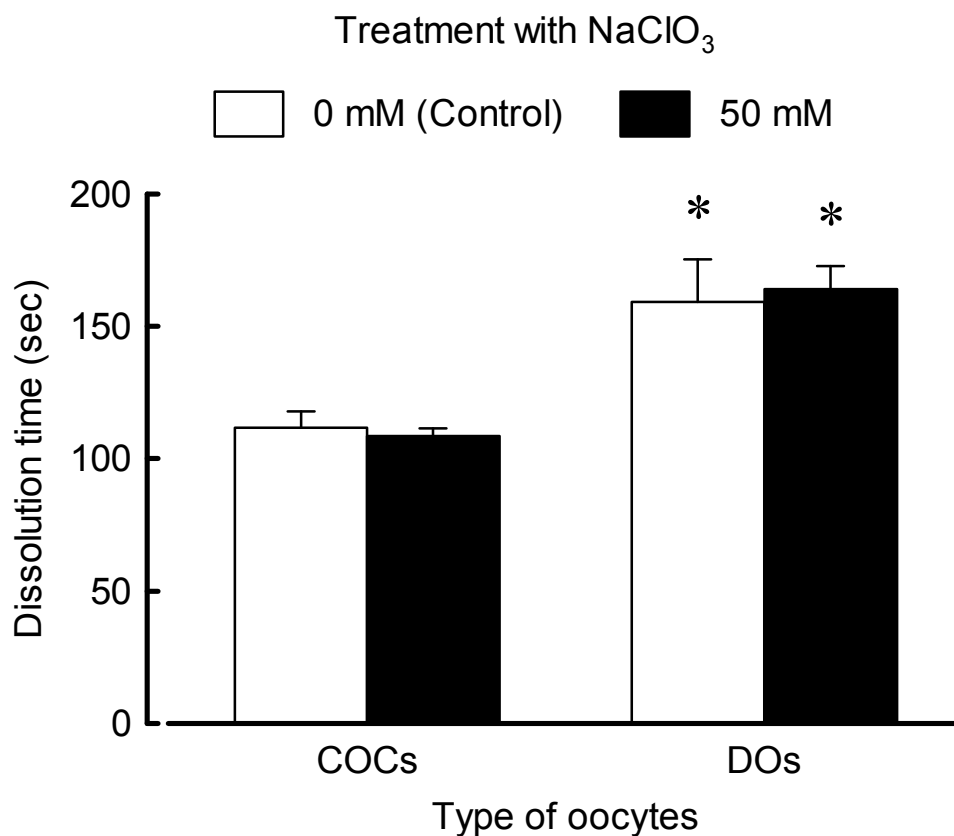
Figure 38. Effect of treatment with 50 mM NaClO$_3$ during 44-h IVM culture in COCs and DOs on dissolution time of ZPs caused by 0.1% protease. Values are expressed as the mean ± SEM. The total number of oocytes examined was 81-102 for each IVM culture period. * Values are significantly different as compared with COCs in the same treatment group (P<0.05).

5.5   Discussion

In the present study, the acidity of ZP glycoproteins significantly increased in COCs and DOs cultured for 44 h compared with immatured oocytes as indicated by 2D gel electrophoresis.   The increase of acidity was consistent with the sulfation of ZP glycoproteins during oocyte maturation, because acidification was prevented in the oocytes treated with a sulfation inhibitor, 50 mM $NaClO_3$ (Figs. 29-32).   These findings strongly suggest that porcine ZP glycoproteins undergo the acidic modifications elicited by sulfation in the final maturation phase of oocytes prior to fertilization, as reported by Rath *et al.* (2005).

The rates of sperm penetration, polyspermy and MPN formation in COCs (Table 15) and DOs (Table 16) were significantly increased in untreated oocytes with the advance of meiotic maturation ($P<0.05$).   Similarly, the number of sperm bound to ZPs (Fig. 33) and the proportion of AR-inducing sperm (Fig. 34) were progressively increased in untreated oocytes during IVM culture period ($P<0.05$).   Although the addition of $NaClO_3$ to IVM medium due to block the sulfation of ZP glycoproteins efficiently decreased the number of sperm bound to ZPs and the rate of AR-inducing sperm in COCs cultured for 28, 32, 44 h and DOs cultured for 24, 28, 44 h, no inhibitory effect of sulfation inhibitor detected on the progressive increase in the sperm penetration rate.   Nonetheless, the incidences of polyspermy were strongly decreased in oocytes treated with $NaClO_3$ during 28, 32 and 44 h (COCs) and during 24, 28 and 44 h (DOs) of IVM.   Under our culture condition, GVBD occurred around 28 and 24 h of maturation culture in COCs and DOs, respectively (Figs. 20 and 21 in Chapter 4).   The current results show that the time course of this sulfation in ZP glycoproteins during oocyte maturation seems more related to the induction of GVBD in both COCs and DOs.

The amount of sulfate is high in the basic structures of *N*-linked oligosaccharides in the porcine ZP (Noguchi and Nakano, 1992), and the sulfated glycans of ZP glycoproteins play important roles in the binding of boar sperm to eggs and penetration by the sperm (Mori *et al.*, 1998).   Töpfer-Petersen and Henschen (1987) 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 (Jones, 1991; Urch and Patel, 1991) and mice (Howes *et al*., 2001). Porcine ZP glycoproteins, fucoidan, dextran sulfate and polyvinyl sulfate potentiate the conversion of proacrosin to β-acrosin (Lo Leggio *et al*., 1994), and the interactions between mouse ZP2 glycoprotein and proacrosin are important for retention of AR sperm on the egg surface during fertilization (Howes *et al*., 2001). 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 (Moreno and Barros, 2000) and mouse (Moreno *et al*., 2002) sperm, and this interaction mediates post-AR binding of spermatozoa to the ZP long enough for penetration to begin in pigs (Jansen *et al*., 1998; Gaboriau *et al*., 2007). Therefore, in the present study, it is likely that the blocking sulfation 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.

The inhibitory effects of treatment with $NaClO_3$ during 44 h of maturation culture on the polyspermy (Table 17), sperm binding to ZP (Fig. 36) and induction of AR in ZP-bound sperm (Fig. 37) were observed in both COCs and DOs after 44 h of maturation culture, and there is no significant difference between COCs and DOs regardless of treatment with $NaClO_3$. The present results demonstrate that the cumulus cells during maturation were not correlated with the sulfation of ZP glycoproteins incorporated in sperm-ZP interactions. It may be assumed that the sulfation of ZP glycoproteins during oocyte maturation would be mediated by oocytes or ZP, not by cumulus cells. Although the ZP dissolution time in 0.1% protease was not altered by treatment with $NaClO_3$ in COCs and DOs (Fig. 35), the ZP dissolution time in DOs is longer than that of COCs after 44 h of IVM culture (Fig. 38). The sperm penetration and the level of the acidic mobility shift elicited by sulfation during IVM in DOs (Table 16 and Fig. 31) were lower than that in COCs. This may partly be due to the zona hardening caused by oxidation of ZP glycoproteins during IVM culture.

The present study is the first to prove that ZP acidification in accordance with the sulfation of ZP glycoproteins during oocyte maturation play a vital role in sperm-ZP interactions. We previously reported that *N*-glycosylation of ZP glycoproteins for periods in excess of 20-24 h after meiotic maturation played an important role in porcine sperm-ZP interactions (Chapter 2). We have confirmed that treatment with $NaClO_3$ does not inhibit the increase in terminal

GlcNAc residues on the ZP by detecting lectin-blotting with S-WGA (unpublished data). Moreover, the blockage of sulfation did not protect against the proteolytic modification of the ZP matrix before fertilization (Fig. 38).

In summary, it can be stated that: 1) Porcine ZP glycoproteins undergo the acidic modification in accordance with sulfation in the final maturation phase of oocytes prior to fertilization. 2) The blockage of sulfation during IVM culture markedly reduced the number of sperm bound to ZPs and the rate of AR-inducing sperm, resulting in the incidence of polyspermy, but not sperm penetration. 3) However, the lower incidences of polyspermy elicited by treatment with $NaClO_3$ were not due to protective proteolytic modifications of the ZP matrix before fertilization. 4) Sulfation of ZP glycoproteins occurred in accordance with GVBD, and the cumulus cells during oocyte maturation would have a very small role in the ZP sulfation. It is concluded that sulfation of ZP glycoproteins during oocyte maturation is indispensable for the acquisition of sperm-ZP interactions, responsible for sperm binding to the ZP and induction of the AR in sperm bound to the ZP.

Chapter 6

General Discussion

In porcine oocytes fertilized *in vitro*, remarkably low normal fertilization rates, resulting from a high rate of polyspermy, have retarded the advancing studies on the application of porcine IVF. This polyspermy, often reaching levels greater than 50%, remains to be solved (Nagai *et al*., 1984; Mattioli *et al*., 1988; Wang *et al*., 1994; 1998). Polyspermic penetration *in vitro* appears not to be due to a delayed or incomplete cortical granule exocytosis but more likely to a delayed ZP reaction (Wang *et al*., 1998).

In oocytes maturation, it is well known that besides the nucleus, oocyte organelles and the cytoplasm must undergo a final maturation process before they become fully competent for fertilization. Moreover, as with changes of the oocyte nucleus, the cytoplasm and other cell organelles, ZP synchronously undergoes biochemical and structural modifications in the final maturation phase of oocyte prior to fertilization (ZP maturation). To date, the physiological and histological mechanism and its consequences have been only partly understood and require further research (Rath *et al*., 2005). Therefore, the present study investigated that the role of ZP modifications during meiotic maturation in sperm-ZP interactions of porcine oocytes.

The interactions between sperm and ZP are mediated by multiple complexes involving several sperm plasma membrane proteins and several carbohydrates contained in ZP glycoproteins (Storey, 1995; Shur, 1998; Thaler and Cardullo, 2002; Rodeheffer and Shur, 2004). The GalTase on the sperm surface at least partly mediates gamete adhesion by binding to its appropriate carbohydrate substrate in ZP (Shur, 1986). The sperm surface HABP1 present in rat, mice, bull and human sperm plasma membrane is also responsible for the sperm-ZP interactions (Ranganathan *et al*., 1994). On the other hand, as the aspect of ZP, several carbohydrates, including fucose, GalNAc, mannose and galactose in ZP glycoproteins, participate in the sperm-ZP interactions in rat, hamster, and/or mouse (Shalgi *et al*., 1986; Boldt *et al*., 1989; Ponce *et al*., 1994).

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Fucose residues have been suggested to be involved in the sperm-oocyte interaction in different species from invertebrate to human (Ahuja, 1982; Tesarik *et al*., 1993; Miranda *et al*., 1997).   The human and porcine sperm-oocyte interaction was mediated by mannose residues in ZP glycoproteins (Mori *et al*., 1989; Tulsiani *et al*., 1990; Tesarik *et al*., 1991; Benoff *et al*., 1993a-c; Miranda *et al*., 1997; Maegawa *et al*., 2002; Song *et al*., 2007).   Terminal galactose residues have been implicated in sperm-oocyte binding in many different mammalian species (Benoff, 1997; Shalgi and Raz, 1997).

In pig, *N*-linked carbohydrates chains of ZP3α play a major role in the primary sperm binding to ZP (Yonezawa *et al*., 1995).   Yurewicz *et al*. (1991) also showed that *O*-linked carbohydrates chains isolated from porcine ZP3 competitively blocked the sperm-binding to intact ZP.   In mouse, the *O*-linked carbohydrate chain of ZP3 is involved in mediating the sperm binding to ZP (Berger *et al*., 1989).   The present results revealed that the presence of Con A, WGA and S-WGA, possessing a specific affinity for GlcNAc residues, during IVF strongly counteracted the sperm bound to ZP and the sperm penetration (Tables 6 and 7 in Chapter 2).   From these findings, it was clear that the sperm-ZP binding and the sperm penetration were mediated by GlcNAc residues in porcine ZP glycoproteins.   Similar observations on the involvement of zona GlcNAc in the fertilization of hamster and human oocytes have been reported (Mori *et al*., 1989; Miranda *et al*., 1997; 2000; Zitta *et al*., 2004).   Loeser and Tulisiani (1999) demonstrated that mannose and GlcNAc ligands in human, and mannose, GlcNAc and GalNAc ligands in mouse are important in the interaction of the opposite gametes and induction of AR.

In the present study, the terminal GlcNAc residues in porcine ZP were specifically detected in ZP3 glycoprotein by lectin-blotting with WGA and S-WGA, and these relative intensities of terminal GlcNAc residues were increased with the advance of oocyte maturation.   These progressive increases of GlcNAc residues were significantly blocked in oocytes treated with 10 μg/ml tunicamycin during IVM culture (Fig. 4 in Chapter 2).   The treatment with tunicamycin for 20 h after the onset of normal IVM culture for 24 h was needed to reach at the same levels of the penetration rate, and the number of sperm bound to ZP and AR induction in ZP-bound sperm

compared with that of control group in COCs (Table 8, Figs. 8 and 9 in Chapter 2), indicating that the first 24 h of IVM culture is critical to the new glycosylation of ZP glycoproteins in COCs. In contrast to COCs, the new glycosylation of ZP glycoproteins in DOs during the first 36 h of culture is indispensable for acquirement of the capacity of oocytes to accept spermatozoa (Table 9 in Chapter 3). These different results suggest that the *N*-glycosylation of ZP glycoproteins during oocyte maturation is partly supported by the presence of cumulus cells, because the longer culture period in DOs than COCs was required for completion of the new *N*-glycosylation to obtain the sperm penetration at the same levels of oocytes treated without tunicamycin. Furthermore, treatment with tunicamycin during IVM of COCs significantly decreased in the number of sperm bound to ZP and the percentages of AR-inducing sperm (Figs. 8 and 9 in Chapter 2, $P<0.05$), but had no effects on MPN formation (Table 8 in Chapter 2). These similar results were also found in DOs (Figs. 12 and 13, Table 9 in Chapter 3, $P<0.05$). Therefore, it is suggested from the results in Chapters 2 and 3 that new *N*-glycosylation of GlcNAc residues in ZP glycoproteins during oocyte maturation is indispensable for the initial steps in fertilization of porcine oocytes.

In the present study, there was a difference in the relative intensity of the lectin-blotting between the GlcNAc residue labeled with S-WGA (1.05 ± 0.03) and WGA (1.14 ± 0.03) in matured oocytes treated with tunicamycin (Fig. 4 in Chapter 2). This difference would be due to the fact that S-WGA has a specific affinity only to GlcNAc residues, but WGA could bind to sialic acid residues in addition to GlcNAc residues (Debray *et al*., 1981). Perhaps sialylation may be concomitant with glycosylation of ZP glycoproteins during maturation of porcine oocytes. It is reported that the sialic acid of ZP glycoproteins are physiologically involved in regulating the binding of sperm to ZPs in porcine (Lo Leggio *et al*., 1994) and bovine (Velásquez *et al*., 2007) oocytes.

The number of sperm bound to ZP and sperm penetration rates were significantly declined in the oocytes inseminated in the presence of SSA and MAL II, recognizing α-2, 6-linked and α-2, 3-linked sialic acid, respectively, compared with that in the absence of lectin (control; $P<0.05$) (Tables 10 and 11 in Chapter 4). In addition,

the relative intensities of sialic acid residues detected by SSA lectin-blotting significantly increased in ZP glycoproteins derived from matured oocytes in the control IVM medium compared with that in oocytes freshly isolated from follicles, but this increased sialic acid residues was potently removed by treatment with neuraminidase (Fig. 15 in Chapter 4).   The significant reductions in the sperm penetration (Tables 12 and 13 in Chapter 4), the number of sperm bound to ZPs (Fig. 22 in Chapter 4) and the percentage of AR induction in sperm bound to ZPs (Fig. 23 in Chapter 4) by treatment with neuraminidase were also observed in COCs cultured for 28, 32 and 44 h and DOs cultured for 24, 28 and 44 h ($P<0.05$).   Under our culture conditions, GVBD in COCs was accomplished at 28 h of IVM culture period, whereas the maturation time for 24 h was required to accomplish GVBD in DOs.   Therefore, it is suggested that porcine ZP glycoproteins undergo the sialic modifications in the final maturation phase of oocytes prior to fertilization, and the sialylation phase of ZP glycoproteins during oocyte maturation is compatible with the induction of GVBD independently of the absence or presence of cumulus cells.

In the present study, 2D gel electrophoresis demonstrated an obvious shift in the mobility of ZP glycoproteins in response to acidity during the oocyte maturation process.   This mobility shift was broadly prevented in the oocytes treated with a sulfation inhibitor, 50 mM $NaClO_3$ (Figs. 29-32 in Chapter 5).   Porcine ZP3 glycoproteins are highly heterogeneous mainly due to differences in the amount of sulfated N-acetyllactosamine and sialic acid in the acidic chains (Nakano et al., 1990). Gunaratne (2007) mentioned that not only N-glycosylation, but also sialylation and sulfation of ZP glycoproteins were required for the induction of AR in sperm bound to ZP.   The inhibitory effects of treatment with $NaClO_3$ during IVM on the number of sperm bound to ZPs and the rate of AR-inducing sperm were observed in COCs after 28, 32 and 44 h of maturation culture, and in DOs after 24, 28 and 44 h of maturation culture.   The present findings indicate that sulfation of ZP glycoproteins during oocyte maturation contributes to the porcine ZPs acquiring the capacity to accept sperm, and is assessed in accordance with GVBD induction independently of the absence or presence of cumulus cells as well as sialylation.

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Although the blocking *N*-glycosylation and the desialylation in ZP glycoproteins during oocyte maturation dramatically decreased in the incidences of sperm penetration and polyspermy, the number of sperm bound to ZP and percentage of AR-inducing sperm, the blockage of sulfation had no inhibitory effect on sperm penetration rate. However, the incidences of polyspermy were strongly decreased in oocytes treated with $NaClO_3$ during 28, 32 and 44 h (COCs) and during 24, 28 and 44 h (DOs) of IVM (Tables 15 and 16 in Chapter 5). In short, the blocking sulfation of ZP glycoproteins during oocyte maturation significantly suppressed in polyspermic fertilization with no detrimental effect on sperm penetration and MPN formation. Therefore, it seems that the blocking sulfation by treatment with $NaClO_3$ during oocyte maturation is an efficient for promoting normal fertilization process in terms of an effective decrease in the incidence of polyspermy during IVF of porcine oocytes.

Polyspermy is one of the unsolved problems associated with pig oocytes matured and fertilized *in vitro* that causes early embryo death (Niwa, 1993). Depending on the species, the polyspermic block resides either at ZP, or the egg plasma membrane, or both. Polyspermy is primarily blocked by zona changes in hamster, goat, ovine, bovine oocytes, by oolema changes in rabbit oocytes and by both in mouse, rat, guinea pig, and cat oocytes (Yanagimachi, 1994). Moreover, proteinases, ovoperoxidase, *N*-acetylglucosaminidase and neuraminidase are thought to bring about changes in the ZP. After sperm-egg fusion, cortical granules (CG) release into perivitelline spaces (cortical reaction), causing the ZP to become refractory to sperm binding and penetration (zona reaction). The CG proteinase exerts the zona sperm receptor modification and catalyzes the proteolysis of ZP2 as a consequence of a decrease in solubility of the ZP (zona hardening) (Barros and Yanagimachi, 1971; Gwatkin *et al*., 1973; Wolf and Hamada, 1977; Moller and Wassarman, 1989). Interestingly, according to the recent findings reported by Velásquez *et al*. (2007), the neuraminidase released from the CG during cortical reaction of bovine oocytes and this neuraminidase would participate in polyspermic block by removing sialic acid from ZP. In the present study, desialylation of ZP glycoproteins during oocyte maturation decreased the sperm penetration, sperm binding to ZP and AR induction (Chapter 4).

However, whether the neuraminidase released from CG during cortical reaction is still unclear. Therefore, further study is needed to examine the release of neuraminidase from CG following egg activation in porcine oocytes.

Chapter 7

Summary and Conclusions

In Chapter 2, to investigate whether *N*-glycosylation of ZP glycoproteins occurred during meiotic maturation of porcine oocytes and whether this *N*-glycosylation had influence upon fertilization, COCs were cultured in IVM medium supplemented with or without tunicamycin, *N*-glycosylation inhibitor, for various intervals. After maturation, oocytes were inseminated with pre-incubated spermatozoa to examine sperm-ZP interactions and some oocytes were assessed for ZP hardness. *N*-glycosylation of ZP3 glycoprotein in porcine COCs occurred during the first 24 h of IVM, and the increases in the number of sperm bound to ZP, acrosome-reacted sperm, sperm penetration rate, and level of polyspermy, but not the zona hardening properties, were observed in accordance with this glycosylation. The increase in terminal GlcNAc residues in ZP glycoprotein through new *N*-glycosylation during the first 24 h of meiotic maturation played a critical role in ZP acquiring the capacity to accept sperm in porcine COCs.

In Chapter 3, to examine whether there is a correlation between the cumulus cells during meiotic maturation of porcine oocytes and *N*-glycosylation of ZP glycoproteins responsible for sperm-ZP interactions, DOs matured in an absence or presence of tunicamycin for various intervals were co-incubated with pre-incubated spermatozoa for IVF. The first 36 h of *N*-glycosylation of GlcNAc residues in porcine

ZP during IVM was indispensible for sperm-ZP interactions.   Since the longer culture period in the absence of tunicamycin after the onset of IVM culture periods was needed to obtain the sperm penetration at the same levels of untreated oocytes in DOs rather than COCs, the cumulus cells are partly involved in ZP glycosylation during oocyte maturation.

In Chapter 4, to investigate the role of sialylation of ZP glycoproteins during meiotic maturation in sperm-ZP interactions, oocytes were cultured for various culture periods in IVM medium.   After maturation, oocytes were treated with neuraminidase for 1 h to remove the sialic acid residues from ZP.   After treatment, some oocytes were inseminated with pre-incubated spermatozoa for observation of fertilization parameters and the others were used for ZP digestion.   The lectin-blotting assay and 2D gel electrophoresis clearly demonstrated that ZP acidification occurred in accordance with the sialylation of ZP glycoproteins in oocytes matured for 44 h.   The number of sperm bound to ZPs and the percentage of AR in sperm bound to ZPs were suppressed in the desialylated COCs after 28, 32 and 44 h, and the desialylated DOs after 24, 28 and 44 h of IVM culture, resulting in a significant reduction in penetration and polyspermy rates. Moreover, the sialylation was not associated with a protective proteolytic modification of the ZP matrix before fertilization.   These results indicate that the time course of this sialylation in ZP glycoproteins is correlated with the induction of GVBD during oocyte maturation, and the cumulus cells during oocyte maturation are not essential in the sialylation of ZP glycoproteins responsible for sperm-ZP interactions.

In Chapter 5, to investigate the role of sulfation of ZP glycoproteins during meiotic maturation in sperm-ZP interactions, oocytes were cultured for various intervals in IVM medium in the absence or presence of a sulfation inhibitor, 50 mM $NaClO_3$. As indicated by 2D gel electrophoresis, the increase of acidity was consistent with the

sulfation of ZP glycoproteins during oocyte maturation, and the ZP acidification was prevented in the oocytes treated with NaClO₃. The blocking of ZP sulfation by NaClO₃ treatment during IVM in COCs and DOs markedly abolished the incidence of polyspermy with no inhibitory effect on penetration, however the number of sperm bound to ZPs and the rate of AR-inducing sperm were decreased, and the time course of ZP sulfation was related to the induction of GVBD, irrespective of the presence of cumulus cells.   These results support the hypothesis that sulfation in ZP glycoproteins during oocyte maturation is critically important in regulating the fundamental steps of sperm-ZP interactions.

In conclusion, it is clear from the present study that porcine oocytes undergo ZP modifications during maturation, and these findings are the first to demonstrate that the ZP modifications such as *N*-glycosylation, sialylation and sulfation of ZP glycoproteins during meiotic maturation is essential in sperm-ZP interactions of porcine oocytes.   *N*-glycosylation of GlcNAc residues in ZP glycoproteins was indispensible for sperm-ZP interactions, and such *N*-glycosylation occurred during the first 24 and 36 h of IVM of porcine COCs and DOs, respectively.   ZP acidifications elicited by sialylation and sulfation of ZP glycoproteins during oocyte maturation contributed to the porcine ZP acquiring the capacity to accept sperm.   These ZP acidifications were temporally compatible with the induction of GVBD during oocyte maturation, but did not require for the presence of cumulus cells.   The inhibition of *N*-glycosylation and the removal of sialic acid residues in ZP glycoproteins during oocyte maturation dramatically decreased in the incidences of sperm penetration and polyspermy, the number of sperm bound to ZP and percentage of AR-inducing sperm.   However, the blocking sulfation of ZP glycoproteins during oocyte maturation significantly suppressed in polyspermic fertilization with no detrimental effect on sperm penetration

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and MPN formation.

It has been reported that the biochemical and morphological alterations of ZP occur during meiotic maturation, fertilization and egg activation, and it seems that the opposite gametes interactions would be mediated by other types of carbohydrate residues such as fucose and mannose in ZP glycoproteins during meiotic maturation. Therefore, further studies are required to unravel the anticipated involvement of various carbohydrate residues in sperm-ZP interactions responsible for their possible role in sperm-egg recognition, AR induction and secondary binding.

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