Modification of the zona pellucida sperm-binding protein 3 (ZP3) in pigs during folliculogenesis

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Abstract: Polyspermic fertilization is still one of major problems during in vitro fertilization in pigs. The zona pellucida is an extracellular matrix surrounding mammalian oocytes, plays an important role during fertilization and forms polyspermy block after fertilization as well. The porcine zona is composed of three glycoprotein called ZP2, ZP3 and ZP4. The purpose of this study is to evaluate modifications of the zona pellucida sperm-binding protein 3 (ZP3) of oocytes with different developmental competence. This protein is involved in binding of the spermatozoa to the oocyte. Using western blot analysis, it was found no significant differences in expression of ZP3 protein in oocytes with lower developmental competence (from small follicles), in compared to oocytes with higher developmental competence (from medium follicles). But it was detected band in area > 50 kDa, which could play an essential role during fertilization of oocytes.

Key-Words: zona pellucida, polyspermic fertilization, ZP3 protein, porcine oocytes

Introduction

The zona pellucida (ZP) is an extracellular matrix that surrounds all mammalian oocytes and mediates species-specific binding of spermatozoa to the oocyte during fertilization [1]. Following fertilization, zona pellucida form the zona block to prevent polyspermy and protects the preimplantation embryo during passing down through the oviduct [2].

The porcine zona pellucida is composed of three highly specific glycoproteins modified by many posttranslational modifications including glycosylation and sulfation [3]. The glycoproteins are called as ZP2 (also named ZPA and ZP1), ZP3 (also named ZPC and ZP3α) and ZP4 protein (also named ZPB and ZP3β) [4]. ZP3 and ZP4 protein probably form long fibres of the ZP architecture, whereas ZP2 protein serves as crosslinker. The thickness of porcine ZP is 16 μm [3].

After fertilization, the ZP is modified by the contents of cortical granules and it occurs to proteolytic cleavage of ZP2 protein and to formation of disulfide bonds as well. This process is known as zona hardening and constitutes a major block to prevent polyspermy [5].

Polyspermic fertilization one of major problems during in vitro fertilization of porcine oocytes because it is lethal for embryo development. The polyspermy rate in vitro can reach 65% [6].

It has been shown that boar spermatozoa evince a high affinity for the ZP3-ZP4 heterocomplex but not for free glycoprotein subunits [7]. In study Yonezawa et al. [8] assumed the carbohydrate structures of ZP4 in the porcine ZP3-ZP4 complex are responsible for sperm-binding activity in this complex. The question of why ZP4 shows the sperm-binding activity only in the heterocomplex with ZP3 needs to be clarified in the future.

The ZP3 protein (46 kDa) belong to the ZPC subfamily and its immature form is composed of 421 amino acid (AA). The mature form of this protein is composed of 310 AA [9].

This study is aimed to modification of ZP3 protein of oocytes with different developmental competence during folliculogenesis - according to ability of oocytes to undergo normal fertilization and zona hardening. Oocytes were derived from small and medium follicles in selected phase of estrous cycle.

Material and Methods

Oocyte donors

Cycling pubertal gilts Landrace×Czech Large White crossbred were used as oocyte donors (age 8.5–11 months, weight 130–150 kg). The ovaries were recovered at local slaughterhouse and transported to the laboratory within 2 h at 39°C. In the laboratory, the ovaries were evaluated by morphological status (shape and size of follicles and of corpus luteum) and divided into the four phases of folliculogenesis: in the early (days 1–5), middle (days 6–10) and late (days 11–14) luteal phases and in the early (days 15–16) follicular phase.

Oocyte collection

Oocytes were recovered from antral follicles by aspiration (5–9 mm) and cutting of the ovarian cortex (2–4 mm), respectively. Oocytes from larger follicles (≥10 mm) were not included in this study because of the dominant follicles phase. Oocytes were divided in 6 subpopulations:

1. Early luteal phase (days 1–5), small follicles (2–4 mm) – subpopulation called S 1–5
2. Middle luteal phase (days 6–10), small follicles (2–4 mm) – subpopulation called S 6–10
3. Late luteal phase (days 11–14), small follicles (2–4 mm) – subpopulation called S 11–14
4. Late luteal phase (days 11–14), medium follicles (5–9 mm) – subpopulation called M 11–14
5. Early follicular phase (days 15–16), small follicles (2–4 mm) – subpopulation called S 15–16
6. Early follicular phase (days 15–16), medium follicles (5–9 mm) – subpopulation called M 15–16

Oocyte maturation

All healthy cumulus-oocytes complexes with dark, evenly granule ooplasm and at least two cumulus layers by Hulinska et al. [10] were used for the oocyte maturation. The oocytes were matured in 500 µl of TCM-199 medium (Earle's salts) with addition of 0.20 mM sodium pyruvate, 0.57 mM cysteamin, 50 IU ml–1 penicillin, 50 µg ml–1 streptomycin, 10% BFS (bovine fetal serum; Sigma Chemicals Co., Prague, Czech Republic) and gonadotropins (P.G. 600 15 IU ml–1; Intervet, Holland) in a 4-well muti-dish (Nunc, Intermed, Denmark) at 39°C in atmosphere of 5% CO2. The oocytes were matured for 44 h in vitro. After maturation, neither the maturate oocytes (MII) nor the groups of immature oocytes (GV) were vortexed in the presence of hyase to remove cumulus cells.

Western blot

To the samples of 25 oocytes from each subpopulation of oocytes before maturation and after maturation were added lysis buffer and 10% SDS (sodium dodecyl sulfate; Serva, Heidelberg, Germany). The samples were centrifuged and sonicated followed by addition of sample buffer (0.5 M tris-HCl, 10% SDS, glycerol, bromophenol blue, dH2O, 2-mercaptoethanol; Serva) and the samples were boiled. These samples were loaded in polyacrylamide gel (Serva) and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed in Mini-Protean Tetra System (Bio-Rad, Hercules, CA, USA). Dual Xtra was used as marker (Bio-Rad).

After SDS-PAGE, the activation of PVDF membrane by methanol was performed followed by composition of western blot sandwich and semi-dry western blotting (Mini-Protean Tetra System; Bio-Rad) for 1 h. Then the PVDF membrane was blocked using 5% powdered milk in washing buffer (100 mM NaCl, 20 mM tris-HCl, 0.075% Tween) for 1 h and the membrane was incubated with primary antibody ZP3 N-20 (1 : 1000 dilution, Santa Cruz) over night at 4°C. The PVDF membrane was washed three times for 5 min and two times for 10 min and incubated with secondary antibody Rabbit anti-Goat HRP (1 : 2000 dilution, Santa Cruz) for 1 h. ZP3 protein was visualized using ECL (Enhanced Chemiluminescence; Santa Cruz) and were processed using NIS-Elements ar 3.0 software.

Statistical analysis

The results were statistically analyzed by the T-test using STATISTICA Cz, version 10 software (StatSoft, Inc., Prague, Czech Republic). Differences at $P < 0.05$ were considered statistically significant.

Results and Discussion

Modifications of ZP3 protein during folliculogenesis

Using western blot, it was detected modifications of ZP3 protein of oocytes with different developmental competence (Fig. 1). Days 1−14 introduce luteal phase of estrous cycle and days 15−16 introduce follicular phases of estrous cycle.

Band of the subpopulation of oocytes S 1−5 was always weaker in compared to the other subpopulations and by this subpopulation does not occur band > 50 kDa, both before maturation and

after in vitro maturation. Band > 50 kDa was appeared gradually according to the days of estrous cycle and follicle size and it is located there also after maturation. Moreover, bands of individual subpopulations after maturation seemed to be shorter in compared to bands before maturation.

It was found no significant differences in expression of ZP3 protein of immature oocytes recovered from small follicles in compared to medium follicles. It was found no significant differences in expression of ZP3 protein of mature oocytes recovered from small follicles in compared to medium follicles as well. The expression of ZP3 was evaluated by the mean intensity of this protein (Tab. 1). The individual subpopulations of oocytes were evaluated as unit (small or medium follicles) because mean follicle size does no change and developmental competence of oocyte was evaluated by follicle size.

Fig. 1 Modifications of porcine ZP3 protein during folliculogenesis: (A) subpopulations of immature oocytes, (B) subpopulations of mature oocytes.

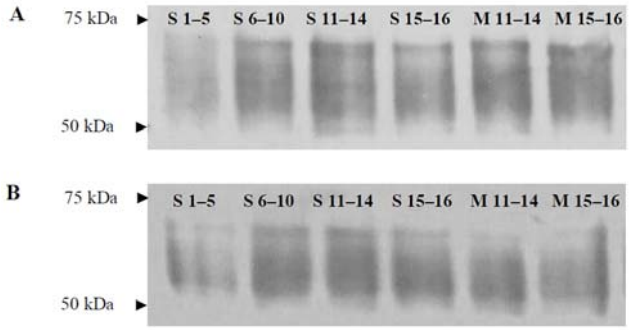

Table 1 Effect of meiotic maturation and developmental competence on expression of porcine ZP3 protein. Values with different superscripts by the same stage of meiotic maturation are significantly different ($P < 0.05$).

Stage of meiotic maturation	Follicle size	No. of measurements	Mean intensity of ZP3 protein ± SEM
GV (0 h)	S (2−4 mm)	12	31.84 ± 6.63^{a}
GV (0 h)	M (5−9 mm)	6	30.66 ± 12.29^{a}
MII (44 h)	S (2−4 mm)	8	24.41 ± 5.82^{a}
MII (44 h)	M (5−9 mm)	4	23.24 ± 6.79^{a}

Antosik et al. [11] divided oocytes in 4 groups according to their morphology of cumulus-oocytes complexes (COC) and western blot analysis was performed. It was detected increased expression of ZP3 protein in oocytes of grade I in compared to other grades (II–IV). This data suggest that not only follicle size but also COC morphology are important factors that affect the expression of ZP3 protein in pigs.

In present study are used only healthy COC with homogenous cytoplasm and at least two layers of cumulus cells.

Our results suggest that there is no difference in expression of ZP3 protein of oocytes from small and medium follicles. But in our lab was previously demonstrated [10] that oocytes recovered from small follicles have significantly higher polyspermy rate in compared to oocytes from medium follicles. The monospermy rate in oocytes from small follicles increased significantly from the early luteal to the late luteal phase and remained unchanged in the early follicular phase. A similar tendency was observed in the total efficiency of fertilization.

It is possible that band in area > 50 kDa is responsible for increasing of the monospermy rate in small oocytes and also for increasing of the efficiency of fertilization.

Conclusion

It is generally known that to polyspermy in pigs occurs more often in conditions in vitro than in vivo. In vitro fertilization of porcine oocytes and also polyspermic fertilization are therefore hot issue.

The ZP3 protein is one of proteins of the porcine zona pellucida and together with the ZP4 protein is involved in sperm-binding activity of zona. The precondition that expression of ZP3 protein varies by oocytes with different developmental competence was not correct. In this study, it was found no significant differences in expression of ZP3 protein of ocytes recovered from small follicles in compared to medium follicles, both before maturation and after maturation. However, it seems to be that before in vitro maturation occurs to modification of ZP3 protein manifested by band in area > 50 kDa. This band may play an important role during fertilization. Further studies are needed to confirm this hypothesis.

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