Praca oryginalna *Original paper*

Localization of zona pellucida glycoprotein 3 (pZP3) and integrin-beta-2 (ITGB2) in porcine oocytes cultured in vitro*)

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Summary

It has been clearly demonstrated in several studies that zona pellucida is modificated during oogenesis and folliculogenesis, important stages of mammalian oocytes maturation. However, little is known to date about differential expression and various distributions of proteins involved in fertilization, e.g. zona pellucida glycoprotein 3 (pZP3), integrin-beta-2 (ITGB2) within the porcine oocytes. Since the morphology of the female gamete significantly influences the ability of oocytes for maturation and fertilization, this study aimed to investigate the distribution of pZP3 and ITGB2 in four morphologically different porcine oocytes using confocal microscopic observations.

The porcine COC's were morphologically evaluated in a four graded scale with special relation to colorization of cytoplasm and cumulus cell layers, cultured in culture medium NCSU-23 for 44 h at 38°C, and then subsequently fixed with anti-pZP3 and anti-ITGB2 antibodies, and analyzed using confocal microscopic observations.

Consequently, we found that pZP3 protein was localized in oocytes graded as I and II in zona pellucida and cytoplasm. In oocytes graded as III and IV, pZP3 was distributed in the cytoplasm. Regarding the ITGB2, in oocytes graded as I the zona pellucida localization was observed. In the other grades of oocytes a strong cytoplasmic expression of ITGB2 was detected.

In conclusion, the expression of both pZP3 and ITGB2 proteins as well as the differential distribution of these proteins within the female gamete are associated with the morphological type of porcine oocytes.

Keywords: zona pellucida glycoprotein, integrin, oocytes, procine

The morphology of oocytes is the main factor determined the *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and a proper embryo growth and development (4, 9, 11, 18, 19, 28).

The quality of oocytes is mainly determined by proper steps of maturation. The *in vitro* manipulation procedures lead to the improved efficiency of artificial reproductive techniques applied in many species of mammals, including pigs, bovine, goats, dogs and cats (3, 6, 11, 14, 16, 22, 24). However, the differential efficiency of IVM in some species, including dogs, leads to the use of those methods to a smaller degree (8, 15, 29). The main reason for increased research on the combination of new media supplements used in *in vitro* culture is to mimic the *in vivo* conditions. The IVM efficiency is determined by the number of oocytes that reach the MII stage and are fully competent to successful monospermic fertilization, zygote formation, as well as a proper development of embryos. These parameters differ between every species of mammals, from a higher one in bovine and pigs to approximately only 20% of oocytes that reach the MII stage in canine species. Therefore, as it is suggested in several reports, the research on *in vitro* cultivation (IVC) or IVM efficiency has an increased interest in reproductive biology. There are many reports that indicate the role of new

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selected *in vitro* media supplements which have increased the number of oocytes reaching the MII stage, although only a few reports have been published as yet in the field of the role of IVC of oocytes in the expression of genes or proteins responsible for important steps of maturation, fertilization of embryo development (1, 2, 23). Similarly, only a few reports indicated the influence of IVC on the localization of selected proteins within the oocytes.

Zona pellucida (ZP) is formed by specific proteins, also called *zona* glycoproteins, such as ZP1, ZP2, ZP3 and ZP4, described in pigs as porcine *zona pellucida* glycoproteins (pZP) (3, 12, 20, 21). This unique structure that surrounds the oocytes membrane is responsible for each step of fertilization, including: gamete interactions, induction of acrosome reaction within the spermatozoa, as well as a block to polyspermy (17, 20, 21). pZP3 proteins play a crucial role during the initial steps of fertilization, during male-female gamete interaction, and are also described as the primary sperm receptor. Following sperm-oocyte binding, ZP3 activates the sperm acrosome reaction and, therefore, it also activates several other metabolic pathways that are responsible for a proper *zona* penetration by spermatozoa during fertilization.

Integrins belong to the family of cell membrane glycoproteins that are involved in several important biological functions of the cells, including cell adhesion and recognition. The functionality of integrins as cell adhesion molecules needs attention to discover a new function of these proteins involved in fertilization (26, 27, 30). Although several reports have indicated the role of integrins, including integrin-beta-2 (ITGB2), in fertilization, there are only a few reports describing a different localization of this protein within the porcine oocytes.

Since it was previously reported that pZP3 and ITGB2 proteins may be translocated within the oocytes between the *zona pellucida* and oocyte's cytoplasm, in this study the different localization of pZP3 and ITGB2 proteins was analyzed, using a confocal microscopic observation, in porcine oocytes of different morphology after *in vitro* maturation.

Material and methods

Animals. A total of 25 puberal crossbred Landrace gilts at the mean age of 155 days (range 140-170 days) and the mean weight of 110 kg (95-125 kg) were used in this study. The experiment was performed on gilts during their natural estrous. All animals were checked for estrous once a day for 15 minutes using a fence-line contact with a mature boar. After the commencement of estrous, detection continued on consecutive days, and on day 17 of the subsequent estrous cycle the gilts were slaughtered. The experiments were approved by the local Ethics Committee.

Collection of porcine ovaries and COCs. The ovaries and reproductive tracts were recovered from gilts immediately after the slaughter and transported to the laboratory. The COCs were isolated from follicles larger than 5 mm (> 5 mm), using a caliper. The follicles were opened by individual puncturing with a 20-G needle attached to a 5-ml syringe into a sterile Petri dish. Recovered cumulus-oocyte complexes (COCs) were washed three times in modified PBS supplemented with 36 µg/ml pyruvate, 50 µg/ml gentamycin, and 0.5 mg/ml bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA). They were selected under an inverted Zeiss microscope (Axiovert 35, Lübeck, Germany), counted, and morphologically evaluated with special care using the four grade scale suggested by Jackowska et al. (11). All four morphologically different groups of COCs graded as I, II, III, and IV were considered for use in the subsequent steps of the experiment.

Assessment of oocyte developmental competence by brilliant cresyl blue (BCB) test. Before the cultivation, COCs were washed twice in modified Dulbecco PBS (DPBSm, Sigma-Aldrich,) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin (Sigma-Aldrich), 0.4% [w/v] BSA, 0.34 mM pyruvate, and 5.5 mM glucose (DPBSm). COCs were then treated with 26 µM brilliant cresyl blue (BCB; Sigma-Aldrich, St. Louis, MO, USA), diluted in DPBSm at 38.5°C under 5% CO_2 in air for 90 min. After the treatment, the oocytes were transferred to DPBSm and washed twice. During the washing procedure, the oocytes were examined under a stereomicroscope and classified as either having stained blue (BCB^+) or remained colorless (BCB^-). Only BCB^+ oocytes, which had completed their growth phase and may have reached developmental competence, were used in the experiment.

In vitro maturation of porcine COCs. The selected grade I COCs were cultured in Nunclon™Δ 4-well dishes (Nunc, GmbH, Co. KG, Germany) in 500 µl standard porcine *in vitro* maturation (IVM) medium (NCSU23) supplemented with 10% (v/v) filtered porcine follicular fluid, and gonadotropin supplements at final concentrations of 2.5 IU/ml human chorionic gonadotropin (hCG; Ayerst Laboratories, Inc., Philadelphia, PA, USA) and 2.5 IU/ml equine chorionic gonadotropin (eCG; Intervet, Whitby, ON, Canada). Cells were cultured for 44 h at 38°C under 5% CO_2 in air.

Confocal microscopic observation of localization of ITGB2 and pZP3 proteins in oocytes. COCs after BCB staining and IVM (n = 100) were incubated with 300 µg/ml with bovine testicular hyaluronidase Sigma-Aldrich Co. (St. Louis, MO, USA) for 2 min at 38°C to remove cumulus cells. Oocytes were fixed with 2.5% paraformaldehyde in PBS and 0.2% Triton-X 100 for 30 min at room temperature (RT) and washed three times in PBS/PVP (0.2%). To block nonspecific binding, samples were incubated at 3% BSA in PBS with 0.1% Tween20 for 30 min at RT. Oocytes were incubated for 12 hours at 4°C with goat polyclonal anti-pZP3 (N-20) antibody (Ab) or goat polyclonal anti-integrin β2 (C-20) Ab (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 500 in PBS/1.5% BSA/0.1% Tween20. After several washes with PBS/0.1% Tween 20, the samples were incubated for 1 hour at RT with fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG Ab produced in rabbit diluted 1 : 200 in PBS/0.1% Tween 20. Following this wash in PBS/0.1% Tween 20,

the oocytes were mounted on glass slides in the antifade drop and observed under a confocal system LSN 510 on Carl ZEISS microscope Axiovert 200M. FITC was excited with at 488 nm by an argon laser, and emissions were imaged through a 505-530 nm filter.

Results and discussion

Using a confocal microscopic observation, we compared the expression of pZP3 and ITGB2 proteins in four morphologically different groups of porcine oocytes. As a result, we found that both of these proteins were expressed in all four groups of oocytes, but with a different pattern, intensity of the expression and distribution. In 85% of group I and II of oocytes, the pZP3 protein was distributed in *zona pellucida* and cytoplasm. In 79% of group III oocytes, the pZP3 expression was decreased in *zona pellucida* and strongly expressed in the cytoplasm. Group IV was characterized by an increased pZP3 expression in the cytoplasm. In this group of oocytes, specific cytoplasm retractions were also observed. Regarding the ITGB2 localization and expression, we found an increased expression of this protein in the *zona pellucida* in the 89% of group I of oocytes. We detected significant differences between group II and III, where ITGB2 was localized rather in the cytoplasm than in *zona pellucida* (72%/28%). The group IV oocytes were characterized by the expression of ITGB2 only in the cytoplasm (91%). Moreover, the oocytes from this group were of a smaller size and had retractions of the cytoplasm.

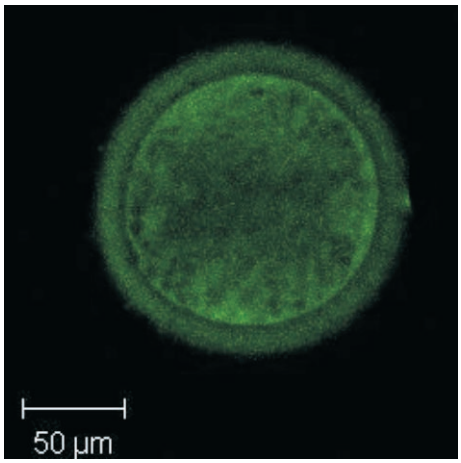

Fig. 1A-I

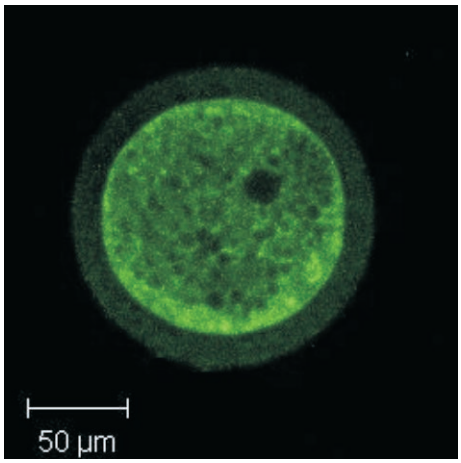

Fig. 1B-II

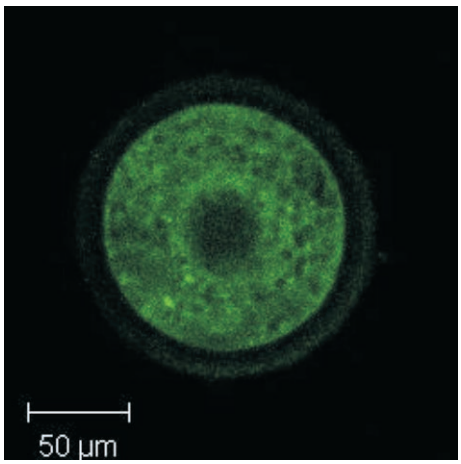

Fig. 1C-III

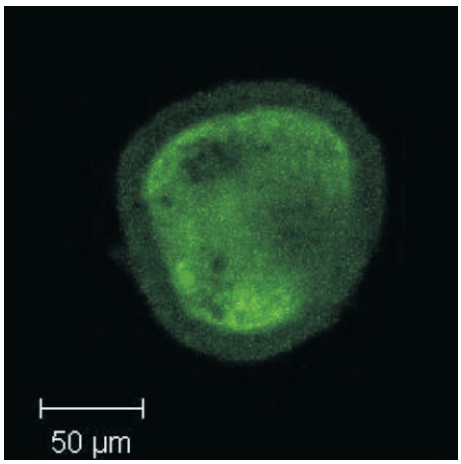

Fig. 1D-IV

Fig. 1. Confocal microscopic observation of pZP3 distribution in oocytes after *in vitro* maturation (IVM)
The porcine oocytes (n = 100) were isolated from puberal gilts ovaries after slaughter and then subsequently cultured in tissue culture medium (NCSU-23) for 44 h at 38°C, and morphologically evaluated using the four graded scale (I, II, III, IV), according to cumulus cells layers and colorization of cytoplasm. After *in vitro* maturation (IVM) the oocytes were stained with porcine pZP3 (goat polyclonal anti-pZP3 Ab, N-20), (Fig. 1A-I, 1B-II, 1C-III, 1D-IV). The treated oocytes were labeled for 40 min with FITC-conjugated anti-goat IgG Ab at a 1 : 200 dilution in PBS. Bars are 50 µm.

During mammalian oogenesis and folliculogenesis the *zona pellucida* is permanently modified. Avilés et al. (5) investigated the oligosaccharide contents in the ovarian *zona pellucida*. They showed that differences in the composition of carbohydrate content of *zona pellucida* is highly related to the activation of oligosaccharides metabolic pathways taking place during folliculogenesis. Furthermore, during each step of folliculogenesis the *zona* glycoproteins are replaced by one new protein, hence the folliculogenesis is crucial for a proper ZP protein composition. However, the most important conclusion from these experiments is that vesicular aggregates in the ooplasm constitute a secretor activity of *zona* glycoproteins.

This experiment, similarly to those previously reported, showed that in matured porcine oocytes the *zona* glycoprotein is expressed in „good" and morphologically poor cells. However, the distribution of this protein is highly related to the morphological type of oocytes. In our previous study we found that pZP3 protein may also be differently distributed in immature and mature oocytes as well as in oocytes which were developmentally incompetent or fully competent (13). Similarly, these experiments indicated that the *zona* glycoprotein may be expressed in both *zona pellucida* and the cytoplasm which is a stage of maturity and, as it was shown in the present study, in a morphological type of a porcine oocytes dependent manner. The modification of porcine *zona pellucida* of a single oocyte following the cortical granule (CG) exocytosis was previously reported by Tatemoto et al. (25). As a result, they found that the modification of *zona pellucida* is

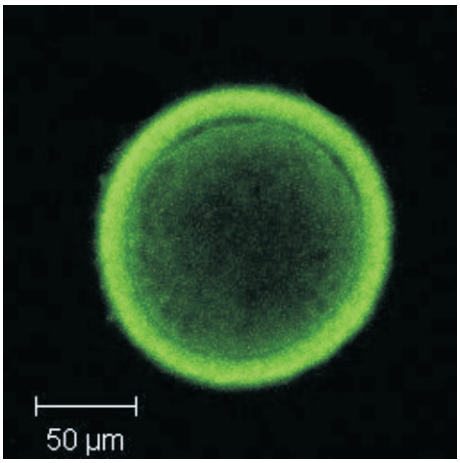

Fig. 2A-I

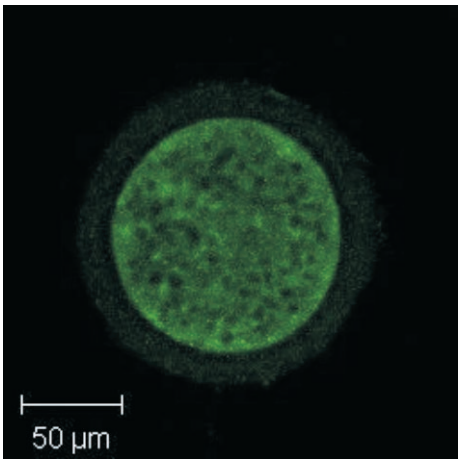

Fig. 2B-II

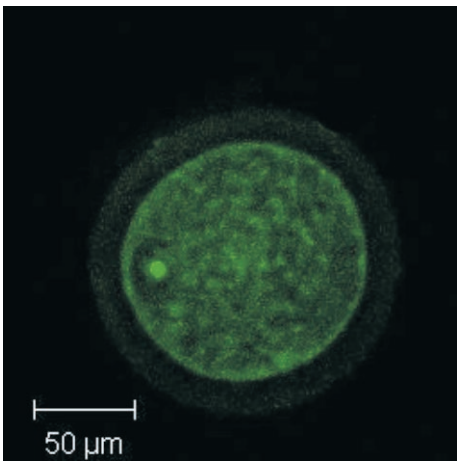

Fig. 2C-III

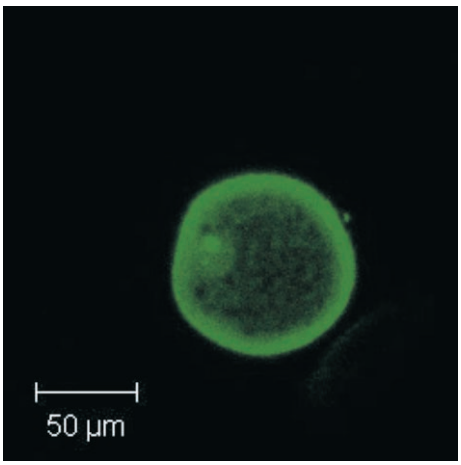

Fig. 2D-IV

Fig. 2. Confocal microscopic observation of ITGB2 distribution in oocytes after *in vitro* maturation (IVM)
After *in vitro* maturation (IVM) the oocytes (n = 100) were stained with porcine ITGB2 (goat polyclonal anti-integrin β2 Ab ,C-20), (Fig. 2A-I, 2B-II, 2C-III, 2D-IV). The treated oocytes were labeled for 40 min with FITC-conjugated anti-goat IgG Ab at a 1 : 200 dilution in PBS. Bars are 50 µm.

related to a decreased amount of ZP1 and ZP2 glycoproteins in the combination with exocytosis of CGs. Moreover, a prolonged period after the oocytes activation seems to be required for a complete *zona* modification.

The experiments showed a specific pZP3 translocation between the *zona pellucida* and oocytes cytoplasm. Bauskin et al. (7) previously showed that human ZP2--specific protease may be released during the cortical granule exocytosis. Moreover, this mechanism was highly related to the stage of meiotic maturity of oocytes, and it was a part of the mechanism of the block to polyspermy. Similarly, our results demonstrated that the porcine ZP3 release is related to the stage of the maturity of oocytes, because the expression of pZP3 protein after IVM was investigated. However, to prove that *zona* glycoproteins expression correlates with the stage of the maturity of porcine oocytes, the immature as well as mature oocytes must be analyzed. In another study, Ducibella et al. (10), using human oocytes as the model, investigated the biochemical changes in the activity and the structure of *zona pellucida*. They showed that changes in ZP biochemistry and the cortical granule quantitation indicated that failed fertilization was associated with cytoplasmic but not nuclear oocytes activation. Hence, it may be suggested that the expression of *zona* glycoproteins may be related to both the cytoplasmic and nuclear activation. Taking into account our results, we suggested that both of these activations during oocytes maturation may also determine the differential distribution of *zona* glycoproteins within the oocytes.

Recently it has been reported by Kempisty et al. (13) that ITGB2 may be expressed in *zona pellucida*, as well as the porcine oocyte's membrane and cytoplasm. Moreover, they showed that a different distribution of ITGB2 is determined by the stage of maturity of oocytes as well as their developmental competence measured by the BCB staining test. They found that a double exposure (before and after IVM) of oocytes to the BCB test leads to the translocation of ITGB2 from *zona pellucida* and oocytes membrane to the cytoplasm. Furthermore, the expression of this protein was significantly decreased after a double exposure to the BCB test as compared to the control and single staining. Hence, it may be suggested that both the expression and distribution of ITGB2 is correlated not only with the stage of maturity or developmental competence, but also with a morphological type of porcine oocytes.

Conclusions

In conclusion, the present study indicates that the expression and distribution of pZP3 and ITGB2 are highly related to the morphological type of porcine oocytes. Moreover, both of these proteins are translocated between *zona pellucida* and oocyte's cytoplasm which is associated with the morphology of the female gamete.

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