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# Expression of Genes Encoding Zona Pellucida Glycoproteins and Cortical Granule Distribution in Porcine Oocytes Isolated from Small and Medium Follicles in Relation to Puberty Status of Donors

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**ABSTRACT**

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The zona pellucida proteins belong to a group of proteins that regulate the processes of gamete recognition, interaction, and fusion, since they are recognized as primary and secondary sperm receptors. It is suggested that cortical granule distribution is significantly associated with the zona pellucida structure and membrane preparation to cortical and acrosome reaction. Therefore, this study investigated the zona pellucida marker gene expression (ZP2 and ZP4 protein) in relation to cortical granules distribution in oocytes and puberty status of donors. Oocytes were collected from adult cyclic sows (isolated from medium and small follicles) and juvenile gilts (isolated only from small follicles). The oocytes were examined by RT-qPCR and by confocal microscopy. The expression of genes for ZP2 and ZP4 protein in oocytes collected from small follicles from

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cycling sows was higher in comparison to oocytes from medium follicles or oocytes collected from small follicles from juvenile gilts ($P < 0.001$). We also observed increased expression of both *ZP2* and *ZP4* mRNA in oocytes collected from small follicles (juvenile gilts) as compared to medium follicles (cycling sows) ($P < 0.001$). Moreover, we found a difference in the distribution of cortical granules. In oocytes from medium follicles, the peripheral localization of cortical granules was twice higher than their central concentration. However, in oocytes derived from small follicles (cyclic sows) cortical granules in comparison to oocytes from medium follicles were more centrally localized ($P < 0.05$). It has been suggested that the donor puberty status and the size of follicles significantly influenced the zona pellucida gene expression and cortical granule localization in porcine oocytes. This is accompanied by fertilization specificity and fertilizability of porcine oocytes *in vitro*.

**Keywords**: pig; ovarian cycle; maturation; developmental competence; folliculogenesis

The mammalian cumulus–oocyte complex (COC) maturation is a compound process that encompasses several steps involving cytoplasmic and nuclear changes. It ultimately leads to the female gamete achieving the MII stage (Kempisty et al. 2011). The proper course of maturation both *in vivo* and/or *in vitro* is regulated by intrinsic and extrinsic factors such as the activity of gap junction connections, the availability of substances for oocyte nutrition, and/or structural and morphological properties of COCs (Kempisty et al. 2014). Moreover COCs expansion and hyaluronic acid expression are valuable markers of COC quality (Zamostna et al. 2016). It has been shown in several studies that the maturation of porcine COCs is highly associated with the size of ovarian follicles (Marchal et al. 2002; Machatkova et al., 2008; Hulinska et al. 2011). Indeed, the COCs collected from follicles of bigger size displayed an increased ability to reach the MII stage. Moreover, it was also reported that follicular size might also influence the fertilization ability of oocytes in pigs (Hulinska et al. 2011).

Cortical granules (CGs) are secretory vesicles present only in female germ cells. In mammals, their diameter ranges from 0.2 to 0.6 μm (Liu 2011). They are continuously formed during the early stages of oocyte growth, and during follicular development and maturation, they translocate from the ooplasm to the cortex until the time of ovulation. This migration of CGs depends on the functioning cytoskeleton and is considered to be critical for successful cytoplasmic maturation, which is characterized by redistribution of organelles. Migration of CGs is a preparatory phase for triggering the cortical reaction, which is a calcium-dependent, exocytotic process started by the sperm–oocyte fusion. It has been experimentally documented that oocytes of prepubertal gilts, prior to the onset of estrus, do not reach optimal developmental competence, resulting in a higher polyspermy rate and significantly lower blastocyst yield than in adult sows despite no differences in nuclear maturation (Marchal et al. 2001; Bagg et al. 2007).

The ability of mammalian oocytes to undergo fertilization is mainly regulated by the expression of genes encoding zona pellucida glycoproteins (ZPs), forming the peptide structure of the zona. It has been well documented that the structure and expression of ZPs significantly influence the first recognition, interaction, and fusion between female and male gametes (Chiu et al., 2008; Wassarman and Litscher 2012; Avella et al. 2014). Therefore, ZPs are recognized as the most important markers of fertilization ability of mammalian oocytes. In rodents, it was shown that knockout models for ZPs (-/-) displayed infertility, identified as an inability to undergo gamete fusion. The structure of the zona pellucida in mice is composed of three major ZPs proteins: ZP1, ZP2, and ZP3 (Rankin et al. 1999). Moreover, in pigs, additional ZP protein ZP4 (also called as ZP3α) has been identified. All of these proteins are involved in fertilization, and ZP2 is implicated as the secondary sperm receptor that participates in gamete fusion soon after the sperm–acrosome reaction. Using an RT-PCR assay of a human oocyte cDNA library, Lefievre et al. (2004) identified the gene sequence encoding the ZP4 protein, also called ZPB. Recent studies describe the function of ZP3 and ZP4 proteins in induction of the acrosome reaction as well as in binding of spermatozoa to zona, which occurs in a time- and dose-dependent manner (Chiu et al. 2008).

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There exists data that identifies the influence of follicle size on *ZPs* gene and protein expression in oocytes in several species of mammals. However, the association between follicle size and/or *ZPs* expression in relation to puberty status of pigs has yet to be investigated. Therefore, the aim of this study was to determine the expression of *ZP2* and *ZP4* mRNAs in oocytes collected from small and medium follicles of cycling sows and small follicles from juvenile gilts. Additionally, the CGs distribution within the cells was also investigated.

## MATERIAL AND METHODS

***Animals.*** The crossbred animals (Landrace × Czech Large White pigs) were used as oocyte donors. Ovaries from adult and juvenile cyclic sows were obtained from a local abattoir and transported to the laboratory within two hours at 31–33°C. The ovaries of each donor were evaluated in terms of corpus luteum morphology and follicle population. For oocyte collection, only ovaries from juvenile gilts and cycling sows from the late luteal to the early follicular phase (before dominant follicle progression) were used as described previously (Hulinska et al. 2011). Only oocytes in germinal vesicle stage were included in this study.

***Sample collection.*** Oocytes were recovered from all follicles located on the ovaries. They were separated from medium follicles with a greater meiotic competence (3–5 mm) and small follicles with a lesser meiotic competence (< 3 mm) by aspiration and cutting of the ovarian cortex, respectively. Oocytes from larger follicles (≥ 5 mm) were not included in this study. From juvenile gilts only oocytes from small follicles were obtained. All healthy COCs with dark, evenly granulated ooplasm and at least two cumulus layers were used for the experiments. After isolation, oocytes were manually denuded from cumulus cells by narrow pipette in TCM-199 medium containing 1% newborn calf serum (NBCS) and 0.1% (w/v) hyaluronidase and transported to 1 ml TRI reagent (T9424) in 1.5 ml Eppendorf-type tube (for RT-qPCR analyses) or to phosphate buffered saline (PBS) with 0.4% bovine serum albumin (BSA) (for cortical granules staining) (all Sigma-Aldrich, USA).

***Immunostaining of cortical granules.*** The methods for staining CGs were based on those reported by Yoshida et al. (1993) and Wang et al. (1997) with a few modifications. Denuded oocytes were fixed in 3.7% paraformaldehyde for 1 h (immediately after denudation) at room temperature, washed in PBS containing 0.4% BSA, and permeabilized with 1% Triton X-100 (Sigma-Aldrich)for 1 h. For CGs staining, oocytes were incubated in 10 µg/ml fluorescein isothiocyanate (FITC) – labelled lectin peanut agglutinin (PNA) in PBS at room temperature for 30 min in a dark box. The nuclear status of oocytes was evaluated by staining the oocytes with a use of Vectashield Antifade Mounting Medium (Vector Lab, USA) containing 1 µM of DNA dye – SYTOX Orange (Invitrogen, USA).

Finally, the oocytes were mounted on slides and observed under a confocal laser microscope Leica TCS SP2 AOBS (Leica, Germany) equipped with Ar and HeNe ion laser. The 488 nm excitation band and 547 excitation band were used for CGs and chromatin visualization, respectively. The 40× Leica HCX PL APO CS objective, pinhole, offsets, gain, and AOBS were adjusted. These parameters were kept throughout the whole experiment. The oocytes were scanned in equatorial optical sections, and microphotographs were saved and processed using the NIS-ELEMENTS AR 3.0 software (Laboratory Imaging, Czech Republic).

***Evaluation of distribution of CGs in immature oocytes.*** A total of 145 immature oocytes (11 batches) were evaluated for CGs distribution. Two parts in each optical section of oocyte were measured: central area that covered 80% and peripheral area that occupied 20% of the optical section (Figure 1). The movement of CGs was assessed by using the index of cortical granules distribution ($I_{cgd}$). The $I_{cgd}$ represents the ratio between the mean intensity signal in the peripheral part of the oocyte and the mean intensity signal in the central part of the oocyte (Figure 1).

***RNA extraction from oocytes.*** Total RNA was extracted from samples using TRI Reagent (Sigma-Aldrich), and RNeasy MinElute cleanup Kit (Qiagen, Germany). The amount of total mRNA was determined from the optical density at 260 nm, and the RNA purity was estimated using the 260/280 nm absorption ratio (higher than 1.8) (NanoDrop spectrophotometer; Thermo Scientific, USA). The RNA integrity and quality were checked on a Bioanalyzer 2100 (Agilent Technologies, Inc., USA). The resulting RNA integrity numbers (RINs) were between 8.5 and 10 with an average of 9.2 (Agilent Technologies, Inc.). The RNA in each

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sample was diluted to a concentration of 100 ng/µl with an OD260/280 ratio of 1.8/2.0.

***RT-qPCR analysis of ZP2 and ZP4 expression in porcine oocytes.*** Reverse transcription was performed using AMV reverse transcriptase (Promega, USA) with Oligo dT (PE Biosystems, UK) as primers at a temperature of 42°C for 60 min (thermocycler UNO II; Biometra, Germany). The primers used were designed using Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, USA). The primers were purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw). RT-qPCR was performed using the Lightcycler 2.0 instrument (Roche Life Science, Germany) with version 4.05 software. Using the above-mentioned primers, the SYBR Green detection system was utilized. Each of 20 µl reaction mixtures contained 4 µl template cDNA (standard or control), 0.5 µM of every gene-specific primer, and a previously determined optimal $MgCl_2$ concentration (3.5 µM for one reaction). LightCycler® FastStart DNA Master SYBR Green I mix (Roche Diagnostic, Germany) was used. The real-time PCR program included a 10 min denaturation step to activate the *Taq* DNA polymerase followed by a three-step amplification program: denaturation, annealing, and extension. The specificity of the reaction products was checked by determination of melting points (0.1°C/s transition rate). The results of mRNA expression encoding *ZP2* and *ZP4* genes were quantified in relation to two housekeeping genes, *HPRT* and *ACTB*.

***Statistical analysis.*** One-way ANOVA, followed by Tukey's post-hoc test, was used to compare the results of the real-time quantification of the proliferation index. The experiments were carried out in at least three replicates. The differences were considered to be significant at $P < 0.01$, and $P < 0.001$ for the quantitative analyses of mRNA expression. The software program GraphPad Prism version 4.0 (GraphPad Software, USA) was used for the statistical calculations.

Microphotographs of CGs distribution from confocal microscopy were saved and processed using the NIS-ELEMENTS AR 3.0 software (Laboratory Imaging). The values of $I_{cgd}$ were statistically analyzed by the independent samples *t*-test using STATISTICA CZ, version 10 software (StatSoft, Inc., Czech Republic). Differences at $P < 0.05$ were considered statistically significant.

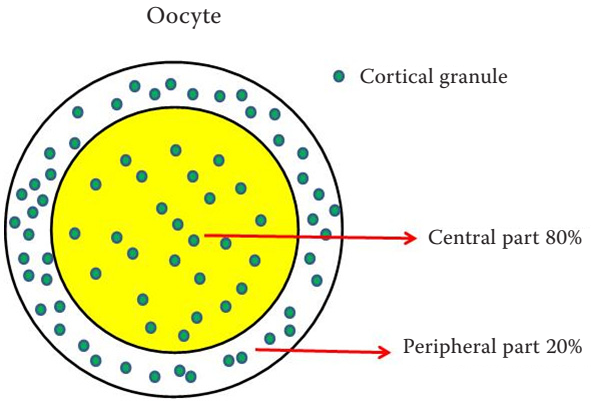


Figure 1. Representative picture of cortical granules (CGs) distribution

The figure shows the basic principle of analyses used for images from confocal microscopy. Each picture has been divided to central and peripheral part of oocyte. On the basis of a manually selected marginal area, we used NIS ELEMENTS AR 3.0 software for automatic selection only 20% under the selected region of interest. We then evaluated the average intensity of the marginal area (AIMA) and average intensity of the central area (AICA) and calculated the value of the index of cortical granules distribution ($I_{cgd}$) as $I_{cgd}$ = AIMA/AICA

## RESULTS

Utilizing RT-qPCR, we analyzed *ZP2* and *ZP4* mRNA expression in 100 denuded porcine oocytes (three times) isolated from medium follicles cycling sows (MFCS), small follicles juvenile gilts (SFJG), and small follicles cycling sows (SFCS). We found an increased expression of *ZP2* mRNA in oocytes isolated from small follicles (cycling sows) as compared to medium follicles (cycling sows) ($P < 0.001$). Moreover, the expression of the *ZP2* transcript was higher in oocytes collected from small follicles (juvenile gilts) as compared to oocytes from medium follicles (cycling sows) ($P < 0.001$). We observed a statistical difference in *ZP2* gene expression between oocytes isolated from small follicles (juvenile gilts) and oocytes from small follicles (cycling sows) ($P < 0.01$) (Figure 2A). The *ZP4* transcript level was also increased in oocytes collected from small follicles (cycling sows) as compared to medium follicles (cycling sows) and small follicles (juvenile gilts) ($P < 0.001$ for both groups, respectively). Furthermore, we

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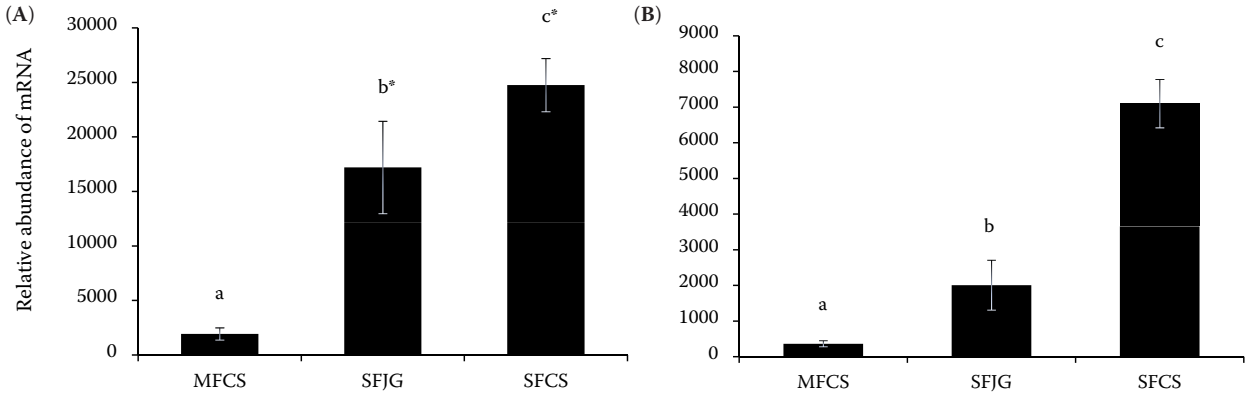


Figure 2. Relative abundance of *ZP2* and *ZP4* transcripts in porcine oocytes

Porcine oocytes after isolation were immediately used to isolate RNA, which was then reverse-transcribed into cDNA. Presence of *ZP2* (**A**) and *ZP4* (**B**) transcripts was evaluated by RT-qPCR analysis

MFCS = oocytes isolated from medium follicles of cycling sows, SFJG = oocytes from small follicles of juvenile gilts, SFCS = oocytes from small follicles of cycling sows

Results are presented as mean ± SEM with the level of significance of $P < 0.001$ ($^*P < 0.01$)

$^{a–c}$values with different superscripts are significantly different

observed an increased expression of *ZP4* mRNA in oocytes collected from small follicles (juvenile gilts) as compared to medium follicles (cycling sows) ($P < 0.001$) (Figure 2B).

Distribution of CGs was analyzed in totally 145 oocytes. Oocytes were derived as described before and selected into 3 groups – MFCS, SFJG, and SFCS. As shown in Figure 3, the difference of the $I_{cgd}$ values between oocytes with higher and lower meiotic competence derived from medium and small follicles, respectively, was statistically significant ($P < 0.05$). The value of $I_{cgd}$ in SFCS oocytes, with lower meiotic competence, ($I_{cgd}$ = 1.78 ± 0.07) shows a tendency for central localization of CGs in the cytoplasm of oocytes derived from small follicles. Conversely, in MFCS oocytes (with higher meiotic competence), the peripheral concentration of CGs was more than twice higher ($I_{cgd}$ = 2.24 ± 0.09) than their concentration in the central part (Figures 3 and 4). In small follicles from juvenile animals (SFJG), the distribution of CGs was lower but similar to SFCS, and we did not find significant differences between $I_{cgd}$ in juvenile oocytes and the other oocyte groups.

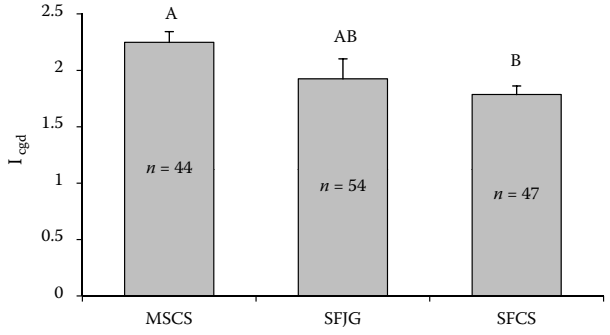


Figure 3. Index of cortical granules distribution ($I_{cgd}$) in porcine oocytes

Different values of $I_{cgd}$ in immature oocytes derived from small follicles of juvenile animals (SFJG) and cyclic animals. From cyclic sows, ovaries were recovered from small follicles (SFCS) and medium follicles (MFCS). Cortical granules were visualized with FITC-PNA, and oocytes were examined by confocal microscopy

$^{A–B}$values with different superscripts are significantly different ($P < 0.05$)

## DISCUSSION

The proper developmental capability of mammalian COCs is achieved during the stages of folliculogenesis and oogenesis. During these phases of oocyte growth, the female gametes reach maturation (measured by reaching MII stage) and fertilization capability, which is reflected in the ability of gametes to recognition, interaction, and fusion. Mammalian oocytes are surrounded by a protein membrane called zona pellucida, which is built from zona pellucida proteins 1, 2, 3, and 4 (ZP1, ZP2, ZP3, ZP4) (Wassarman 2008). Generally, these proteins are recognized as the primary and

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secondary sperm receptors, playing a significant role in gamete fusion during successful monospermic fertilization (Topfer-Petersen 1999). It has been previously shown in several species that oocytes lacking ZPs proteins are unable to fertilize (Harada et al. 2003; Wassarman and Litscher 2012; Huang et al. 2014).

Ovarian follicle size significantly influences the ability of oocytes to mature and the gamete fertilizability. It was also demonstrated that oocytes isolated from large and medium follicles are characterized by their increased ability to grow and develop (Eppig et al. 2002). Moreover, oocytes recovered from follicles of a larger size have a higher probability to form a zygote and develop embryos in the preimplantation stage (Gandolfi et al. 1997; Hulinska et al. 2011).

The CGs are cytoplasmic organelles of oocytes, secreting factors that protect the oocytes from polyspermy during acrosome and cortical reactions. It was demonstrated that the cortical reaction might be associated with alternations in the zona pellucida structure, also called the zona reaction (Sun 2003). Therefore, in this study, we focused on the CGs structure in correlation with *ZPs* mRNA expression in pigs. Additionally, we aimed to determine if pig ovarian follicle size and puberty status of donor females are orchestrated by CGs distribution and *ZP2*/*ZP4* transcript levels.

Although the structure and function of zona pellucida proteins in normal and knockout animal models are well recognized, the expression of *ZPs* in porcine oocytes in relation to ovarian follicle size is not entirely known. We have previously described the association between the follicle size and expression of *ZP1*, *ZP2*, *ZP3*, and *ZP4* (also referred to as *ZP3α*) in porcine oocytes isolated from pubertal gilts (Kempisty et al. 2009). In these experiments, COCs were collected from small (< 3 mm), medium (3–5 mm), and large (> 5 mm) follicles and cultured *in vitro* for 44 h. We found significantly increased expression of *ZP2* and *ZP3* mRNA in oocytes isolated from medium follicles as compared to small follicles. However, when looking at all transcripts, the highest levels were detected in oocytes collected from large follicles. These results were also confirmed on a protein level using Western blot and a confocal microscope analysis. It was suggested that the expression of markers responsible for the fertilizability of porcine oocytes is highly associated with and regulated by the ovarian follicular size in pubertal gilts.

There is also data that indicates that the CGs distribution within oocytes is related to applied gamete's preservation methods. Recently, Wang et al. investigated human ovarian cortex cryopreservation and its effect on single follicle development (Wang et al. 2016). They analyzed the expression of selected genes, including *ZP3* mRNA, in oocytes and CGs using single-cell mRNA assays. They observed that *ZP3* mRNA was downregulated during both slow freezing and vitrification procedures. Single follicle growth was significantly higher in the fresh (control) group as compared to slow freezing and vitrification groups. They concluded that follicle damage that occurred during *in vitro* culture, resulting in lower mRNA expression, must be verified in the future during both slow freezing and vitrification procedures.

In the current study, we found an increased expression of both *ZP2* and *ZP4* mRNA in oocytes isolated from small follicles of both juvenile gilts

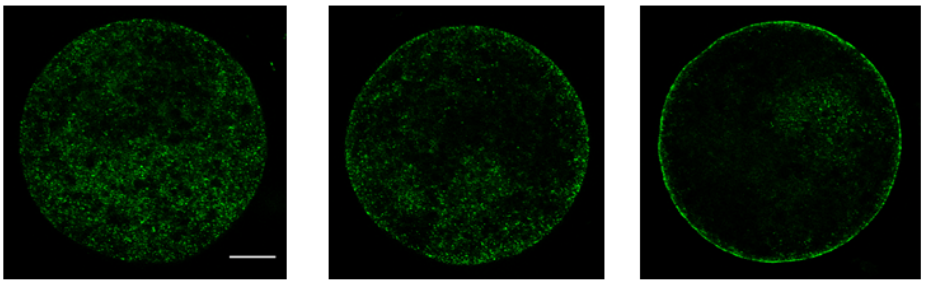

Figure 4. Localization of cortical granule (CG) within porcine oocytes isolated from follicles in different size
Representative images of CG localization in oocytes from small follicles of juvenile animals (SFJG) (**A**), from small follicles of cyclic sows (SFCS) (**B**), and from medium follicles of cyclic sows (MFCS) (**C**). Oocytes were stained with PNA-FITC; scale bar = 20 μm

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and cycling sows as compared to oocytes from medium follicles of cycling sows. These experiments show that puberty status of donors does not always correspond to the expression of fertilizability markers. Additionally, in the separated group of cycling gilts, the follicle size determined the expression of *ZPs* transcripts. Surprisingly, the highest expression of genes in oocytes isolated from small ovarian follicles was detected. The increased expression of both *ZPs* mRNAs was observed in cycling sows as compared to juvenile gilts, which may suggest higher fertilizability of these oocytes. We conclude that the donor puberty status significantly regulates fertilizability of oocytes in pigs.

The cytoplasmic maturation of oocytes is influenced by the size of the follicle from which the oocyte has been recovered. It was previously reported that porcine oocytes, after isolation from small and medium follicles, differ in ATP content, formation of metabolic units, and number of lipid droplets (Milakovic et al. 2015). The distribution of CGs is a very important cytoplasmic marker of oocytes. Cortical granules, as well as zona pellucida, are important components of a polyspermy block. It was previously reported that porcine oocytes derived from small follicles (with lower maturation competence) have a higher incidence of polyspermy penetration during *in vitro* fertilization as opposed to oocytes from bigger follicles (with high maturation capacity) (Hulinska et al. 2011). Our results indicate that the distribution of CGs in germinal vesicle (GV) oocytes is different during folliculogenesis. In oocytes from medium follicles, we found a higher distribution of CGs in peripheral areas of oocytes contrary to those from small follicles in cyclic sows. It was previously reported that CGs migrate to the cortex in pig oocytes during *in vitro* maturation (Wang et al. 1997). These results indicate the beginning of cytoplasmic maturation in GV oocytes before their aspiration from medium follicles. However, we did not observe significant differences in CGs distribution in oocytes isolated from small follicles between juvenile gilts and cycling sows. These results indicate an undetermined status of these oocytes before their selection for subsequent development in the ovarian cortex.

In the present paper the cytoplasmic parameters of oocytes in animals showing different puberty status were assessed. These results can contribute to better understanding the cytoplasmic changes of oocytes during folliculogenesis.

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