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Zona pellucida glycoprotein 3 (pZP3) and integrin β2 (ITGB2) mRNA and protein expression in porcine oocytes after single and double exposure to brilliant cresyl blue test

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Abstract

Brilliant cresyl blues (BCB) staining test is a useful tool in assessing the competence of cumulus-oocyte-complexes (COCs) in several mammalian species. It is mostly used to select gametes after they are recovered from the ovary or before and after IVM to isolate those oocytes that reach developmental competency. However, there is evidence that double exposure to BCB test may lead to impaired fertilization or even have a toxic effect on cells.

The aim of the present study was to investigate the expression pattern of sperm-egg interaction molecules in oocytes after single and double exposure to BCB test.

Follicles were dissected from porcine ovaries after slaughter and aspirated COCs were cultured in standard porcine IVM culture medium (TCM 199) for 44 h. The BCB test was applied to COCs before and after IVM. In developmentally competent oocytes, assessed by determining the activity of glucose-6-phosphate dehydrogenase (G6PDH; BCB test), real-time quantitative PCR reaction methods, western blot and confocal microscopy analysis were applied to determine the transcript levels of porcine *zona pellucida* glycoprotein 3 (pZP3), and integrin beta 2 (ITGB2), as well as the levels of pZP3 and ITGB2 proteins. In the control group, assessment of the expression of the investigated genes was performed before and after IVM without BCB test.

We observed a significantly higher level of pZP3 mRNA in oocytes after single exposure to BCB test compared to control before and after IVM ($P < 0.001$), and to double staining ($P < 0.05$). The level of ITGB2 mRNA was also increased in gametes after single exposure to BCB test as compared to control before and after IVM ($P < 0.001$, $P < 0.01$, respectively), and double staining ($P < 0.05$). Western blot analysis demonstrated a higher level of pZP3 protein in oocytes after single staining with BCB as compared to control both before and after IVM ($P < 0.001$, $P < 0.05$, respectively) and double staining ($P < 0.05$). Confocal microscopic observations have revealed the same pattern of increased level of pZP3 and ITGB2 expression after single exposure to BCB test. In both cases we detected specific cytoplasmic localization of both proteins. The ITGB2 protein has *zona pellucida* and membrane localization in control oocytes before IVM. After IVM and after single exposure to BCB, ITGB2 was also strongly detected in the cytoplasm. In both cases, after double exposure to BCB both proteins were detected only partially in the cytoplasm.

Our results suggest that (i) single exposure to BCB increased the expression of sperm-oocyte interaction genes, (ii) double exposure to BCB leads to only partial expression of pZP3 and ITGB2 in oocyte cytoplasm, (iii) the BCB staining test itself may

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be a cause of specific pZP3 translocation from the *zona pellucida* to the cytoplasm, and that (iv) *in vitro* maturation of oocytes may increase ITGB2 expression and translocation from the *zona pellucida* to the cytoplasm.
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1. Introduction

Fertilization is a complex and unique process which needs several biochemical mechanisms, which are dependent upon species specific gamete recognition, egg penetration by sperm, and successful gamete fusion. The eggs are surrounded by an extracellular matrix, which is specific in morphological composition and biochemical ultrastructure among various species and phylogenetic groups. However, this extracellular matrix always contains several glycoproteins that form the structure of the *zona pellucida*, called *zona pellucida* glycoproteins (ZPs). There are three major glycoproteins described as ZP1, ZP2, and ZP3 [1–4]. In pigs however, an additional glycoprotein called pZP3 α (pZP4) has been described [5,6]. Several reports support that ZPs play a role in fertilization in almost all species of mammals. However, most of the findings come from mouse model experiments [7–10]. Therefore, this study was aimed to present additional information based on a porcine model. Sinowatz et al [11] used immunohistochemical and *in situ* hybridization studies to show the contribution of ZP2 and of ZP3 in the matrix assembly for both oocytes and surrounding follicular cells. ZP2 is recognized as a sperm receptor, however maximal sperm binding is achieved until ZP2 forms heteromultimeric complexes with ZP3 [12]. The origin and structural organization of the mammalian ZP glycoproteins is still controversial. Several experiments have indicated that ZP3 mRNA and protein are synthesized in secondary follicles in both oocytes and follicular cells. In the tertiary and preovulatory follicles the mRNA and ZP3 protein are mainly found in the cytoplasm of corona radiata cells. These results indicated that ZP3 expression and the spreading or cell specific localization of ZP3 may be an important factor in the formation of the matrix from oocytes and surrounding follicular cells [11]. Moreover, the role of ZP3 glycoprotein in fertilization was intensively investigated in several species of mammals. In pigs it was shown that ZP3 may play a crucial role in fertilization and that this protein is influenced by factors such as the age of donors, follicular size, or oocyte morphology [1,3,4]. Moreover, it was demonstrated that human ZP3 protein may be a useful tool in studying the initial stage of the human fertilization process and might have clinical applications in the diagnosis of human male infertility [13]. A further study revealed that specific oligosaccharide chains on the ZP3 glycoprotein are essential for sperm receptor activity [14]. Therefore, the role of ZP3 glycoprotein in immature and mature porcine oocytes was investigated.

Integrins form specific oocyte membrane receptors for their ligands: fertilin α, β and cyritestin. The $\alpha6\beta1$ integrin was the first integrin identified in the cell membrane. Several lines of experiments using mouse $\alpha6\beta1$ $(-/-)$ knock out model indicated that this integrin may play a subordinate role in the process of sperm-egg interactions, as other integrins also identified in the oocyte support their function as oocyte receptors [15–17]. By contrast, other reports have indicated the main role of integrins in fertilization [18–20]. Miller et al [18] suggested that integrins are essential proteins for sperm-oocyte adhesion rather than for gamete fusion.

It has previously been reported that the brilliant cresyl blues (BCB) test is one important indicator in the selection of developmentally competent oocytes [21–25]. This test is based on the determination of glucose-6-phosphate dehydrogenase (G6PDH) activity, which is synthesized in growing oocytes but is inactive in the oocytes that have completed their growth phase. Decreased expression or loss of expression of G6PDH is highly associated with oocytes that have become competent. These oocytes fail to enzymatically break down BCB and thus stain positively blue (BCB^+) [22,25]. The oocytes that have not reached their competency are colorless (BCB^-). Wongsrikeao et al [25] demonstrated that double staining with BCB (before and after IVM) affects fertilization and embryonic development. Moreover, single application of BCB (before IVM) may be used as a test to select gametes for IVF. However, the molecular principle of selecting competent oocytes by using the BCB test, with respect to the expression of genes responsible for oocyte fertilization potential, is still unknown. Therefore, the aim of this study was to determine the porcine *zona pellucida* glycoprotein 3 (pZP3) and integrin $\beta2$ (ITGB2) transcript and protein levels after single and double exposure to BCB.

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