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Transcript abundance, glutathione and apoptosis levels differ between porcine oocytes collected from prepubertal and cyclic gilts

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ABSTRACT

It is well known that puberty has a strong impact on oocyte developmental competence *in vitro*; however, the reason for this phenomenon at the cellular level has not been clarified yet. It is hypothesized that cytoplasmic maturation is responsible for oocyte quality and may be impaired in prepubertal gilts. Previous results on mitochondrial DNA copy number and mitochondria and cortical granule distribution showed that cytoplasmic maturation is a complex trait and should include multithreaded analysis. Therefore, the aim of the present research was to analyze the transcript abundance of developmentally important genes (*BMP15*, *GDF9*, *GSTA2*, *ATP5A1*, *EEF1A1*, *BAX*, *BCL2*) followed by investigation of the glutathione and apoptosis level in oocytes of prepubertal and cyclic gilts. We found differences in relative transcript abundance of *BMP15* and *GDF9* genes after IVM, whereas different concentrations of glutathione were noted before IVM (5.3 vs. 2.9 pmol, respectively). The glutathione level was equalized after IVM (10.3 vs. 9.1 pmol), whereas the incidence of apoptosis remained similar before (3.9% vs. 1.1%) and after IVM (4.5% vs. 1.9%) being higher in prepubertal oocytes. A potential impact of gilt puberty on oocyte quality has been therefore masked by the significant effect of IVM. Because the maternal effect genes, *BMP15* and *GDF9*, play key roles in regulation of folliculogenesis and oocyte–cumulus interaction, their upregulation in oocytes of cyclic gilts may result in increased developmental competence. On the basis of findings from this and our previous research, we suggest that the reduced quality of oocytes from prepubertal females is a complex phenomenon and is not related to a single marker trait.

1. Introduction

There is a lot of published evidence concerning factors affecting oocyte and embryo quality, but there are relatively few studies that focus on the effect of sexual maturity of the donor female on the developmental competence of oocytes. Studies performed on oocytes from prepubertal females (pig, cattle, goat, sheep, rhesus monkey) revealed a reduced oocyte quality. This reduction resulted in a decreased proportion of embryos reaching more advanced preimplantation stages [1–10]. The majority of reports, though, compare oocytes from adult females with those from prepubertal heifers or gilts. Prepubertal females represent a vast majority of oocyte donors used for scientific experiments. The use of prepubertal oocytes with reduced developmental competence negatively affects the efficiency of several biotechniques such as IVF or SCNT [11].

The effect of sexual maturity on the quality of oocytes is described in a few studies. Menino et al. [12] collected Day-2 embryos from the same gilt at the first and third estrus cycle and cultured them *in vitro* till the blastocyst stage. Embryos derived from oocytes ovulated at the third cycle

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more often reached the blastocyst stage. On the other hand, oocytes ovulated at the first estrus were more often aneuploid and were characterized by a higher embryo mortality (33.3% vs. 10.6% for third estrus oocytes) [13,14]. The extent of embryo mortality depends more on embryo quality than on the local conditions in the female genital tract. This was reported by the progesterone and estrogen concentration measurement and embryo transfer in gilts at the first and third estrus [15]. In prepubertal gilts, the developmental competence of oocytes increased with the follicle diameter, whereas the quality of oocytes from cyclic gilts was not affected by the follicle size [16,17]. It is the mechanisms that are responsible for the reduced quality of oocytes from prepubertal gilts that need further study.

It is evident, that oocytes and embryos from gilts which have not reached sexual maturity display a reduced developmental competence both *in vitro* and *in vivo*. Despite a similar meiotic competence of oocytes from prepubertal and cyclic gilts, fewer blastocysts are produced from prepubertal oocytes. The suggestion is that nuclear maturation is not a reliable marker of oocyte quality. Other factors or mechanisms may be responsible for the reduced quality of oocytes from prepubertal gilts. A hypothesis about insufficient cytoplasmic maturity and about the direct or indirect impact of the oocyte growth environment cannot be excluded [17,18]. The better oocyte quality of adult sows was also confirmed by analysis of SCNT embryos [11,19]. The oocytes of the adult females were much better recipients of somatic cell nuclei. This finding proves that cytoplasmic maturation is essential for shaping the developmental competence of the oocyte and the embryo [2,20–24]. Cytoplasmic maturation includes such intracellular processes as transcription, protein synthesis, and distribution of mitochondria and cortical granules. These processes reflect the degree of oocyte preparation for fertilization and the correctness of cellular mechanisms [25–27]. Recently, Paczkowski et al. and Yuan et al. [20,28,29] noted a decreased quality of prepubertal oocytes confirmed by the reduced expression of genes involved in metabolism and genes engaged in response to oxidative stress.

Our previous research showed that cytoplasmic maturation is a complex phenomenon and should be analyzed on several levels. Additionally, we have found different fatty acid profiles in follicular fluids (FFs) of prepubertal and cyclic gilts, which might influence the developmental competence of oocytes *in vitro*. Because mitochondrial DNA copy number and cortical granule redistribution did not differ between the prepubertal and cyclic oocytes [30], we decided to search for other cytoplasmic factors potentially responsible for the reduced quality of the oocytes from prepubertal gilts. We investigated the relative transcript abundance of seven genes related to oocyte quality, glutathione concentration, and the frequency of apoptosis.

2. Materials and methods

Unless otherwise stated, all chemicals and reagents used in this study were purchased from Sigma–Aldrich (Germany). The ovaries utilized in our experiment were collected *postmortem* from slaughtered gilts.

2.1. Recovery of cumulus–oocyte complexes

The methodology used for collecting cumulus–oocyte complexes (COCs) and the methodology used for IVM have been described previously [30]. Briefly, COCs were collected from the ovaries of commercially slaughtered crossbred gilts. The gilts were 5 to 6 months old and weighed approximately 100 to 110 kg. The ovaries were transported to the laboratory in a thermo isolated flask within 2 hours after the animals had been slaughtered. On arrival, the ovaries were divided into two groups on the basis of their morphology: prepubertal (lack of corpus luteum, several 2–5-mm follicles) and cyclic (presence of corpus luteum or albicans and some 2–5-mm follicles). A syringe was used to aspirate the COCs, which were then placed in HEPES-Tyrode albumin lactate pyruvate medium and morphologically evaluated under a stereomicroscope. Only COCs with evenly granulated cytoplasm and at least three compact cumulus cell layers were selected. Half of the collected COCs were analyzed directly after collection. The other half were subjected to IVM and analyzed afterward. Four groups of COCs were investigated: (1) prepubertal (without IVM), (2) prepubertal IVM, (3) cyclic (without IVM), and (4) cyclic IVM. Each of the investigated parameters was analyzed in three replicates (three independent pools of oocytes) before IVM and in three replicates after IVM.

2.2. In vitro maturation

In vitro maturation was carried out in the basic NCSU23 medium supplemented with 10% (v:v) porcine FF, 10 U/mL pregnant mare's serum gonadotropin (Folligon; Intervet), 10 U/mL hCG (Chorulon; Intervet), 0.1 mg/mL cysteine, and 50 μg/μL gentamicin sulfate. The maturation protocol involved two steps: (1) an initial 20 hours of culture in hormone-supplemented NCSU23 medium and (2) an additional 24 hours of incubation in freshly made hormone-free medium. Incubation was performed in 500-μL IVM medium (approximately 70 COCs per droplet) in four-well plates (NUNC) at 39 °C in a 5% CO_2 humidified atmosphere. Preparation of the FF included the aspiration of the FF from the ovarian follicles (3–6 mm) and the transfer to the conical tubes. The collected FF was centrifuged at 3000 rpm for 30 minutes, and aliquots of the resulting supernatant were frozen in liquid nitrogen in Eppendorf tubes. Follicular fluid was stored for 1 month and used to supplement the IVM medium.

2.3. Relative transcript abundance

The gene expression level was inferred by measuring the messenger RNA level (relative transcript abundance, RA) in samples containing 25 oocytes, in six independent replicates in each of the four experimental groups. The isolation of RNA was done according to the protocol for the mirVana PARIS Kit (Ambion, Life Technologies). The oocytes were lysed in cell disruption buffer and denaturing solution. After extraction using acid phenol:chloroform, the RNA was transferred to column and washed several times. Total RNA was resuspended in 100 μL DEPC water. RNA was precipitated using NF Pellet Paint Co-Precipitant (Novagen,