IDENTIFICATION OF THE LIPID DROPLETS OF IMMATURE PORCINE OOCYTES DURING THE DIFFERENT STAGE OF FOLLICULOGENESIS

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ABSTRACT

The meiotic and growth competence of oocytes originating from larger follicles is greater then of oocytes derived from small follicles. Lipid droplets stored in oocytes have been shown to be accumulated in oocytes during follicular development, and have large influence on quality of oocytes during the process of meiotic maturation, fertilization and preimplantation development.

The principal objective of this research was to investigate the lipid compound of the porcine immature oocytes during the luteal and follicular phases of folliculogenesis. The oocyte subpopulations for this experiment were obtained from small (<5 mm) and medium (6-9 mm) follicles. The oocytes were stained with Nile Red to quantify cytoplasmic lipid droplets as small (≤10µm in diameter) and large (>10µm in diameter). We observed following parameters in immature oocytes: total area covered with lipid droplets (%), total number of lipid droplets per oocyte, number of small lipid droplets and number of large lipid droplets.

The total of area covered with lipid droplets is similar in oocytes derived from small and medium follicles. The total number of all lipid droplets and number of small lipid droplets were significantly higher ($P < 0.05$) in oocytes derived from small than medium follicles. We found that the total number of lipid droplets is significantly higher in the late luteal phase in oocytes derived from medium follicles, than all stages of luteal phase in oocytes derived from small follicles during the process of folliculogenesis. We also noted that the number of small lipid droplets was significantly higher in oocytes derived from the late luteal phase in oocytes derived from medium follicles in comparison to oocytes derived from the middle and the late stage of the luteal phase in oocytes derived from small follicles. The number of large lipid droplets was significantly higher in the late luteal phase in oocytes derived from medium follicles compared to the late luteal and the early follicular phase in the oocytes derived from small follicles. In the early luteal phase in the oocytes derived from small follicles the number of large lipid droplets was significantly higher in comparison to the early follicular phase in oocytes derived from small follicles.

In conclusion, the lipid composition in the immature porcine oocytes varied during the different phases of folliculogenesis. The number of small lipid droplets was higher in oocytes derived from medium follicles than in oocytes derived from small follicles.

Key words: porcine, oocytes, folliculogenesis, lipid droplets, Nile red

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INTRODUCTION

The oocyte is the largest cell in the female mammal, is known to contain substantial endogenous energy stores to use during meiotic maturation, fertilization and preimplantation development (Ambrousi et al., 2009). For successful maturation of the oocytes and progression of early embryos, the amount of cytoplasmic lipids may large influence (Jeong et al., 2009). This is especially manifest in oocytes of domestic mammals, where very high levels of lipids have been described (McEvoy et al., 2000).

The lipids are a structural component of a cell membrane and cytoplasm (Gajda 2009), the majority of them in form of triglycerides and assembled in lipid droplets (LD) a few times surrounded by a phospholipid monolayer (Ostermeyer et al., 2001). The porcine oocytes contains 156 ng of lipids (McEvoy et al., 2000) are categorized by a high level of the lipid content in compared with other animal species (Nagashima et al., 1994). Lipid plays an important role in energy storage, cell structure and in modifying the physical properties and metabolic function of biological membranes (Kim et al., 2001).

In a recent years, a new and reliable method was developed to evaluate the lipid content of single mammalian oocytes. This method can be performed by staining the oocytes with a fluorescent probe Nile Red which is the most specific probe used for identification of intracellular lipid droplets (Genicot et al., 2005).

The amount of emitted fluorescent light correlated with the lipid content. This technique could be used to analyze the lipid content of oocytes from different sized follicles, originating from different donors, or cultured in various conditions.

In the present study, lipid droplets were observed in immature porcine oocytes to examine the changes in the number of their cytoplasmic inclusion. The aim of this research was to compare the lipid content of immature porcine oocytes, derived from small and medium follicles in germinal vesicle stage (GV). Ìn this research the following parameters were examined: total area covered with lipid droplets, total number of the lipid droplets in an individual oocytes and number of small and large lipid droplets.

MATERIAL AND METHODS

The porcine ovaries were obtained from a slaughterhouse are selected according to luteal (early, middle and late) and follicular (early and late) phases of folicullogenesis.

Oocyte staining

The immature (GV) oocytes derived from small and medium follicles by aspiration and cutting of ovarian cortex. Oocytes in germinal vesicle stage were denuded of cumulus cells manually in TCM-199 medium containing 1% NBCS (Newborn calf serum) and 0.1% (w/v) hyaluronidase (Sigma Aldrich). After washing all processed oocytes fixed in a 500 µl 3.7% paraformaldehyde solution for 60 min at room temperature. They were washed in PBS and permeabilized with 1% TRITON X-100. The lipids of the oocytes were stained in PBS supplemented with 0.04% BSA and 1µM Nile red (Sigma Aldrich) for 10 min at room temperature.

After staining oocytes were washed in PBS and mounted on glass slides, without oocyte compression, using Vectashield medium (Vector Lab) containing 1 µM of DNA dye (TO-PRO 3, Invitrogen) for identification of nuclear stage. The oocytes were stored below 0ºC until examined.

Oocyte examination

The oocytes were examined with the use of a laser scanning confocal microscope (Leica TCS SP2 AOBS; Leica, Heidelberg, Germany) equipped with Ar and HeNe lasers. The 488 nm excitation band and 570–667 nm detector were used for lipid droplets visualization and 633 nm excitation band and 635-713 nm detector for detection of chromatin. The 40x Leica HCX PL APO CS objective, pinhole, offsets, gain and AOBS were adapted. These parameters were kept throughout the whole experiment. The oocytes were scanned in equatorial optical section, micro photographs were saved and processed using the NIS – Elements AR 3.00 software.

Statistical analysis

All data were subjected to one-way ANOVA, and the significance of difference among means was determined by the Fisher's least significant difference (LSD) test (StatSoft, Inc. 2009. STATISTICA, data analysis software system, version 9.0.). Differences at $P<0.05$ were considered statistically significant.

RESULTS

Oocytes from small and medium follicles regardless of the phase

The following parameters: total area covered with lipid droplets and total number of large lipid droplets (LD) were approximately similar between the subpopulation of the oocytes derived from small and medium follicles, regardless of the phase. On the contrary, total number of all lipid droplets and number of small lipid droplets were significantly different ($P < 0.05$) between oocytes derived from small and medium follicles, regardless of the phase (Table 1.).

Table 1.Characteristics of oocytes from small and medium follicles regardless of the phase

Follicle size	*n*	*Area of lipid droplets(%)*	*Total number of lipid droplets*	*Small lipid droplets(n)*	*Large lipid droplets(n)*
Small	136	29.16±6.17[a]	338.93±82.22[a]	178.70±61.58[a]	160.24±46.70[a]
Medium	74	30.25±7.18[a]	376.32±94.97[b]	205.72±74.69[b]	170.61±50.17[a]

Data with different superscripts within the same column and inside the same group are significantly different (a–b, $P < 0.05$).

Oocytes from small and medium follicles as related to the phase

The subpopulation of oocytes derived from small follicles varied in the total area of oocytes covered with lipid droplets evaluated during the folliculogenesis, from the early luteal to the early follicular phase (Table 2.). The total area of oocytes covered with lipid droplets is lower in the late luteal phase in oocytes derived from the small follicles than in the late luteal phase in oocyte derived from medium follicles. During the early follicular phase area covered with lipid droplets in oocytes derived from medium follicles is higher than in oocytes derived from small follicles. The total area of oocytes covered with lipid droplets did not significantly differ throughout either the luteal or the follicular phase.

Table 2. The total area of oocytes covered with lipid droplets in the luteal and the follicular phase

	n	*Phase*	*Stage*	*Total area of lipid droplets %*
Small	45	Luteal	early	29.28±7.19[a]
	34		middle	29.16±5.50[a]
	28		late	29.43±5.85[a]
	29	Follicular	early	28.69±5.79[a]
Medium	41	Luteal	late	31.15±6.75[a]
	33	Follicular	early	29.13±7.65[a]

The total number of lipid droplets is higher in the early follicular phase in oocyte derived from medium follicles in comparison to oocytes derived from small follicles. In the late luteal phase total number of lipid droplets in oocytes derived from small follicles is lower than in oocytes derived from medium follicles. The total number of lipid droplets is significantly higher in the late luteal phase in oocytes derived from medium follicles, than in all stages of luteal phase in oocytes derived from small follicles (Table 3.).

Table 3. The total number of lipid droplets in the luteal and the follicular phase

	n	*Phase*	*Stage*	*Total number of lipid droplets*
Small	45	Luteal	early	350.64±83.05[a]
	34		middle	339.82±87.33[a]
	28		late	330.93±78.78[a]
	29	Follicular	early	327.45±79.75[a]
Medium	41	Luteal	late	387.71±98.84[b]
	33	Follicular	early	362.18±88.17[a,b]

Data with different superscripts within the same column and inside the same phase are significantly different (a–b, $P < 0.05$).

The number of small lipid droplets is higher in the early follicular and late luteal phases derived in oocytes from medium follicles in comparison to oocytes derived from small follicles. (Table 4.). Correspondingly, small lipid droplets of the cytoplasm area was significantly higher in the late luteal phase in oocytes derived from medium follicles comparing to the middle and the late luteal phase of folliculogenesis in oocytes derived from small follicles.

Table 4.The number of small and large lipid droplets in the luteal and the follicular phase

	Phase	*Stage*	*n*	*Total number of small lipid droplets*	*Total number of large lipid droplets*
Small	Luteal	early	45	182.29±59.16[a,b]	168.36±52.38[a,b,c]
		middle	34	172.88±62.21[a]	166.94±46.42[a,b,c]
		late	28	175.14±66[a]	155.79±42.64[a,b,d]
	Follicular	early	29	183.38±62.64[a,b]	144.07±38.19[d]
Medium	Luteal	late	41	209.12±79.15[b]	178.59±47.34[c]
	Follicular	early	33	201.48±69.72[a,b]	160.70±52.51[a,b,c,d]

Data with different superscripts within the same column and inside the same phase are significantly different (a–b-c-d, $P < 0.05$).

According to our data, the average number of large lipid droplets is decreased consequently during the luteal phase in oocytes derived from small follicles, and increased in late luteal phase in oocytes derived from medium follicles. The number of large lipid droplets was significantly higher in the late luteal phase in oocytes derived from medium follicles compared to the late luteal and the early follicular phase in the oocytes derived from small follicles. In the early and the middle luteal phase the number of large lipid droplets was significantly higher in comparison to the early follicular phase in oocytes derived from the small follicles.

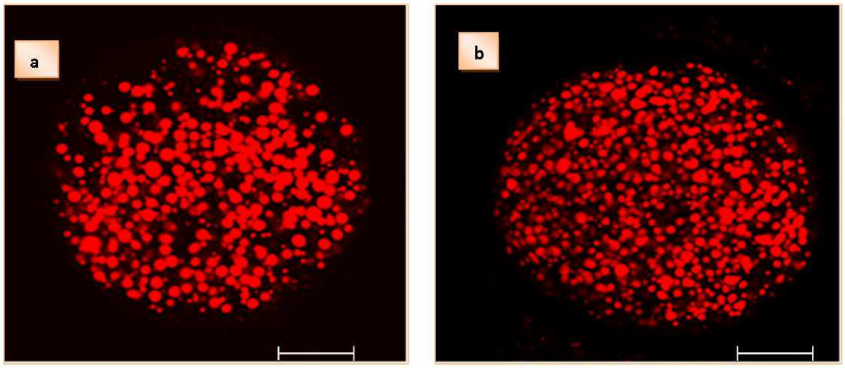

Fig. 1. Representative picture of immature porcine oocytes stained with Nile red: a) oocyte derived from small follicle; b) oocyte derived from medium follicle (scale bar represents 20 µm)

DISCUSSION

The ovarian follicle is the basic structural and functional unit of the mammalian ovary that provides the micro environment necessary for oocyte growth and maturation (Lucci et al., 2007). The size of follicles from which oocytes are derived has a significant effect on the quality of oocytes, their maturation, fertilization and development. Marschal et al. (2002) reported that the developmental competence increases in parallel with follicular size. Lipid droplets are metabolically active organelles that participate in lipid homeostasis, cell signalling, and intracellular vesicle trafficking (Robenek et al., 2006). The lipid composition is an important parameter linked to quality of oocytes. In our research we found out that the total number of lipid droplets was higher in the luteal and the follicular phase in oocytes derived from medium follicles, in comparison to the luteal and the follicular phase in oocytes derived from small follicles (Table 3). The oocytes from small follicles in the late luteal phase positively influences the efficiency of their fertilization as compared to other phases of folliculogenesis (Hulínská et al., 2011).

Lipid rich oocytes were shown to possess greater developmental competence (Jeong et al., 2009). The first detailed lipid analysis of immature porcine oocytes provided Homa et al. (1986). The large amounts of lipid droplets make the development of cryopreservation methods for porcine embryos much more problematic than for many other mammals (Zhou et al., 2009). The lipid environment of porcine oocytes in the immature stage, may be adapted to the highly requirements of the cell, promoting growth and development with a potential role in the regulation of maturation (Homa et al., 1986). Cran et al. (1985) described the presence of the lipid droplets in porcine oocytes and reported the number and size of lipid droplets of immature and matured porcine oocytes. According to Sturmey et al. (2006) lipid droplets tended the show peripheral distribution in immature porcine oocytes. The large number of small lipid droplets accumulated in mature porcine oocytes were an energy source for fertilization and early embryo development (Niimura et al., 2002). In the present

investigation, number of small lipid droplets in immature porcine oocytes was constantly higher than number of large lipid droplets, during the luteal and the follicular phase (Table 4).

Several authors have already been used Nile red to evaluate the lipid content in mammalian oocytes and embryos (Genicot et al., 2005, Leroy et al., 2005, Romek et al., 2011). This study demonstrates the utility of Nile Red as a stain to detect intracellular lipid droplets by confocal microscopy using Nile Red staining (Figure 1.). It is also possible to combine, the quantification of lipids and the evaluation of the size and distribution of lipid droplets within oocytes and embryonic cells.

CONCLUSION

In conclusion, the lipid composition in the immature porcine oocytes varied during the different phases of folliculogenesis. The total number of lipid droplets and number of small lipid droplets in oocytes derived from medium follicles is significantly higher in comparison to oocytes derived from small follicles regardless of the phase.

Our findings demonstrate that the total numbers of lipid droplets was significantly higher in the late luteal phase in oocytes derived from medium follicles, than all stages of luteal phase in oocytes derived from small follicles during the process of folliculogenesis. The total area covered with lipid droplets is similar in oocytes derived from small and medium follicles. The number of small and large lipid droplets were greater in oocytes derived from medium follicles than in oocytes derived from small follicles related to the phases of folliculogenesis.

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