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Relationship between morphometric parameters and oocyte competence of *Sus Scrofa*

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Abstract: The aim of the current study was to establish whether morphometrically analysis (weight and size) of the porcine ovaries affects the quality of oocytes. Twenty-one ovaries were measured by – weight (gr), length (cm), and width (cm) and divided into three groups (n=7). We also compared oocyte diameter (including ZP, excluding ZP and thickness of ZP), according to ovarian weight between groups. Twenty COCs from each group of ovaries were tested in BCB solution. It was found that in the values between the weight of the ovaries and their dimensions, a significant difference was observed between groups 1 and 3 regarding the length of the ovary ($P<0.01$). The width of the second group was significant greater than the ovaries of group one ($P<0.01$). The analysis of oocytes (n=14) from each group showed that in the first group, the percentage of BSB positively stained oocytes was the highest (70%) compared to the other two groups where the positive oocytes were 60 % and 30% for 2 and 3 group, respectively. Our study indicates that the morphometric parameters are not always related to the oocyte quality namely that small ovaries (<5gr) also show good oocyte competence by BCB staining and we concluded that smaller ovaries can be used as a research model too.

Keywords: porcine ovaries; morphometric analysis; oocytes

INTRODUCTION

Oocyte quality has a significant influence on the success of in vitro maturation (IVM), *in vitro* fertilization (IVF), embryo development, and possible implantation (Goto et al., 1988; Sirard et al., 2006; Hoshino, 2018; Murin et al., 2019). Lower oocyte quality and occurrence of morphological anomalies are related to a lower rate of IVM, IVF, and IVC (*in vitro* compartmentalization), which means, that oocyte quality is a key attribute of effective in vitro manipulations and treatment of infertility. This fact is a reason why oocyte collection is based on a strictly qualitative assessment of the current state of oocytes (Vassena et al., 2003; Krisher, 2004). Oocyte quality can determine the probability of success of in vitro techniques. Various non-invasive methods for oocyte quality evaluation have been practiced and suggested. The most commonly used non-invasive methods are for example propidium iodide or Brilliant cresyl blue staining (Murin et al., 2019). The right approach to evaluation can provide valuable information for the selection of oocytes with higher developmental competence and may maximize the success of embryo development (Wang and Sun, 2007; Mtango et al., 2008).

Pig ovaries are usually obtained from slaughterhouses, which is convenient, but variations between animals and their handling can significantly affect the quality of the tissue arriving in the laboratory. For example, porcine oocytes are highly susceptible to temperature stress, and transient temperature shocks can occur in vivo during slaughter (Tong et al., 2004), or ovaries be exposed to low temperature after removal (Yuge, 2003). These stresses result in poor maturation and subsequent embryo development and are often outside the control of the researcher. A simple, reliable means of identifying cumulus–

oocyte complexes (COC) with high maturational competence before IVM culture would reduce an important source of variability and aid the improvement and standardization of subsequent IVM conditions. As with many mammalian species, porcine oocytes are usually graded by visual assessment of morphological features such as the thickness and compactness of the cumulus investment, ooplasm homogeneity (Garg et al, 2012), and morphometric analysis in oocytes (oocyte diameter, cytoplasm and thickness of Zona Pellucida) (Coticchio et al., 2004) and last but not least the size of follicles (Hyttel et al., 1997; Hendriksen et al., 2000). It is generally accepted that the selection of more competent porcine oocytes can be done by the incubation of COCs in brilliant cresyl blue (the BCB test), avoiding negative effects on their further development after fertilization in vitro (Ishizaki et al., 2009; Wongsrikeao et al., 2000). According to various authors among the porcine oocytes evaluated by the brilliant cresyl blue (BCB) test, more competent BCB+ gametes were larger (113.08 μm) than less competent BCB- gametes (100.29 μm) (Roca et al., 1998). Studies are still being conducted to develop techniques/procedures that provide greater reproductive performance in farm animals, including pigs. In this sense, the study of gilts‘ reproductive organs (ovaries) for assessing the presence of abnormalities and/or other parameters that may affect future animal fertility is important. Therefore, special attention has been recently placed on developing a protocol for the non-invasive selection of competent oocytes, which coincides with the aim of the current study to establish whether morphometrically analysis (weight and size) of the ovaries affects the quality of oocytes with the use of the Brilliant cresyl blue (BCB) test. We also compared oocyte diameter (including ZP, excluding ZP, and thickness of ZP) according to ovarian weight between groups.

MATERIALS AND METHODS

The collected ovaries from the Sus Scrofa species, from a slaughterhouse in Kostinbrod town were placed in a transport medium at room temperature and within 1-2 hours and were delivered to the laboratory of “Analyzes and Techniques in Reproductive Biology” - IBIR-BAS. The ovaries were cleaned of fat and washed twice with phosphate buffer PBS. After the assessment by their appearance, the morphometric parameters of the ovary were measured – weight (gr), length (cm) and width (cm) and divided into three groups (n=21) according to their weight- about 3 gr, 4 gr, and 5gr. The length of each ovary was determined as the maximum distance from pole to pole along an axis parallel to the ovarian mesenteric attachment. The width was determined as the furthest distance along an axis vertical to the longitudinal axis (Fig. 1). By puncturing the ovarian follicles, oocytes from each group were aspirated using an 18G needle and a 5-10 ml syringe. The contents of the syringe were collected in a 15 ml tube at 38 ° C. Using microsurgical techniques, the collection of follicular fluid from each ovary separated for this purpose was done individually, using a sterile Pasteur pipette under a stereomicroscope (Micros, Austria 10x). The follicular fluid was transferred to Petri dishes with wash buffer (PBS, pH = 7.4, temperature 4 °C).

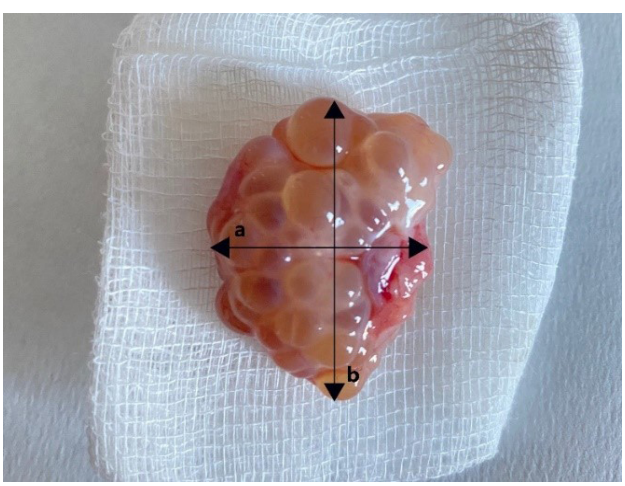

Figure. 1 Dimentions that were measured during mormorphometry evaluation of pig ovary: a- the furthest distance along an axis vertical to the longitudinal axis; b- the maximum distance from pole to pole along an axis parallel

Oocytes from each group were cultured in vitro in a humidified atmosphere (95% air: 5%CO2) at 38.5 °C for 24h in maturation medium (Medium 199, with Earle′s salts, with stable glutamine, 25mM HEPES) supplemented 10% fetal bovine serum. Twenty COCs from each group of ovaries were washed twice in PBS (FertiPro NV, Beernem, Belgium) and tested in BCB brilliant cresyl blue solution (CellaVision®, RALdiagnostics, SA) in concentration 13 μM with subsequent incubation for 90 min at 39 °C in a humidified 5% CO2 atmosphere (Kempisty et al.., 2011). After incubation, the oocytes were transferred to PBS supplemented with Medium 199 (Earle′s salts, L-glutamine and 25 mM HEPES) and washed twice. The washed oocytes were examined under stereomicroscope and classified into two groups: those with blue-stained ooplasm were evaluated as BCB positive (BCB+), and those with a colourless ooplasm– as BCB negative (BCB–) - based on glucose-6-phosphate dehydrogenase activity (G6PD) characteristic for growing oocytes (El Shourbagy et al.,2006).

Statistical analysis

The Statistical analysis was processed with the IBM SPSS 19. Descriptive data for each group were presented as "Mean ± Standard Error". The results of the comparison of groups were assessed by Student's test (T-Test) at a significant level of * P <0.01.

RESULTS AND DISCUSSION

To determine the influence of ovarian dimensions on oocyte diameter, we evaluated the ovaries and initially classified them into 3 groups as mentioned in the experiment above. Table 1 shows the relationship between the weight of the ovaries, their sizes on the one hand, and the morphometric parameters of the oocytes on the other between the three formed groups.

Regarding the relationship between the weight of the ovaries and their dimensions, significant differences were observed between groups 1 and 3 regarding the length of the ovary (P<0.01). The width of ovary of the second group was significant greater than that of ovaries of group 1 (P<0.01). With regard to the relationship between the weight of the ovaries and the morphometric indicators of the oocytes, the following proportional relationships were found - the smallest values were found in group 1 about the diameter of the oocyte with zona pellucida, while the ovaries with the largest weight in group 3, were measured significant biggest oocytes in diameter with zona pellucida. Figure 2 presents positively and negatively stained oocytes in the three studied groups. The analysis of an equal number of oocytes (n=14) from each group showed that in group 1, the percentage of BSB positively stained oocytes was the highest (30%) compared to the other two groups where the positive oocytes were 60 % and 70% for 2nd and 3th group, respectively. We focused on detection of colorless cytoplasmic membrane, which was characterized by unstained ooplasm. Unstained (BCB-) were designated as poor quality. Bearing in mind the BSB test as an indicator of the maturity of the oocytes, our results demonstrated that in the ovaries with the greatest weight (5gr) had the best morphometric indicators because the observed percentage of

Table 1. Relationship between ovary weight and oocyte morphometric parameters

Groups (Weight of the ovary, gr) (n=7)	*Width of the ovary (cm), n=7*	*Length of the ovary (cm), n=7*	*Oocyte diameter (μm), n=20*	*Oocyte diameter without ZP (μm), n=20*	*Thickness of Zona Pellucida (μm), n=20*
group 1 (3gr)	2,27±0,23*	2,22±0,24*	77,28±2,26*	57,60±1,80*	9,00±0,36*
group 2 (4gr)	2,74±0,17*	2,04±0,06	94,27±0,64	74,30±0,71	10,15±0,20
group 3 (5gr)	2,64±0,16	1,98±0,13*	130,48±1,28*	104,78±0,51*	12,41±0,38*

*Data are presented as mean±SE; Superscript index * is labeled based on the t-test result. The same index means a significant difference in the column /P value < 0.01/*

mature oocytes (70%) was the highest. Followed by the second group which showed 60% positively stained oocytes.

Oocytes still in the growth phase have high G6PDH activity and show colorless cytoplasm (BCB). The enzyme G6PDH converts the dye into a colorless form. Oocytes that have completed their growth have low levels of G6PDH and show blue cytoplasmic staining (BCB+). BCB+ oocytes have a significantly higher rate of blastocyst development than BCB- oocytes, suggesting that BCB+ oocytes have higher developmental competence (quality) compared to colorless oocytes of reduced quality when it comes to IVM.

Most of the existing studies have focused and analyzed on follicular growth, oocyte maturation, and early embryonic development, but there are still few studies on the morphometric analysis (weight, width, length) of the ovaries and their relationship with oocytes.

A recent study evaluated differences in ovarian characteristics, and IVM between two swine breeds. The authors have observed a larger ovary length between breeds ($p = 0.01$), whereas no significant difference was observed in ovary weight. Their results indicated differences with regard to ovarian characteristics as well as to cumulus expansion, and nuclear and cytoplasmic oocyte maturation at 20 hr. Nevertheless, the two breeds showed similar maturation results at 48 hr (Jochems et al., 2021). Similar to our study Pawlak et al., 2011 evaluated the size of the oocytes and compared the BCB+ and BCB- oocytes from cycling gilts and pre-pubertal gilts. They demonstrated that BCB- oocytes were almost equally distributed between the two examined size categories (≥120 μm: 44.5%, 73/164; <120 μm: 55.5%, 91/164). With regard to oocyte size, some of the porcine oocytes acquire meiotic competence with a diameter of 100–115 μm, whereas most of oocytes with a diameter ≥120 μm are fully competent (Hunter, 2000). An interesting comparative finding, in several mammalian species reported the change in oocyte diameter is not directly proportional to the follicular diameter, even at the early stages of follicular growth (Griffin et al., 2006).

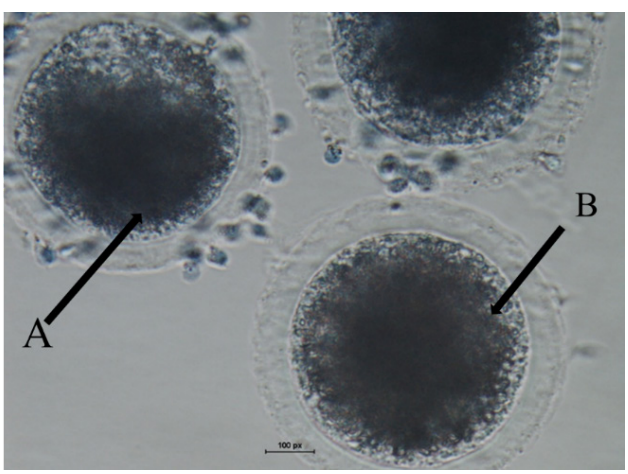

Figure 2. Classification of porcine oocytes after BCB staining. (A- arrow) BCB+ oocyte group; (B-arrow) BCB- oocyte, (magnification X 40)

CONCLUSION

We found that the morphometric parameters (weight, width, length) are not always related to oocyte quality and small ovaries (<5gr) also show good meiotic competence by BCB staining. They can also be used as a research model and probably have the potential for practical application in the increasingly common development of fertility preservation in farm animals.

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