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Quantitative and Morphological Study of Preantral Follicles from Prepubertal Gilts

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ABSTRACT

***Background*:** The development of animal reproductive biotechniques is intended of raising reproduction efficiency. The mammal ovary contains thousands of follicles, of which approximately 99.9% are eliminated by means of the atresia, apoptosis and cellular necrosis process. In other to reduce this follicular loss, the methods for isolation and characterization of preantral follicles have been studied as a premise to culture systems of these structures. The purpose of this study was quantify and evaluate the quality of preantral ovarian follicles from prepubertal gilts after mechanical isolation procedure. Furthermore, it aims to analyze the preantral follicular histological morphology.

***Materials, Methods & Results*:** Ovaries (n = 20) from prepubertal gilts were divided in two halves and used each one for isolation and histological processes. The tissue chopper previously regulated for the performance of seriated cuts at 200 μm intervals was used for mechanical isolation. The marker bisbenzimidine Hoechst 33342 was added to the follicular pool for evidence the presence of granulosa cells around oocyte and the viability of the isolated PAF was evaluated by using propidium iodide. For the histological evaluation, the ovarian halves were fixed, dehydrated and diaphanized, and after enclosed in paraffin, each of them was divided into 5 blocks and sectioned in series 6 μm thick. At each 1000 μm interval, 2 slides were removed per block, totaling 200 slides that were dyed with periodic acid Schiff (PAS) and hematoxylin. The total PAF calculated in situ for each follicular stage (primordial, primary and secondary) was estimated according with the preantral follicles number evaluated on histological sections. The number of isolated preantral follicles per ovary was (599,160 ± 74,089), presented positive correlation ($P < 0.05$) between ovarian weight. After isolation, 76.4% of preantral follicles were viable ($P < 0.05$), while 64.3% had granulosa cells around the oocyte ($P < 0.05$). The average number of PAF estimated in histological cuts was 131,938 ± 14,615 per ovary, oscillating between 67,599 and 291,898, out of which 117,968 (89.41%) primordial follicles, 3,448 (2.61%) primary follicles, and 10,522 (7.98%) secondary follicles. The verified follicles diameter was primordial (33.1 μm), primary (47.6 μm) and secondary (79.1 μm).

***Discussion*:** The PAF quantification estimated in situ per ovary was different from results obtained on mechanical isolation, showing that used techniques do not complement. However, both procedures highlighted the specie reproductive potencial. The greater percentage of oocytes was included in primordial follicles (89.4%; $P < 0.05$). Follicular diameter and the number of granulosa cells increased ($P < 0.05$) according to the development stage. These data are important to define which interval of cut can be adopted before utilizing the mechanical isolation procedure with tissue chopper, avoiding sections damages to the follicular structure, which contributes to the smaller viability of isolated PAF. In summary, the results reported that it is possible to obtain a great number of viable PAF from of prepubertal gilts ovaries after mechanical isolation, providing material to supply the demand for studies relating to folliculogenesis and in vitro culture techniques.

Keywords: primordial follicles, oocyte, porcine, viability.

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INTRODUCTION

The mammal ovary contains thousands of follicles, of which approximately 99.9% are eliminated by means of the atresia, apoptosis and cellular necrosis process. Antral follicles development has been described in many species, but referred information to preantral follicles (PAF) physiology is not totally understood.

It is known that primordial follicles been activated and develop on primary, secondary and tertiary follicles. However, the factors that promoter or inhibit the activation of these follicles are partially elucidated, as well those related with follicular growth control. This way, the preantral phase comprises a considerable scope for scientific investigation.

The research on techniques that enable the recuperation of ovarian PAF and further *in vitro* development is essential. Such objective can be attained by means of the Manipulation of Oocytes Enclosed in Preantral Follicles (MOEPF) biotechnique, which presented successfully production of embryos in different species, such as mice [10,27], swine [38] and bubaline [16]. Hence, the recuperation of a great number of oocytes coming from the same animal will enable to increase the production of uniform populations of zootechnically superior animals or endangered species. In addition, it is an investigation model for complex mechanisms involved in the initial folliculogenesis [35] and allows the development of media for *in vitro* cultivation [4,9,12,22,23,32,34].

The purpose of this study was quantify and evaluate the quality of preantral ovarian follicles from prepubertal gilts after mechanical isolation procedure. Furthermore, it aims to analyze the preantral follicular histological morphology.

MATERIALS AND METHODS

Source of Ovaries

Ovaries (n = 20) of hybrid prepubertal gilts from commercial crossing, aged between 4 and 6 months, were used. After slaughter, ovaries were collected, placed in plastic tubes containing phosphate buffered saline (PBS) at 4ºC [6] and carried to the laboratory.

Ovarian morphometry, mechanic isolation and preantral follicles quantification

Ovaries were morphometrically evaluated as to length (cm), width (cm), thickness (cm) and weight (g) using a pachymeter and a precision scale. Then, superficial antral follicles were punctured for the elimination of follicular liquid, and each ovary was longitudinally sectioned into two parts. One half was used for the mechanical isolation procedure, and the other for the histological processing, for the quantification of preantral follicles enclosed in the ovarian tissue (*in situ*).

The tissue chopper[1] previously regulated for the performance of seriated cuts at 200 µm intervals was used for mechanical isolation. To obtain an efficient fragmentation the cuts were done in the longitudinal, transversal and oblique axes. Next, the fragments obtained were put in a 20 mL PBS suspension, mechanically dissociated through successive pipetting procedures and filtered in 500 µm and 100 µm diameter nylon meshes [11].

Two samples of 100 µL samples were removed and put in a Petri plate for the quantification of follicles under the inverted microscope. To estimate the total number of PAF in each ovary, the following formula was adopted [3]:

Totally PAF = (PAF sample 1 + PAF sample 2)/2 x 400*

*average found in samples 1 and 2 that corresponds to the ovary half was multiplied by 10 to calculate the amount in 1 mL. The result obtained was multiplied by 20 (final suspension). To estimate the total amount per ovary this was multiplied by 2, therefore: 10 x 20 x 2 = 400

Qualitative analysis of isolated preantral follicles

The marker bisbenzimidine Hoechst 33342[2] (B2261) was added to the follicular pool for evidence the presence of granulosa cells around oocyte, on final concentration of 10 µg/mL, incubated for 30 min at 37°C, which was assessed on epifluorescence microscope.

The viability of the isolated PAF was evaluated by using propidium iodide[3] (P3566) on final concentration of 10 µg/mL, prepared for analysis as previously described. PAF were classified as note viable when the nucleus was marked in red, indicating the absorption of fluorochrome [26].

Histological process, follicle quantification (in situ) and morphology

For the histological evaluation, the halves of ovaries were fixed, dehydrated and diaphanized, and after enclosed in paraffin, each of them was divided into 5 blocks and sectioned in series 6 µm thick. At each 1000 µm interval, 2 slides were removed per block, totaling 200 slides that were dyed with periodic acid Schiff (PAS) and hematoxylin.

Quantification and measurements were carried out using digitalized images (HL Image® 97) obtained in light microscope with X 400. To measure oocytes and the nuclei, the basal slide was considered as the external limit and the diameter was taken as the average of the larger and shorter axes in straight angle at the sections where the nucleus and the nucleolus were observed.

Follicles were classified as: primordial (oocytes surrounded by on layer of flattened granulosa cells), primary (single layer of cubical granulosa cells) and secondary (two or more layers of cubical granulosa cells) [36].

The total PAF calculated *in situ* for each follicular stage (primordial, primary and secondary) was estimated according to the formula [13]:

NT = (Nf x Cp) / (St x do)

NT= Total number of follicles *in situ*;

Nf = Number of follicles counted in each follicular stage;

Cp = Ovary length (µm);

St = Total number of cuts evaluated;

do = Average oocyte diameter of each evaluated follicular stage (µm).

Statistical analysis

Statistic tests were carried out with the software Bioestat® 5.0, data were presented as percentage and mean (±SEM). Tukey test analyzed the follicular, oocytarian and nuclear diameters, and compare them among follicular classes. The relating follicular population distribution, viability and presence of granulosa cells were evaluated by the Chi-square test. Pearson correlation coefficient was used on the variables ovarian weight and number of mechanically isolated follicles per ovary. Significance was set at $P < 0.05$.

RESULTS

Ovarian morphometry, isolated preantral follicles number and follicular quality

The average weight of ovaries was 3.33 ± 0.15 g, ranging between 2.49 and 4.38 g. The PAF average per ovary isolated by means of the mechanical procedure was 599,160 ± 74,089, with a 30,400 to 1,200,800 variation between samples. It was observed a positive correlation (r = .4762; $P < 0.05$) between ovarian weight and average number of isolated follicles (Figure 1). After isolation procedure, there were a high ($P < 0.05$) percentage of viable PAF (76.4%) with granulous cells (64.3%) around the oocyte (Table 1).

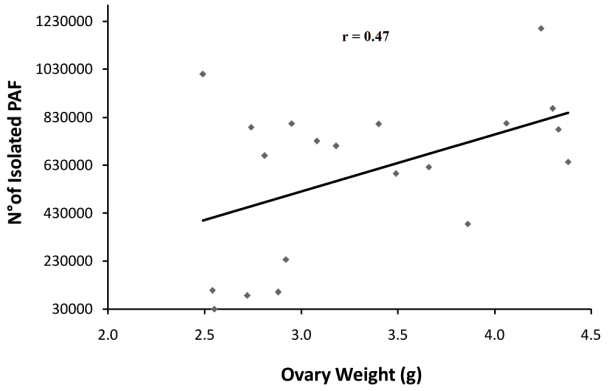

Figure 1. Relationship between ovarian weight (g) and average number of mechanical isolated preantral follicles from prepubertal gilts. Pearson Correlation Coefficient: r = 0.47.

Follicular morphometry and histological quantification (in situ)

The average number of PAF estimated in histological cuts was 131,938 ± 14,615 per ovary, oscillating between 67,599 and 291,898, out of which 117,968 (89.41%) primordial follicles, 3,448 (2.61%) primary follicles, and 10,522 (7.98%) secondary follicles (Figure 2). The follicular and oocytarian diameters increased ($P < 0.05$) as follicles evolved to further stages. However, there was no difference ($P > 0.05$) between nuclear diameters of oocytes obtained from primordial and primary follicles, or from primary and secondary follicles. The average number of granulosa cells differed ($P < 0.05$) among follicular classes, and increased according to the development stage (Table 2).

Table 1. Quality (%) and average ± (SEM) of preantral follicles recovered from prepubertal gilts ovaries after mechanical isolation procedure.

	Follicular Quality		Mechanical Isolation	
	Granulosa cells n = 443	Viable n = 556	PAF Isolated/ Ovary	* PAF Predict Viable/Ovary
PAF Number (%)	285 (64.3)	425 (76.4)	599,160	457,758

*Calculated in function of the isolated preantral follicles average and viability percentage.

Table 2. Morphometry (mean ± SEM) and percentage distribution of histological evaluated preantral follicles from prepubertal gilts.

Follicle class	Ø Diameter (µm)			N° Granulosa cells	Population (%)
	Ø Follicle	Ø Oocyte	Ø Nucleus		
Primordial n = 102	33.04 ± 1.64^{x}	24.24 ± 1.15^{x}	15.09 ± 0.76^{x}	5.88 ± 0.53^{x}	89.41^{a}
Primary n = 100	47.59 ± 2.70^{y}	33.02 ± 1.59^{y}	17.35 ± 1.43^{xy}	16.45 ± 1.72^{y}	2.61^{b}
Secondary n = 102	79.11 ± 5.75^{z}	41.88 ± 2.51^{z}	19.44 ± 1.01^{y}	46.27 ± 6.58^{z}	7.98^{c}

Within a column, means without a common superscript differed ($P < 0.05$).

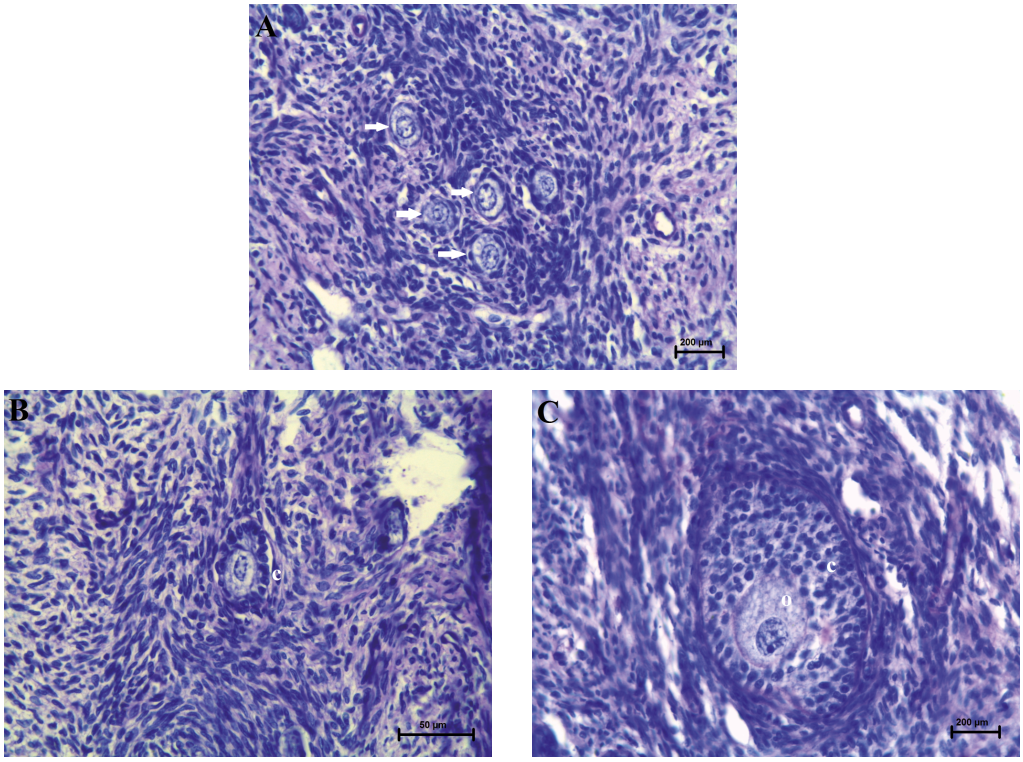

Figure 2. Histological ovarian sections stained with periodic acid Shiff (PAS) and hematoxylin. (A) Primordial follicles with flattened granulosa cells (white arrows). (B) Primary follicle with single layer of cubical granulosa cells (c). (C) Secondary follicle, oocyte (o) and several layers of cubical granulose cells (c).

DISCUSSION

Among the morphometric characteristics evaluated, the average weight of ovaries (3.33 g) was similar to that reported by other studies [25,31], which observed a variation from 3.43 g to 31.9 g for prepubertal gilts and for adult females, justified by age and the presence of structures such as corpus luteum and follicular cysts.

The average number of recuperated PAF per ovary (599,160 ± 74,089) showed a linear relation with the ovarian weight. Previously reported results [14,15,31], have shown high variation in isolated preantral follicles number, even when used structures identifying advanced techniques [31], possibly due to differences between species and classes evaluated [15].

The PAF quantification estimated in situ per ovary (131,938) was different from the results obtained on mechanical isolation (599,160), demonstrating that employed techniques do not complement. However, both procedures highlighted the specie reproductive potencial. Studies to estimate bovine preantral follicles with the same methodology presented discrepant results [20,30].

Works have shown this oscillation, regardless of the isolation type adopted, either enzymatic or mechanical, from hundreds to thousands of PAF can be separated per ovary as reported in bovine (52,100) [20], ovine (28,420) [3], feline (12,500) [18], canine (25,200) [2] and capuchin monkeys (45,625) [7].

The use of enzymes might damage the basal membrane and rupture junctions between follicular cells, harming the follicular viability in culture system [8,37], since paracrine factors exchanged between these cells and the oocyte are fundamental for the induction and regulation of follicular differentiation, favoring the development of an oocyte qualified for fertilization and further embryogenesis [1,28,36]. The follicular quality, evaluated by the presence of granulosa cells and viability, has shown that only 35.6% of PAF were denuded, and that 76.4% were viable after mechanical isolation without the use of enzymes. Groups of primary follicles that preserved the integrity of the basal membrane after enzymatic digestion had smaller denuded rates (1.0% vs. 38.7%), greater granulosa cells preservation during in vitro growth (46.7% vs. 22.5%), and smaller oocytes degeneration rates (27.9% vs. 91.6%) [8]. Concerning follicular viability, inferior results were reported in wild feline [18] and with bubaline fetuses [29] after mechanical procedure, with viability rates of 50% and 60%, respectively. For goats, the comparison between mechanical and enzymatic procedures for PAF isolation of fetuses ovaries, showed higher efficiency of cellular viability ($P < 0.05$) with enzymatic isolation (84.6% vs. 75.0%) [21].

In mammal females, the oogonia stock is established in the fetal phase by means of a sequence of complex and controlled events that shall occur until the formation of the secondary oocyte capable of fecundation [24,28,36]. However, it is known that there is a loss of this follicular population from the initial activities of meiosis. In swine, the estimated number of oogonia is 1,200,000, and the follicular loss at birth reaches 58% [17]. This fact can be verified in the great number of oocytes enclosed in primordial follicles (89.4%) in relation to primary and secondary follicles.

The average diameter of primordial (33.1 μm), primary (47.6 μm) and secondary (79.1 μm) follicles verified in situ was similar to the previously reported variation between 35 and 100 μm for preantral follicles in swine [33]. Smaller average values for primordial (20.0 μm) primary (24.4 μm) and secondary (44.2 μm) follicles were described for goats [19]. This size difference between follicular classes might occur due to the difference among species, and as the result of the histological processing that can cause the retraction of tissues in up to 25% [5]. These data are important to define which interval of minimum cut can be adopted before utilizing the mechanical isolation procedure with tissue chopper, avoiding sections damages to the follicular structure, which contributes to the smaller viability of isolated PAF.

In summary, the results reported that it is possible to obtain a great number of viable PAF from of prepubertal gilts ovaries after mechanical isolation, providing material to supply the demand for studies relating to folliculogenesis and in vitro culture techniques.

SOURCES AND MANUFACTURERS

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***Declaration of interest*.** The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of the paper.

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