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Study of preantral follicle population in situ and after mechanical isolation from caprine ovaries at different reproductive stages

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Abstract

The purposes of this study were to estimate the population of caprine preantral follicles, and to evaluate quantitatively and qualitatively the efficiency of a specific mechanical method for the isolation of preantral follicles from mixed breed goats at different reproductive stages. On average, 37,646±4277 preantral follicles were present in goat ovaries, and 13,631±2399 preantral follicles were obtained after isolation. The number of preantral follicles isolated or in situ was not significantly affected by the reproductive stage. The mean recovery rate per ovary ([number of isolated follicles/number of in situ follicles]×100) of isolated follicles was 36.2%. The distribution of follicles in situ was 67.8% primordial, 25.8% primary and 6.4% secondary; the respective distribution after isolation was 93.8%, 5.2% and 1.0%. In this study, many polyovular follicles were also observed, mainly in prepubertal goat ovaries. Histological analysis showed that few preantral follicles were atretic in situ (4.83%±0.35) or after the isolation procedure (4.67%±0.65) in the three reproductive stages. The percentage of atretic follicles was not affected either by the mechanical method or by the reproductive stage. It is concluded that a large number of preantral follicles can be successfully isolated mechanically, with a high recovery rate and a low rate of follicular atresia, irrespective of the reproductive stage of the caprine female.

Keywords: Preantral follicle; Follicle isolation; Histology; Polyovular follicle; Goat

1. Introduction

At birth, the mammalian ovary contains a large store of follicles of which only a small number will be used during the reproductive lifespan of the female. The total follicular population in the mammalian ovary consists of a large reserve of quiescent primordial follicles and a much smaller number of follicles in the growth phase (Cahill and Mauléon, 1981). There are on average 130,000 and 80,000 primordial follicles, respectively, in bovine (Erickson, 1966b) and ovine (Land, 1970) ovaries. Moreover, the preantral follicular population may be affected by many factors including age (Erickson, 1966a and Erickson, 1966b), breed (Cahill et al., 1979), hormone concentration and reproductive state (Erickson et al., 1976). Despite this great population of preantral follicles, the vast majority becomes atretic during their growth and maturation. This represents an enormous loss of genetic material.

Development of techniques for rescue and in vitro growth of preantral follicles would be an addition to the methods already available for animal reproduction (e.g. artificial insemination, embryo transfer, in vitro fertilization and cloning), since it would supply a large and uniform population of oocytes from genetically superior animals (Betteridge et al., 1989). In the last decades, various techniques for isolating preantral follicles have been reported for pigs (Greenwald and Moor, 1989; Lazzari et al., 1992), cows (Figueiredo et al., 1993; Nuttinck et al., 1993; Hulshof et al., 1994), cats (Jewgenow and Pitra, 1993; Jewgenow and Göritz, 1995) and ewes (Amorim et al., 1998b).

In goats, a specific mechanical method for the isolation of a great number of intact preantral follicles per ovary was recently developed (Lucci et al., 1999). However, the efficiency of this method for the isolation of preantral follicles from caprine ovaries at different reproductive stages is unknown. In general, the efficiency of isolation methods is difficult to determine for lack of comparative studies between the number of preantral follicles present in the ovary and the number of preantral follicles isolated from the same ovary. In addition, for goats no information is available concerning the population of preantral follicles present in the ovaries.

The aims of the present study were: first, to estimate the population of caprine preantral follicles in situ, and secondly, to perform quantitative and qualitative analysis of the efficiency of the mechanical method for the isolation of preantral follicles from goats at different reproductive stages, using as a control the number of preantral follicles present in situ.

2. Materials and methods

2.1. Source of ovaries

In this study, 18 ovaries from prepubertal (2–3 months of age; n▪6), non-pregnant (n▪6) and pregnant adult goats (1–3 years old; n▪6) were collected at a local slaughterhouse. It is important to note that only one ovary from each animal was taken, and that all the ovaries were from mixed breed goats. The ovaries were washed in 70% alcohol for approximately 10 s and then, twice in 0.9% saline solution. Finally, each ovary was transferred to 10 ml of phosphate buffered saline (PBS) and transported to the laboratory within 1 h in a thermoflask filled with water at 39°C.

2.2. Experimental protocol

To evaluate qualitatively and quantitatively the efficiency of the specific mechanical method for the isolation of caprine preantral follicles, described below, each ovary was cut longitudinally into two halves. One half was used to isolate preantral follicles and the other one was used to study the preantral follicle population in situ (Fig. 1).

Preantral follicles were isolated from the ovarian halves by applying the specific mechanical procedure developed for the isolation of caprine preantral follicles described previously by Lucci et al. (1999). Briefly, the ovarian tissue was cut into small fragments using a tissue chopper (The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK) adjusted to 75 µm. The ovarian fragments were placed in PBS plus 5% caprine serum and then pipetted 40 times with a large pasteur pipette (diameter ∼1600 µm) and 40 times with a smaller pipette (diameter ∼600 µm). The suspension was filtered successively through 500 and 100 µm nylon mesh filters. The number of isolated preantral follicles in the suspension containing fragments <100 µm was counted and classified using an inverted microscope (Zeiss). The follicular diameters were measured with an ocular micrometer. Preantral follicles were classified according to the stage of development as primordial (one layer of flattened or flattened-cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte), or secondary (oocyte surrounded by more than one complete layer of cuboidal granulosa cells) (Hulshof et al., 1994).

To evaluate the quality of the isolated preantral follicles, they were prefixed in 2% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer, for 30 min at room temperature. The follicles of each class were embedded in drops of 200 µl of 4% agar solution (agarose, type VII, Sigma, St. Louis, MO, USA). The agar drops were fixed in the same fixation solution described above for 3 h. The drops were dehydrated, embedded in paraffin wax, sectioned serially at a thickness of 7 µm and stained with periodic acid schiff (PAS) and haematoxylin. Follicular quality was evaluated based on the integrity of the basement membrane, cellular density, presence or absence of pycnotic bodies and integrity of the oocyte. Based on these parameters, preantral follicles were classified as morphologically normal or degenerated follicles.

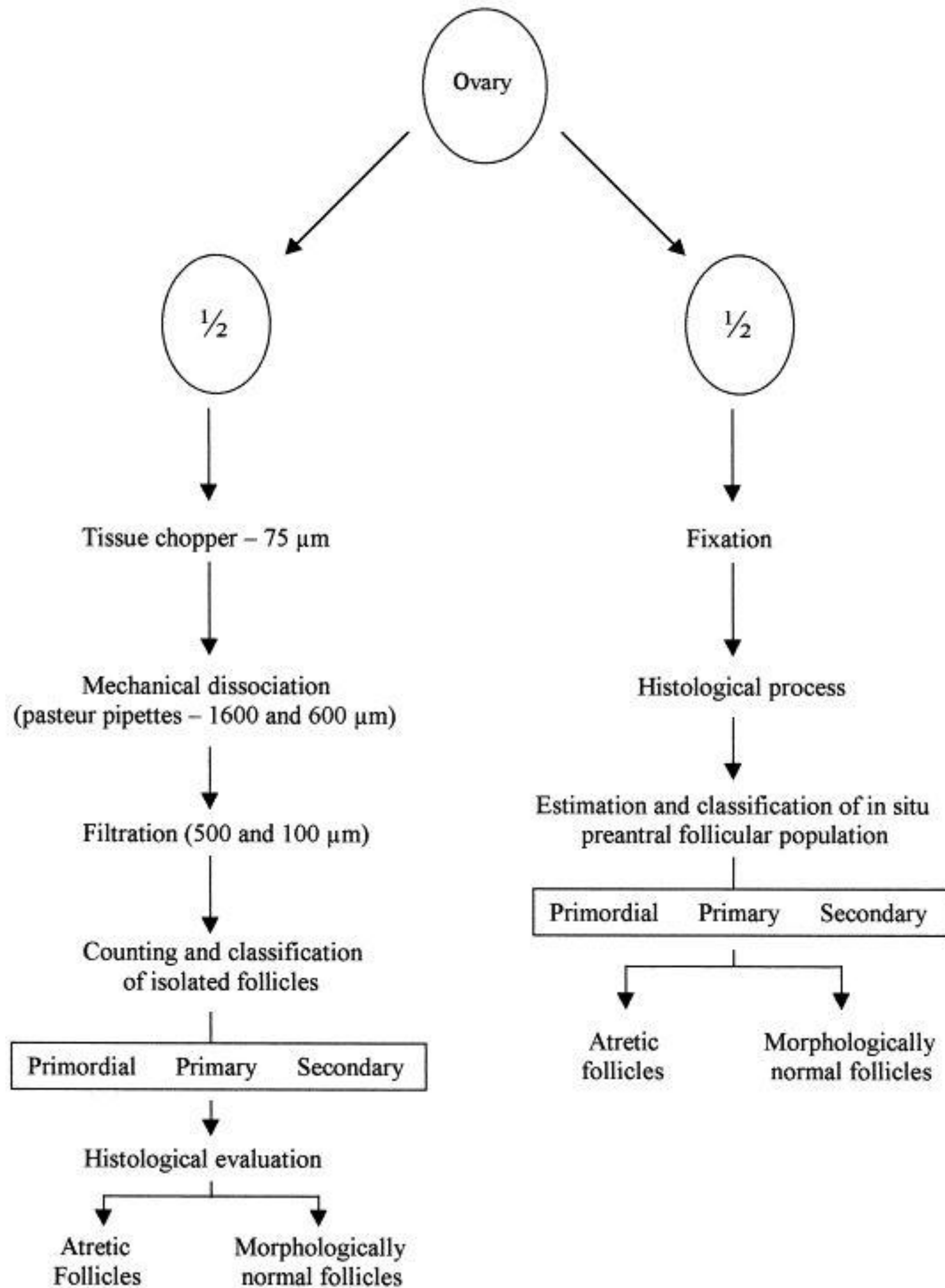

Fig. 1. Experimental protocol to study caprine preantral follicle population in situ and after isolation procedure.

In order to estimate the preantral follicular population in caprine ovaries, the ovarian halves that were not used to isolate preantral follicles were fixed in 10% formaldehyde and processed for histology. Tissue was serially sectioned at 7 µm and stained with PAS and haematoxylin. The number of preantral follicles was estimated by counting the follicles in every 120th section (Cahill et al., 1979) using the nucleus of the oocyte as a marker and the

correction factor described by Gougeon and Chainy (1987). Preantral follicles were classified based on their morphology (primordial, primary and secondary) and quality (morphologically normal and degenerated follicles) as described above. The diameter of the follicles and the oocytes was determined when the nucleolus of the oocyte was observed.

For each ovary, the recovery rate (RR) of follicles isolated by the mechanical method was calculated as follows: RR per ovary=(number of isolated follicles/number of in situ follicles)×100.

2.3. Statistical analysis of data

Analysis of variance and Fisher's PLSD test (Stat View for Macintosh) were used to compare: (1) the mean number of preantral follicles isolated and in situ among the different reproductive stages (prepubertal, non-pregnant and pregnant adult animals), (2) the mean number of preantral follicles that were isolated and observed in situ in each reproductive stage, and (3) the mean follicular recovery rate among reproductive stages. Data were log transformed if Hartley's test indicated heterogeneity of variance. The diameter of preantral follicles, in situ and isolated, were compared by Scheffe's test (Stat View for Macintosh). Comparisons between the percentages of atretic preantral follicles isolated and in situ in each reproductive stage and among reproductive stages were analyzed by Chi-square (Instat for Macintosh). Values were considered statistically significant when $p<0.05$.

3. Results

3.1. Number of preantral follicles isolated and in situ

Table 1 shows the number of preantral follicles in situ and isolated and the recovery rate of preantral follicles for prepubertal, non-pregnant and pregnant adult goats. Large individual variation was observed in the number of isolated and in situ preantral follicles in all the three reproductive stages. In all reproductive stages the number of isolated follicles was significantly lower that those observed in situ. No significant differences were observed among reproductive stages, neither for the number of isolated and in situ preantral follicles nor for the recovery rate. The mean recovery rate per ovary ([number of isolated follicles/number of in situ follicles]×100) varied from 28.3% (prepubertal) to 45.7% (pregnant goats).

Table 1
Number of in situ and isolated preantral follicles per ovary and follicular recovery rate in prepubertal, non-pregnant, and pregnant adult goats (mean ± s.e.m.)

Reproductive stage	Number of preantral follicles per ovary (range)			Recovery rate (%)
	N (ovaries)	In situ	Isolated	
Prepubertal	6	45,127 ± 7784[a] (23,623–66,477)	11,933 ± 3029[b] (3024–22,064)	28.3 ± 7.5
Non-pregnant adults	6	32,204 ± 4583[a] (20,497–44,659)	12,017 ± 3379[b] (1764–22,288)	34.7 ± 7.3
Pregnant adults	6	35,608 ± 9329[a] (20,122–80,739)	16,945 ± 5899[b] (1456–35,000)	45.7 ± 14.2
Total	18	37,646 ± 4277[a] (20,122–80,739)	13,631 ± 2399[b] (1456–35,000)	36.2 ± 5.8

[a,b] Values with different superscripts in the same row are significantly different ($p < 0.0001$).

Table 2
Mean number and percentage of primordial, primary and secondary follicles in situ and isolated per ovary of prepubertal, non-pregnant and pregnant goats (mean ± s.e.m.)

Follicle class	Number and percentage of preantral follicles per ovary (mean ± s.e.m.)								Recovery rate (%)
	Prepubertal		Non-pregnant adults		Pregnant adults		Total		
	In situ	Isolated	In situ	Isolated	In situ	Isolated	In situ	Isolated	
Primordial	$32{,}175 \pm 6279^{aA}$ $(69.2\% \pm 3.5)^{aD}$	$11{,}297 \pm 2958^{bA}$ $(94.1\% \pm 1.8)^{bD}$	$20{,}779 \pm 4116^{aA}$ $(61.9\% \pm 6.4)^{aD}$	$11{,}326 \pm 3384^{bA}$ $(90.7\% \pm 4.0)^{bD}$	$26{,}887 \pm 8369^{aA}$ $(72.1\% \pm 6.3)^{aD}$	$16{,}427 \pm 5746^{bA}$ $(96.5\% \pm 0.4)^{bD}$	$26{,}613 \pm 3697^{aA}$ $(67.8\% \pm 3.2)^{aD}$	$13{,}010 \pm 2358^{bA}$ $(93.8\% \pm 1.5)^{bD}$	50.2 ± 7.8^{G}
Primary	$10{,}668 \pm 1317^{aB}$ $(26.1\% \pm 3.7)^{aE}$	555 ± 184^{bB} $(5.0\% \pm 1.7)^{bE}$	9702 ± 1226^{aB} $(31.4\% \pm 4.4)^{aE}$	532 ± 197^{bB} $(7.8\% \pm 2.9)^{bE}$	6515 ± 1483^{aB} $(20.0\% \pm 4.3)^{aE}$	453 ± 156^{bB} $(3.0\% \pm 0.3)^{bE}$	8962 ± 847^{aB} $(25.8\% \pm 2.5)^{aE}$	513 ± 98^{bB} $(5.2\% \pm 1.2)^{bE}$	5.9 ± 0.5^{H}
Secondary	2284 ± 532^{aC} $(4.7\% \pm 0.4)^{aF}$	98 ± 21^{bC} $(0.9\% \pm 0.1)^{bF}$	1722 ± 480^{aC} $(6.7\% \pm 2.3)^{aF}$	159 ± 52^{bC} $(1.5\% \pm 0.9)^{bF}$	2207 ± 396^{aC} $(7.9\% \pm 2.4)^{aF}$	65 ± 29^{bC} $(0.5\% \pm 0.2)^{bF}$	2071 ± 263^{aC} $(6.4\% \pm 1.1)^{aF}$	107 ± 22^{bC} $(1.0\% \pm 0.4)^{bF}$	5.5 ± 1.9^{H}
Total	45,127 ± 7784 (100%)	11,933 ± 3029 (100%)	32,204 ± 4583 (100%)	12,017 ± 3379 (100%)	35,608 ± 9329 (100%)	16,945 ± 5899 (100%)	37,646 ± 4277 (100%)	13,631 ± 2399 (100%)	36.2 ± 5.8

[a,b] Values with different superscripts in the same row within reproductive stage are significantly different ($p < 0.0003$).
[A,B,C] Values with different superscripts in the same column are significantly different ($p < 0.0001$); concerning to number of follicles.
[D,E,F] Values with different superscripts in the same column are significantly different ($p < 0.0001$); concerning to percentage of follicles.
[G,H] Values with different superscripts in the same column are significantly different ($p = 0.0005$); concerning to recovery rate.

Reproductive stages did not affect the proportions of primordial, primary and secondary follicles observed in situ or after mechanical isolation. However, the proportions of preantral follicle classes obtained after mechanical isolation were significantly different to those observed in situ in all the three reproductive stages. The mean number of primordial, primary and secondary follicles in situ and after isolation and their corresponding proportions in the three reproductive stages are shown in Table 2. Taking the reproductive stages together, for the preantral follicle population in situ and after isolation, the proportions of primordial, primary and secondary follicles were 67.8% and 93.8%, 25.8% and 5.2%, 6.4% and 1.0%, respectively. The mechanical method isolated mainly primordial follicles. The mean recovery rate per follicular class was 50.2%, 5.9% and 5.5%, respectively for primordial, primary and secondary follicle classes (Table 2).

3.2. Morphometric analysis of preantral follicles in situ and after isolation

Histological analysis in situ showed that primordial follicles have an oocyte surrounded by one layer of squamous or squamous–cuboidal granulosa cells; primary follicles have a single layer of cuboidal granulosa cells, and secondary follicles have an oocyte surrounded by two or more layers of cuboidal granulosa cells (Fig. 2). Freshly isolated primordial, primary, and secondary follicles in the suspension were spherical and appeared healthy under the inverted microscope. After isolation, the oocytes of primary and secondary follicles were not always clearly visible because of granulosa cells covering them.

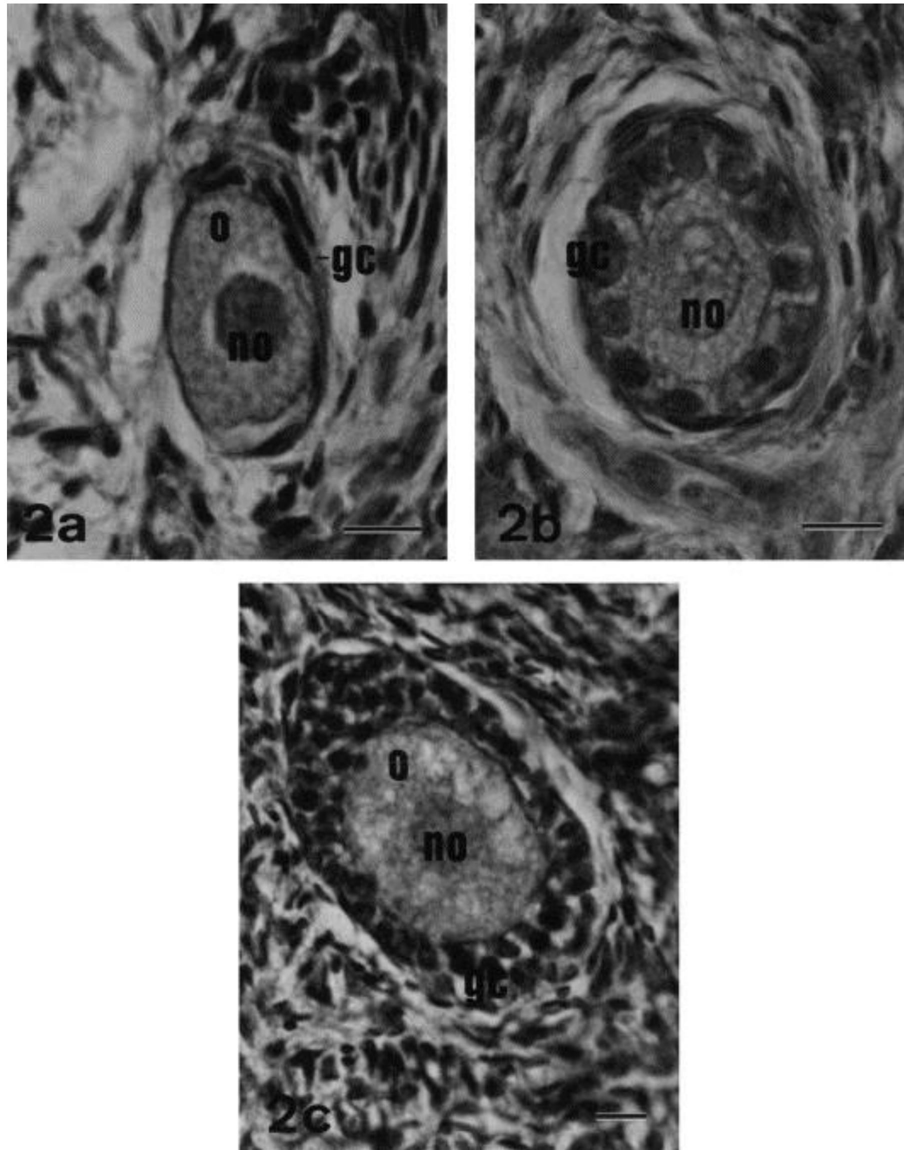

Fig. 2. Histological sections of the ovary, showing (a) primordial, (b) primary and (c) secondary follicles. O: Oocyte; NO: Nucleus of oocyte; GC: Granulosa cells. Bars=10 µm.

Table 3 shows the diameter of primordial, primary and secondary follicles in situ and after isolation. The diameters of isolated primary and secondary follicles were significantly greater than those observed in situ. A significant difference among the diameters of the three follicular classes was also observed, both in situ and after isolation.

Table 3.

Diameter in µm (mean±s.e.m.) of primordial, primary and secondary follicles in situ and after isolation

Follicular class	Mean diameter (µm) (ranger)			
	N		*N*	Isolated
Primordial	162	20.05±0.31^{aA}	322	21.51±0.21^{aA} (12.90-32.25)
Primary	162	24.42±0.32^{aB}	81	32.40±0.57^{bB} (25.80-51.60)
Secondary	48	44.24±3.61^{aC}	29	58.94±3.55^{bC} (32.25-122.55)

^{a,b} Values with different superscripts in the same row are significantly different ($p < 0.0001$)

^{A,B,C} Values with different superscripts in the same column are significantly different ($p < 0.0003$)

In regard to histological evaluation of the goat ovaries, the analyzed sections showed that primordial follicles were always located in the outer part of the cortex. Primary follicles were found in the outer and inner parts of the cortex, while secondary follicles were distributed only within the inner part of the cortex.

Polyovular preantral follicles were commonly observed (Fig. 3), more frequently in primordial, but also in primary and secondary follicles, and mainly in ovaries of prepubertal animals. In prepubertal, non-pregnant and pregnant goats, polyovular follicles were observed, respectively, in 100% (6/6), 50% (3/6) and 17% (1/6) of the ovaries studied. The number of oocytes within the same follicle varied from 2 to 9 for primordial follicles, and from 2 to 3 for primary follicles. Polyovular secondary follicles had no more than two oocytes. The total number of polyovular preantral follicles was not evaluated for lack of parameters of calculation. In isolated follicles, only one case of a polyovular follicle was observed.

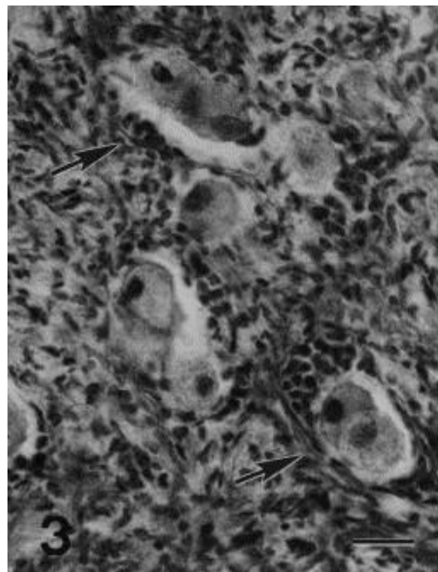

Fig. 3. Histological section of the ovary of prepubertal goat, showing polyovular follicles (arrows). Bar=20 µm.

3.3. Quality of preantral follicles in situ and after mechanical isolation

Histological analysis showed that the vast majority of preantral follicles were morphologically normal (Fig. 4a) and only a few preantral follicles were atretic in situ and after the isolation procedure in the three reproductive stages. Taking the data together, the percentage of atretic follicles was 4.7%±0.7 for isolated follicles and 4.8%±0.4 for follicles in situ. No differences among reproductive stages were observed. Retraction of the oocyte and/or condensation of its nucleus (Fig. 4b) characterized the follicular degeneration more frequently observed. Pycnotic bodies were not observed in granulosa cells in the degenerated follicles, in situ or after isolation. Irregular spaces between the layers of granulosa cells were

found in secondary follicles in situ. The significance of these spaces is unclear, and due to the absence of pycnotic granulosa cells or degenerating oocytes, these follicles were classified as healthy.

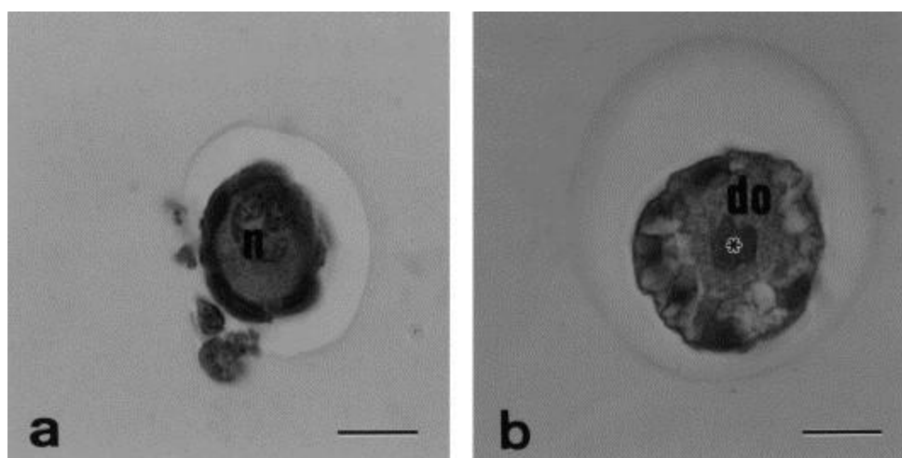

Fig. 4. Histological sections of (a) a morphologically normal follicle and (b) a degenerated follicle, after isolation. N: Nucleus of a normal oocyte, DO: Degenerating oocyte (* marks condensed nucleus). Bars=10 µm.

4. Discussion

This study shows for the first time an estimation of preantral follicular population in caprine ovaries. The results of this study showed that the ovaries of prepubertal and adult goats contain large numbers of preantral follicles (on average 37,646±4277). A similar number of in situ preantral follicles was reported for sheep by Driancourt et al. (1985). Other authors reported greater numbers of preantral follicles in ovaries from ewes (Land, 1970) and cows (Erickson, 1966b). Besides the species differences, preantral follicle population may be affected by many factors including breed (Cahill et al., 1979). It is important to note that all the animals used in the present study were mixed breed goats. Although the age (Erickson, 1966a and Erickson, 1966b) and reproductive stage (Erickson et al., 1976) may also affect the preantral follicular population, in the present study no differences were observed in the number of preantral follicles in situ among prepubertal, non-pregnant and pregnant adult goats. According to Erickson (1966b), in cattle the number of preantral follicles remains stable until the fourth year of life. An influence of puberty on the total number of preantral follicles in cattle was not observed, consistent with the results for goats presented here.

The results showed that the specific mechanical method developed for the isolation of preantral follicles from caprine ovaries can be successfully used to isolate a large number of well preserved preantral follicles from prepubertal, non-pregnant and pregnant goats. Although many papers have shown that mechanical and/or enzymatic methods allow the

isolation of a large number of preantral follicles per ovary, no information is available concerning the efficiency of such methods based on the population of preantral follicles present in the ovary. Taking into consideration that the population of preantral follicle may be affected by many factors, for the best evaluation of the isolation methods, it is important to use the same ovary or at least the contralateral ovary as a control. Using the same experimental design described in the present paper, recent work with ovine ovaries shows that the specific mechanical isolation procedure can recover about 27% of the entire population of preantral follicles from adult ewes, and about 5% from fetal lambs (Amorim et al., 1998a). In our study, the specific mechanical method for the isolation of preantral follicles from caprine ovaries recovered about 36% of the entire population of preantral follicles from goats, irrespective of the reproductive stage (prepubertal, non-pregnant and pregnant adult animals).

In this study, the proportions of primordial, primary and secondary follicles were different in situ than after isolation. The procedure used to isolate preantral follicles in the present study was shown to be more efficient to isolate primordial than primary and secondary follicles. Primordial follicles formed 93.8% of the isolated preantral follicles, whereas primary and secondary follicles formed 5.2% and 1.0%, respectively. Previous studies provided similar results in the isolation of preantral follicles using a tissue chopper (Rodrigues et al., 1998; Lucci et al., 1999; Amorim et al., unpublished results). In contrast, Hulshof et al. (1994), using watchmakers forceps to isolate preantral follicles, observed a low percentage of isolated primordial follicles (12.5%) when compared to primary (57.2%) and secondary (20.7%) follicles. These authors suggested that results be due to primordial follicles being tightly embedded in the tunica albuginea (Greenwald and Moor, 1989), and a mild mechanical treatment is not sufficient to isolate primordial follicles. Large numbers of primordial follicles were easily isolated from pig ovaries using a combined mechanical and enzymatic method (Greenwald and Moor, 1989), suggesting that an enzymatic treatment of the ovaries may be necessary to isolate this class of preantral follicles. We suggest that the use of the tissue chopper is a more powerful method to isolate a great numbers of primordial follicles without additional enzymatic treatment. In general, this mechanical method isolated about 50.2%, 5.9% and 5.5%, respectively, of the total primordial, primary and secondary follicles present in the goat ovaries.

In the present study, the diameters of the freshly isolated preantral follicles were greater than those measured in ovarian tissue sections. This may be due to shrinkage of the tissue after histological procedures. The diameters of isolated primordial, primary and secondary follicles from caprine ovaries were similar to those observed for sheep (Amorim et

al., unpublished results) and goats (Rodrigues et al., 1998), but were smaller than those reported for bovine fetuses (Hulshof et al., 1994). In situ, the diameters of caprine preantral follicles were also smaller than those in ovaries from bovine fetuses (Hulshof et al., 1994). These differences may be species-specific or due to differences in histological techniques. The location of primordial, primary and secondary follicles in the cortex of caprine ovaries was similar to that described to sheep (Hadek, 1958) and monkeys (Koering et al., 1991).

In this study, many polyovular follicles were observed, mainly in ovaries of prepubertal animals and more frequently in primordial follicles. Hadek (1958) reported that polyovular follicles are commonly found in ovine ovaries. Polyovular follicles were also found in bitches (McDougall et al., 1997) and pigs (Greenwald and Moor, 1989). Nuttinck et al. (1993) observed one case of a biovular follicle in a population of isolated bovine preantral follicles. According to Rüsse (1983), in fetal life, the oogonia are found side by side, forming clusters in the gonads of cattle and sheep. The accompanying somatic cells, which are probably the precursors of the follicular cells, are also included in the clusters. Starting from the second third of gestation, the isolation of the oocytes starts with an increase in the number of accompanying somatic cells. We suggest the possibility that the isolation of the oocytes, is sometimes not complete in goats, giving rise to polyovular follicles that persist in postnatal life. McDougall et al. (1997) suggested that a population of oocytes from one germ cell may remain held together in a polyovular follicle. However, the precise role of polyovular follicles in mammalian ovary remains unknown. It would be interesting in the future to investigate the possible development of these follicles in vitro.

The small number of degenerated follicles observed in this study is confirmed by other authors (Cahill et al., 1979; Jorio et al., 1991). The histological analysis of preantral follicles, in situ and of isolated follicles, showed that the degeneration is characterized by oocyte alterations and pycnotic bodies were not observed in granulosa cells. Jorio et al. (1991) described that degeneration of the oocyte is the mode of atresia more frequently observed in preantral follicles. Although pycnosis of granulosa cells occurs in follicles with an antrum, it is almost absent in preantral follicles. Hirshfield (1983) also reported that in secondary follicles, between 75 µm and 150 µm of diameter, the oocyte degeneration was usually the only sign of atresia. In bovine preantral follicles, Figueiredo et al. (1994) found that the follicular degeneration is characterized by a rapid and early disappearance of the oocyte which often occurs before structural follicular changes become visible under the inverted microscope. In a study in vitro, Braw-Tal and Yossefi (1997) related that in some preantral follicles the oocyte degenerated or completely disappeared while granulosa cells looked healthy and continued to proliferate, showing that the oocyte is much more sensitive degeneration events than the

granulosa cells. The secondary follicles with irregular spaces between the layers of granulosa cells were classified as healthy, since they did not have pycnotic granulosa cells or degenerated oocyte. Such classification was supported by the findings of Hirshfield (1983). This author also suggests that these irregular spaces are a sign of premature antrum formation.

In conclusion, this work shows for the first time an estimation of the population of preantral follicles in caprine ovaries. Moreover, a large number of preantral follicles was successfully isolated, with a high recovery rate and a low rate of follicular degeneration, from ovaries of prepubertal, non-pregnant and pregnant goats using a specific mechanical method.

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