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Population estimate of the preantral follicles and frequency of multioocyte follicles in prepubertal and adult bitches

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ABSTRACT

Oocytes from preantral follicles could be an alternative for *in vitro* maturation because most follicles are at the preantral stage. There are few studies that have sought to estimate the number of preantral follicles in bitches. Therefore, the aims of this study were to estimate the population of preantral follicles in the ovaries of small- and medium-sized prepubertal and adult bitches and compare the population of preantral follicles between the right and left ovaries and evaluate the frequency of multioocyte follicles (MOF). Eighty ovaries were collected by elective ovariohysterectomy from 40 healthy bitches. The bitches were divided into four groups: small-size prepubertal bitches (<10 kg, n = 20), medium-size prepubertal bitches (10–20 kg, n = 20), small-size adult bitches (<10 kg, n = 20), and medium-size adult bitches (10–20 kg, n = 20). Immediately after surgery, the ovaries were fixed in Bouin's solution and processed for histology. For each specimen, 70 histologic sections were cut and mounted on slides; then, the number of preantral follicles was estimated using a correction factor. The preantral follicles were classified according to the developmental stage. The data were analyzed using the Kruskal–Wallis test followed by Dunn's test for comparison between groups, and Fisher's exact test was used to evaluate the frequency of MOF ($P \leq 0.05$). Considering the population of preantral follicles from the pair of ovaries, medium-size prepubertal bitches had the highest ($P < 0.05$) population of preantral follicles compared with the small and medium-size adult groups. There was a large variation in the numbers of preantral follicles among individuals of the same weight and within each group. There were differences between medium-size prepubertal and adult bitches regarding the population of preantral follicles in the right ovaries (145,482 ± 110,712 vs. 49,500 ± 44,821; $P = 0.02$); however, no differences were observed between the groups on the basis of comparisons of the number of preantral follicles in the left ovaries ($P > 0.05$). The prevalence of primordial MOF was higher in prepubertal bitches (47% vs. 28%), whereas adult bitches had a higher frequency of secondary MOF (49% vs. 25%; $P < 0.05$). We conclude that medium-size prepubertal bitches had the highest population of preantral follicles compared with small and medium-size adult bitches, and the use of only one ovary per bitch implied contrasting result. The presence of primordial MOF was higher in prepubertal bitches and at the secondary stage in adult bitches.

1. Introduction

Scientific knowledge about the application of biotechnology to the breeding of pet animals has increased. This

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knowledge will provide benefits to pet dogs and endangered wild canids [1].

The low efficiency of *in vitro* maturation of canine oocytes from antral follicles reduces the use of reproductive biotechnologies. The use of oocytes from preantral follicles would present an alternative follicular source as they are greater in quantity and are present at all stages of the reproductive life of the animal [2]. Although oocytes from preantral follicles have lower developmental competence than oocytes recovered from antral follicles, there have been some attempts to recover mature canine oocytes from preantral follicles *in vitro* [3,4].

Biotechnologies applied to reproduction, such as *in vitro* embryo production, cloning, and transgenesis, use oocytes from antral follicles. However, in addition to the difficulty of recovering *in vitro*–matured oocytes from bitches, most of the oocytes are enclosed in preantral follicles [5]. Therefore, advances in the *in vitro* culture of preantral follicles and in the maturation process of the oocytes inside these follicles would improve the results of reproductive biotechnologies and provide a large number of oocytes from the same animal in the future. The *in vitro* maturation of oocytes in the germinal vesicle stage is possible, although they account for a lower proportion compared with the maturation of oocytes harvested from preovulatory follicles [6].

Because of the inherent particularities of canine physiology, canine reproduction biotechnologies cannot be standardized in regards to the collection, selection, and maturation processes of oocytes [7]. Ovarian follicular population studies are needed, especially in regards to the preantral follicle population.

The influence of the phase of the estrous cycle on the *in vitro* maturation of oocytes in bitches is not well established. It was reported that preovulatory hormonal events do not affect the subsequent *in vitro* maturation of high-quality oocytes regardless of the phase of the estrous cycle [8]. Both oocytes collected from bitches at the anestrous and estrous stages were able to mature *in vitro* [6]. In addition, canine oocytes collected during the anestrous stage had lower competence for maturation compared with the *in vitro*–matured oocytes [9].

In this context, the study of the multioocyte follicles would be of great interest. This structure is typically described during the fetal stage and is present in greater numbers in the ovaries of bitches compared with other species. However, there is no information regarding their role during folliculogenesis, and it remains an intriguing physiological phenomenon in mammals [10,11].

Because the *in vitro* maturation of bitch oocytes obtained from antral follicles has not achieved greater developmental rates, the use of oocytes from preantral follicles would present an alternative follicular source in the future. In addition, studies on the preantral follicle population in bitches are rare [12,13]. Therefore, it would be important to compare the number of preantral follicles between the bitches at different reproductive stages. The aims of this study were to estimate and compare the population of preantral ovarian follicles between prepubertal and adult bitches of small and medium size and compare the population between the right and left ovaries and evaluate the frequency of multioocyte follicles in the ovaries.

2. Materials and methods

2.1. Animals and ovary collection

This study was conducted according to the Ethical Principles of Animal Research and under the approval of the Animal Ethics Committee of our Institution, approved at Ethics Committee on Animal Use 24531.2011. Ovaries ($n = 80$) from 40 prepubertal ($n = 20$) and adult mongrel bitches ($n = 20$) were obtained by elective ovariohysterectomy [14]. The females did not present with any macroscopically detectable ovarian or uterine pathology. The body condition score was 3 ± 1 (scale, 1–5) [15]. The ovaries were identified as right or left. Each female was assigned to one of four groups according to weight (small, <10 kg; or medium size, 10–20 kg) and reproductive status (prepubertal or adult bitches). The average age was 8 ± 0.4 months (4–8 months) for the small-size and 8 ± 0.6 months (5–10 months) for the medium-size prepubertal bitches, and 2 ± 0.4 years (1–5 years) for the small-size and 2 ± 0.3 years (1–4 years) for the medium-size adult bitches. The average weight was 5.5 ± 0.6 kg (2.3–8.7 kg) for the small-size and 11.4 ± 0.9 kg (10.8–15.3 kg) for the medium-size prepubertal bitches, and 6.4 ± 0.4 kg (4.5–8.7 kg) for the small-size and 12.7 ± 0.7 kg (10.0–20.0 kg) for the medium-size adult bitches.

2.2. Histologic processing

Immediately after the collection of the ovaries, the ovaries were washed in a 0.9% saline solution and immersed in Bouin's fixative (0.9% picric acid, 9% formaldehyde, and 5% acetic acid) [16] for 24 hours at 4 °C and then placed in 70% alcohol. The ovaries were dehydrated in increasing concentrations of alcohol, diaphonized in xylol, embedded in paraffin, and serially sectioned at 5 μm [17] with a rotating microtome (Leica RM2255; Leica Biosystems Melbourne Pty Ltd., Wetzlar, Germany). In all ovaries, one of each 70 histologic sections was mounted and stained with periodic acid–Schiff and hematoxylin [18]. All procedures were performed by the same operator.

2.3. Classification of preantral follicles and multioocyte follicles

The preantral follicles and the multioocyte follicles were classified according to the developmental stage: primordial (one layer of flattened or flattened cuboidal granulosa cells surrounding the oocyte), primary (one layer of cuboidal granulosa cells surrounding the oocyte), or secondary (oocytes surrounded by two or more cuboidal layers of granulosa cells) [19]. Follicular morphology was evaluated on the basis of integrity of the basal membrane, the cell density, the presence or absence of pyknotic bodies in the nucleus of the oocyte, and the integrity of the oocyte [20]. On the basis of these parameters, only morphologically healthy follicles were evaluated in this study (with an intact basement membrane, intact oocyte, without pyknotic bodies, and considering the number and shape of the granulosa cells). Sections were examined under a light microscope (L2000; Bioval, Biochemistry, São Paulo, Brazil). Using an ocular micrometer, the average diameter of each oocyte nuclei was determined using two follicles of each follicular class (primordial, primary, and secondary) for each section evaluated [18].

2.4. Follicular count

The number of preantral follicles was estimated by counting the follicles in each section, using the nucleus of the oocyte as a reference, and employing a correction factor using the following formula.

$$Nt = (No \times St \times ts)/(So \times do)$$

Nt = total number calculated from a type of follicle in the whole ovary (primordial, primary, or secondary), No = number of follicles observed in all the sections, St = total number of cuts made in the ovary, ts = cutting thickness, So = total number of sections evaluated, and do = mean diameter of the follicles nucleus of each category [21].

The presence of multioocyte follicles was evaluated in all sections during the follicular counting, and the frequency of multioocyte follicles was compared between groups.

2.5. Statistical analysis

The results are presented as the mean ± standard deviation. The BioStat 5.0 program was used to test the normality of the samples, which were not normal [22]. Therefore, a Kruskal–Wallis test, followed by Dunn's test, was used for comparisons between the groups. For comparisons of the frequency of multioocyte follicles between groups Fisher's exact test was used. For all assessments, $P \leq 0.05$ was considered significant.

3. Results

A total of 1337 histologic sections were analyzed from the 80 ovaries examined, and on average, 335,228 follicles were counted, resulting in a total of 6,704,566 preantral follicles. No statistically significant difference was observed in the mean number of preantral follicles between small and medium-size prepubertal bitches ($P = 0.0659$) or between small and medium-size adult bitches ($P = 0.4171$). Medium-size prepubertal bitches had a higher number of preantral follicles ($P < 0.05$) compared with adult females (Table 1). There was a tendency ($P = 0.0633$) for prepubertal bitches to have a higher number of preantral follicles (107,152 follicles) compared with adults (60,461 follicles) regardless of the weight of the bitches.

Some females presented a variation in the number of preantral follicles between the right and left ovaries. Among females that presented with the highest variation, the mean number of preantral follicles was 1461 (right ovary) and 57,707 follicles (left ovary) in small-size prepubertal bitches, 358,482 (right ovary) and 231,279 follicles (left ovary) in medium-size prepubertal females, 76,586 (right ovary) and 212,520 follicles (left ovary) in small-size adult bitches, and 52,423 (right ovary) and 107,267 follicles (left ovary) in medium-size adult females (Figs. 1 and 2).

Considering only the right ovary, the number of preantral follicles from medium-size prepubertal bitches was higher compared with the medium-size adult females ($P \leq 0.05$). However, there was no difference in the population of preantral follicles among groups when only the left ovary was evaluated (Table 2).

Among the ovaries evaluated, 22 of 40 (55%) had multioocyte follicles at the primordial, primary, and secondary stages of development. Multioocyte follicles containing two or three oocytes were more abundant. However, multioocyte follicles with up to 12 oocytes were observed. The prevalence of multioocyte follicles at the primordial stage was higher for prepubertal bitches (47% vs. 28%), but adult bitches exhibited a higher frequency of secondary multioocyte follicles (49% vs. 25%; $P < 0.05$). There was no difference in the prevalence of multioocyte follicles at the primary stage between prepubertal and adult bitches (28% vs. 23%; $P > 0.05$).

4. Discussion

Although other studies have estimated the population of preantral follicles, this study is the first that compares the population of preantral follicles between prepubertal and adult bitches while considering the differences in the reproductive status and the size based on the weight of animals and comparing the right and left ovary follicles [12,13].

In the present study, we did not observe any differences in the mean number of preantral follicles between the small and medium-size prepubertal bitches or between the small and medium-size adult bitches. However, medium-size prepubertal bitches had higher numbers of preantral follicles compared with adult females (Table 1). No

Table 1
Mean ± standard deviation preantral follicles in ovaries of small and medium-size prepubertal and adult bitches.

Groups	Number of preantral follicles per bitch			
	Primordial	Primary	Secondary	Total
Prepubertal small-size (±5.51 kg, n = 20)	$73,046 \pm 72,124^{ab}$	4747 ± 9694^{a}	1045 ± 1673^{b}	$78,838 \pm 83,080^{ab}$
Prepubertal medium-size (±12.65 kg, n = 20)	$125,283 \pm 90,382^{a}$	7324 ± 7622^{a}	2860 ± 2614^{a}	$135,467 \pm 97,127^{a}$
Adults small-size (±6.52 kg, n = 20)	$57,179 \pm 47,104^{b}$	2810 ± 2054^{a}	1652 ± 1272^{a}	6164 ± 48978^{b}
Adults medium-size (±13.23 kg, n = 20)	$53,433 \pm 64,440^{b}$	3812 ± 5249^{a}	2037 ± 1761^{a}	$59,283 \pm 70,238^{b}$

Means followed by different letters in the same column differ ($P \leq 0.05$).

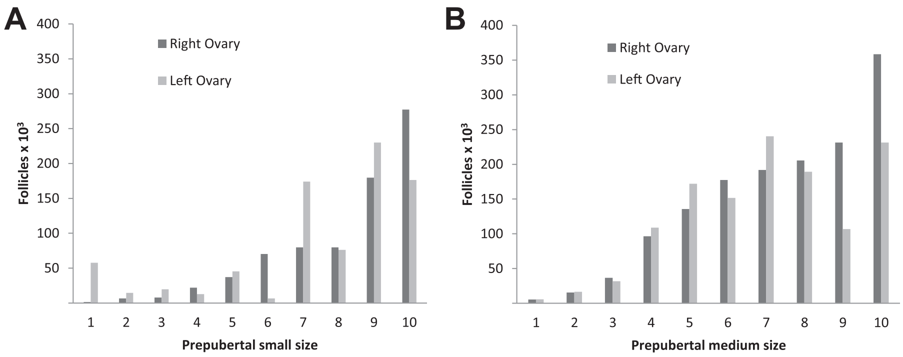

Fig. 1. Variation in the number of primordial follicles in ovaries of prepubertal small (A) and prepubertal medium (B) females.

difference was observed between the groups when considering only the reproductive status. However, the mean number of preantral follicles in prepubertal (107,152 follicles) and adult bitches (60,461 follicles) was higher in our study compared with the values reported in previous studies (Dolezel et al. [12], 474 follicles; Rolo [13], 47,900 preantral follicles). It is important to consider that Dolezel et al. [12] analyzed only 20 fields (50 mm^2 ovary) per ovary by immunohistochemistry and used ovaries from pubertal females, but there was no mention of the weight of the bitches; in addition, Rolo [13] used histologic processing and obtained ovaries from females between 6 months old and 8 years old. Moreover, Rolo (2012) performed 5-μm serial sections, and a histologic section was mounted every 120 sections, which could have contributed to the lower number of follicles reported [12,13]. If we had performed serial closing cuts (<70 sections), we would have increased the chance of counting the same follicle multiple times because the mean diameter of the primordial follicle in bitches is 25 μm [17].

In this study, we collected ovaries from prepubertal and adult bitches less than 5 years old to estimate the follicular population before the first estrus and before the possible decline of ovarian function. Females were classified as small or medium-sized because smaller-sized bitches have a lower interval among estrus and therefore a higher estrus presentation over the year than larger-sized females [23,24]. Dolezel et al. [12] reported that younger females (≤6 years) had a higher number of follicles than older females (7–11 years); however, these authors did not consider the differences in reproductive status or the size of the bitches.

We also observed a variation in the number of preantral follicles between the right and left ovaries from the same females, in addition to a variation within females of the same group (Fig. 1). Individual variations among the ovaries include extremes of 1461 to 358,482 follicles. Therefore, the evaluation of only one ovary per female would have underestimated or overestimated the population of preantral follicles. When only left ovaries were used, no differences were observed between the groups. The evaluation of only right ovaries resulted in differences between medium-size prepubertal and adult bitches (Table 2).

The numerical comparison, not just the statistical significance, for the follicular population in bitches is interesting because the variation between the individuals is extremely large and prevents the use of any statistical model. Moreover, the females used in this study were from a contraceptive program, and for most of them, information about family, reproductive history, or breed standard was lacking.

The primordial follicle class was predominant (92.2%) among the ovaries evaluated, a finding that is consistent with other reports on bitches and bovine females [13,18]. The greater quantity of primordial follicles in the ovary justifies the use of these follicles in assisted reproductive biotechnologies and could explain the difficulty of *in vitro* oocyte maturation because the primordial follicle likely requires more time and more developmental factors for maturation.

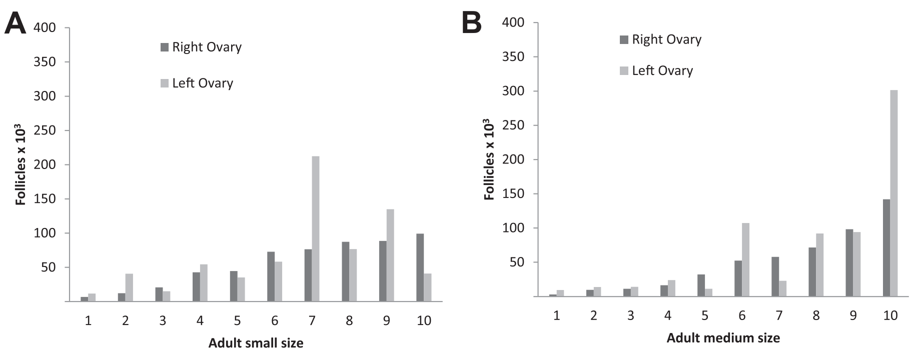

Fig. 2. Variation in the number of primordial follicles in the ovaries of adult small (A) and adult medium (B) females.

Table 2
Mean number ± standard deviation of preantral follicles in ovaries (right and left) of small and medium-size prepubertal and adult bitches.

Groups	Number of preantral follicles per ovary	
	Right	Left
Prepubertal small-size (±5.51 kg, n = 20)	76,245 ± 88,686[ab]	81,432 ± 81,800
Prepubertal medium-size (±12.65 kg, n = 20)	145,482 ± 110,712[a]	125,483 ± 86,197
Adult small-size (±6.52 kg, n = 20)	55,183 ± 34,156[ab]	68,102 ± 61,683
Adult medium-size (±13.23 kg, n = 20)	49,500 ± 44,821[b]	69,067 ± 90,517

Means followed by different letters in the same column differ ($P \leq 0.05$).

There is no consensus regarding the influence of the phase of the estrous cycle of the bitch on the *in vitro* maturation of oocytes. There are reports that oocytes collected from bitches at different stages of the estrous cycle can be matured *in vitro*. Rodrigues and Rodrigues [8] reported no differences in the *in vitro* nuclear maturation of oocytes from bitches at various reproductive stages (follicular phase, diestrous, anestrous, pyometra, and pregnancy). Although they exhibit a lower proportion compared with oocytes collected from preovulatory follicles, oocytes collected from bitches at the anestrous stage were matured *in vitro* [6]. In addition, bitch oocytes matured *in vitro* had a higher metaphase rate (7.6%) compared with oocytes matured *in vivo* (2%) after being transferred to recipients [9]. Despite the lower rate of *in vitro* development of oocytes from preantral follicles, it was reported that they can reach maturation as well as the oocytes from antral follicles. Canine preantral follicles cultivated *in vitro* for a long period with FSH reached the antral stage [3], and a shorter period of culture was suitable for oocyte nuclear maturation [4,25]. Therefore, oocytes from preantral follicles could potentially be used for maturation and *in vitro* embryo production in the future.

In the present study, we observed multioocyte follicles in 22 of 40 ovaries (55%) at all stages of follicular development (primordial, primary, and secondary). Prepubertal bitches had a higher prevalence of multioocyte follicles at the primordial stage (47% vs. 28%), whereas secondary multioocyte follicles were more abundant in adult bitches (49% vs. 25%). The presence of multioocyte follicles in adult ovaries has been reported in mammals, although there is no information about their role in ovarian physiology [18,26–29]. Dog ovaries have a high prevalence of these structures, and despite some inconsistent reports, young bitches present a higher proportion of multioocyte follicles than adult bitches [28].

4.1. Conclusions

In summary, there was a great variation in the number of preantral follicles between the groups (prepubertal and adult, small and medium-sized) and among individuals within the same group. We also reported the presence of multioocyte follicles, which has been described in dogs. Prepubertal bitches had more primordial multioocyte follicles, and more multioocyte follicles at the secondary stage were observed in the adult bitches. It is important to consider the large variability between the right and left ovaries because the use of only the right or left may underestimate or overestimate the ovarian follicular population.

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