Theriogenology 83 (2015) 1015–1020

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

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

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ARTICLE INFO

Article history:
Received 4 June 2014
Received in revised form 13 October 2014
Accepted 1 December 2014

Keywords:
Canine
Histology
Left ovary
Ovariohysterectomy
Right ovary

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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http://dx.doi.org/10.1016/j.theriogenology.2014.12.002

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