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Number and density of equine preantral follicles in different ovarian histological section thicknesses

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ABSTRACT

Regardless of species, advances in preantral follicle culture and cryopreservation and transplant of ovarian tissue techniques are dependent on the number and density of preantral follicles in the ovary. This study tested the effect of different histological section thicknesses on number, classification, and density of equine preantral follicles. An ovarian fragment was obtained from 5- to 10-year-old mares (n = 14) after slaughter, and each fragment was submitted to three histological section thickness treatments: 3, 5, and 7 μm. The area (cm^2) of each ovarian fragment was measured, and the sections were evaluated by light microscopy. The percentage of morphologically normal follicles (89%) was similar ($P > 0.05$) among primordial, transitional, and primary follicles and also among histological section thicknesses. A greater ($P < 0.05$) number of preantral follicles per histological section were seen in the 7-μm (8.0 ± 2.2) than that in the 3-μm (3.4 ± 0.7) treatment. Furthermore, a linear regression analysis reported that the number of preantral follicles increased ($P < 0.05$) when a thicker section treatment was used. However, no association ($P > 0.05$) between follicular density and treatment was observed. The mean number of preantral follicles per fragment (45.3 ± 18.8) and the follicular density (3.0 ± 0.5 follicles per cm^2) were different ($P < 0.05$) among mares. In conclusion, this study on equine preantral follicles reported that (1) a 7-μm histological section thickness might be recommended because it allowed identification of a greater number of preantral follicles per sample, (2) a large individual variation in follicle population and density was detected regardless of histological section thickness, and (3) mares have a low number and density of preantral follicles when compared with those reported for other species.

1. Introduction

The ovarian reserve of preantral follicles and follicular density are important indicators of the reproductive capacity of a species. Moreover, this information can contribute to the development of reproductive techniques (e.g., ovarian biopsy, ovarian tissue cryopreservation, transplantation, and *in vitro* follicle culture) to maintain or improve fertility in mammals.

Although recent studies in horses have reported improvements in maturation and fertilization rates (after

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intracytoplasmic sperm injection) of oocytes originated from immature and mature follicles [1–4], the assisted reproductive technologies (ARTs) have been developed slowly when compared with those used with other species [3,5]. One of the causes for the slow progress of ARTs in horses has been the lack of consistent *in vivo* methods to harvest a large number of immature oocytes [6]. In the horse, distinct from other species, this process requires vigorous flushing and scraping of the follicle wall using ultrasonography [3]. As an alternative method to overcome this limitation, and considering that preantral follicles are of great abundance in the ovary, studies in our laboratory recently validated the use of a transvaginal ultrasound-guided ovarian biopsy pick up method to harvest preantral follicles in mares [7–9].

At this moment, a limited number of studies have reported on equine preantral follicle population; these reports have suggested the presence of a low number of preantral follicles in the ovary when compared with other species [7–11]. Therefore, studies on equine preantral follicle population and density are necessary to facilitate the development of ARTs using immature oocytes enclosed in preantral follicles.

Histological evaluation has been used to determine the features of preantral follicle population, classification, stages of development, and morphology, as well as follicular density [12]. However, this methodology is time consuming and requires the development of technical approaches to overcome this problem [13]. The histological section thickness can influence the evaluation of preantral follicle population. Studies in this area used different section thicknesses (e.g., 3 μm: mice [14]; 5 μm: ovine [15], equine [16]; 6 μm: ovine [17]; 7 μm: caprine [18], bovine [19]; or 10 μm: women [20], equine [8–10]). These studies reported the lack of a standardization for histological section thickness preparations even within the same species (e.g., ovine [21,22]). To our knowledge, there is no comparative study on the use of different histological section thicknesses for evaluation of preantral follicles in any species. Moreover, assessment of preantral follicular density has not been reported in mares.

The purpose of this study was to assess the effect of different histological section thicknesses (3, 5, and 7 μm) for evaluation of equine ovarian fragments to determine (1) the number, density, and morphology (normal, abnormal, and follicular class) of equine preantral follicles; (2) the diameter of follicles, oocytes, and oocyte nuclei; and (3) the number of granulosa cells per follicle class.

2. Materials and methods

2.1. Ovaries

Ovaries from 14 adult mixed-breed mares (5–10 years old) were harvested at an equine slaughterhouse. One fragment (15 × 15 × 1 mm) of a single ovary from each mare was collected and added to fixative Bouin's solution, transported to the laboratory, and after 24 hours placed in 70% ethanol until histological processing. During tissue collection, ovarian fragments with CL and antral follicles were avoided.

2.2. Histological processing and treatments

After standard histological preparation, the samples were cut on a microtome with different section thicknesses (treatments) of 3, 5, or 7 μm, placed on glass slides, and stained with periodic acid–Schiff and hematoxylin. Regardless of the section thickness, every tenth histological section was evaluated to ensure that each follicle was counted only once. After five mounted sections (one section per slide), the section thickness was changed to the next (i.e., 3, 5, and 7 μm). An average of 15 slides per ovarian fragment were evaluated.

2.3. Microscopy and end points

The slides were analyzed using light microscopy (Nikon, Japan) at magnification × 400 and an image capture system (Leica Imaging Software, Wetzlar, Germany). The following end points were evaluated: number of follicles and follicular density per section thickness, per fragment, and per follicle class; follicle morphology; number of granulosa cells per follicle class; and diameters of follicles, oocytes, and oocyte nuclei. Follicle, oocyte, and oocyte nucleus diameters, along with number of granulosa cells, were measured only in morphologically normal follicles. The average of two perpendicular measurements from the outer layer of granulosa cells was used as a measure of follicle diameter. The measurements were made only on follicles with a visible oocyte nucleus. All evaluations and measurements were performed by a single operator.

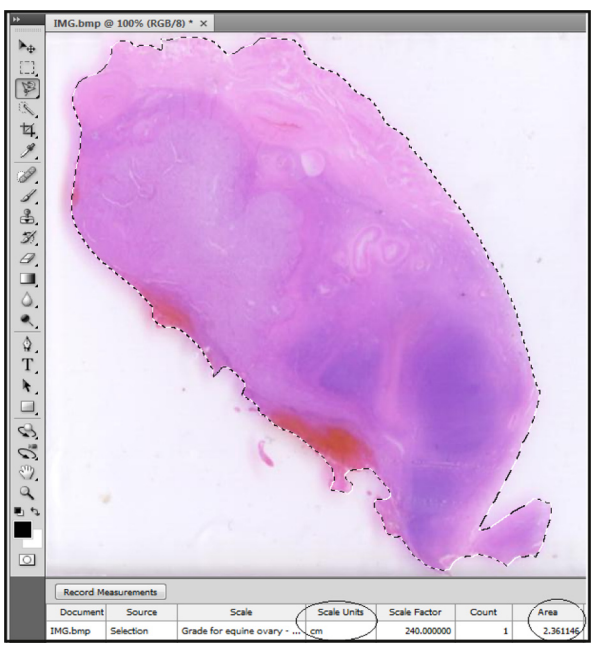

Fig. 1. Equine ovary histological section image scanned on a photo editing program. The border of the section was delimited (dashed line) with a marker tool, and the area was measured after scale calibration (black circle). (For interpretation of the references to color in this figure, the reader is referred to the Web version of this article.)