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Equine preantral follicles obtained via the Biopsy Pick-Up method: Histological evaluation and validation of a mechanical isolation technique

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ABSTRACT

The aims of this study in mares were to: (1) compare preantral follicle parameters between *in vitro* Biopsy Pick-Up (BPU) and scalpel blade collection methods and between histological and mechanical isolation processing (experiment 1); (2) histologically evaluate preantral follicles (experiment 2); and (3) compare histological analysis with a previously established mechanical isolation technique using a tissue chopper (experiment 3) for ovarian cortical fragments obtained *in vivo* using a BPU instrument. In experiment 1, preantral follicles were analyzed (N = 220; 90% primordial and 10% primary). Proportions of primordial and primary follicles did not differ ($P > 0.05$) between tissue collection (BPU vs. scalpel blade dissection) or processing (mechanical isolation vs. histology) methods. Follicle viability and morphology rates were similar ($P > 0.05$) between tissue collection methods, but mechanical isolation produced more ($P < 0.05$) morphologically normal follicles than histology. For experiment 2, preantral follicles (N = 332) were analyzed and primordial and transitional (combined) follicles and oocytes were 36.3 ± 0.3 and 26.1 ± 0.3 μm in diameter, respectively, and primary follicles and oocytes averaged 42.9 ± 1.8 and 31.8 ± 2.1 μm. For experiment 3 (188 preantral follicles), within the same animals, the proportion of primordial versus primary follicles was higher ($P < 0.03$) for histological analysis (98%) compared to tissue chopper analysis (94%), and number of follicles per mg of tissue was not affected ($P > 0.05$) by processing methods. In conclusion, most parameters evaluated for preantral follicles were similar between histological and tissue chopper processing techniques; hence, mechanical isolation efficiently dissociated equine preantral follicles from the ovarian cortex. Therefore, the tissue chopper could be used to isolate large numbers of morphologically normal equine preantral follicles for cryopreservation and/or *in vitro* culture.

1. Introduction

The recent success of *in vitro* culture systems for preantral follicles has enabled researchers to explore early folliculogenesis in several species. Live offspring have been produced from cultured murine preantral follicles fertilized *in vitro* [1–3], and viable embryos have been produced from cultured preantral follicles in several other species (rat [4], pig [5], buffalo [6], sheep [7], and goat [8]). This technology could facilitate the utilization of the large numbers of oocytes within primordial follicles present in the mammalian ovary at birth, a vast majority of which never ovulate, but ultimately degenerate and undergo atresia. It is noteworthy that adult equine ovaries were estimated to contain an average of 35,950 primordial follicles (range, 5600–75,000 [9]).

Little is known about early folliculogenesis in mares, partly because of the difficulty of obtaining preantral

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follicles for *in vitro* culture systems. Since the last equine abattoir in the United States was closed in 2007, the only way to harvest preantral follicles had been from ovaries obtained via ovariectomy or immediately after euthanasia or untimely death. However, the ultrasound-guided Biopsy Pick-Up (BPU) method was recently introduced in our laboratory as a new means to repeatedly harvest ovarian tissue fragments containing large numbers of primordial and primary follicles from living mares without affecting their normal short-term ovarian function or general reproductive health [10].

Preantral follicles can be released from the ovarian stroma of several species using either enzymatic (humans [11], cattle [12], and mice [13]) or mechanical isolation (cattle [14], goats [15,16], and sheep [17]), or a combination of both methods (cattle [18]). To our knowledge, there are only three studies in which equine preantral follicles were isolated from ovarian stroma, and all three employed enzymatic isolation using collagenase [19] with or without DNase [20,21].

A mechanical method using a tissue chopper was recently established in our laboratory, facilitating the isolation of large numbers of viable, morphologically normal primordial and primary follicles from equine ovarian tissue [10]. Previous studies have quantitatively and qualitatively compared the mechanical isolation method using a tissue chopper to *in situ* histological analysis of preantral follicles in sheep [17] and goats [16], but the efficiency of this isolation method has not been studied in horses. Therefore, the purposes of this study in mares were to: (1) compare follicle parameters between *in vitro* BPU and scalpel blade dissection and between follicles mounted on histology slides and mechanically isolated follicles; (2) histologically evaluate preantral follicles; and (3) compare preantral follicle parameters between follicles mechanically isolated using a tissue chopper and *in situ* follicles mounted on histology slides.

2. Materials and methods

2.1. Biopsy Pick-Up method

For each BPU procedure, the mare was restrained in a palpation chute to prevent excessive movement. The tail was wrapped in a plastic palpation sleeve to minimize contamination. The rectum was evacuated and the perineal area was washed with a povidine-iodine scrub and alcohol, and a Foley catheter was placed in the urinary bladder. Approximately 10 minutes before the start of each procedure, analgesia and rectal relaxation were induced with flunixin meglumine (Flunixiject; 1.1 mg/kg iv; Butler Schein Animal Health, Dublin, OH, USA) and hyoscine N-butyl bromide (Buscopan; 0.2 mg/kg iv; Sigma Chemical Co, St. Louis, MO, USA), respectively. Approximately 5 minutes before the start of each procedure, sedation and analgesia were induced with a combination of xylazine (AnaSed; 1 mg/kg iv; Lloyd Laboratories, Shenandoah, IA, USA) and butorphanol tartrate (Dolorex; 0.05 mg/kg iv; Intervet/Schering-Plough Animal Health, Millsboro, DE, USA). Mares were also given penicillin (Agri-Cillin; 6500 U/kg im; AgriLabs, St. Joseph, MO, USA) immediately after each procedure and on each of the next two days after the procedure.

The BPU device was a 48 cm long, automated, spring-loaded instrument with an inner trocar point plunger containing a 15 × 1.6 mm specimen notch surrounded by an outer 16 ga cutting needle (US Biopsy, Franklin, IN, USA). This device was introduced through a needle guide mounted on a probe handle with a 5 to 10 MHz transvaginal ultrasound-guided convex array transducer (Aloka UST-987-7.5) which was used for placement of the biopsy needle within the ovary.

Follicle aspiration was not performed before or during any procedure. The operator chose ovarian areas with few or no follicles or corpora lutea from each ovary to obtain biopsy fragments, with care to avoid the ovulation fossa. The ovary was manipulated transrectally and positioned against the vaginal wall so that the projected needle path could be visualized. The BPU needle was inserted through the vaginal wall and into the ovarian stroma with the trocar point plunger already retracted and the specimen notch covered by the outer cutting cannula. When the needle was properly positioned in the ovary, the inner stylet was advanced to expose the specimen notch. The spring-loaded device was then fired, which propelled the cutting cannula over the specimen notch, thus collecting any ovarian tissue resting within the notch. The BPU needle was then removed from the transvaginal ultrasound extension probe and the specimen notch exposed in order to retrieve the biopsy fragment. The time required for the BPU procedure to be completed in each mare varied from 10 to 40 minutes.

Biopsy fragments (N = 3 to 5 per mare) were removed from the specimen notch using a 25 ga needle and washed in a Petri dish containing fresh α-MEM medium (pH 7.2 to 7.4; Sigma Chemical Co., St. Louis, MO, USA) supplemented with 25 mM HEPES. Biopsy samples were individually placed in an Eppendorf tube containing α-MEM and HEPES, properly labeled, and transported to the laboratory in a styrofoam container at 4 °C (within 5 to 10 minutes after each biopsy procedure).

Transrectal ultrasonography was done 3 days after each BPU procedure and during subsequent estrous cycles to detect potential complications. After each BPU procedure, all biopsy needles were disassembled, cleaned, sharpened, had the firing spring replaced, and then were reassembled, individually packaged, and gas sterilized (ethylene oxide).

2.2. Experiment 1: comparison between in vitro ovarian BPU versus scalpel blade dissected fragments processed by mechanical isolation and histology

2.2.1. Ovaries

Ovaries (N = 2) from an adult (15-year-old) Quarter Horse mare were removed via a ventral abdominal incision immediately after euthanasia, washed in 70% alcohol for 10 seconds, placed in a plastic bag containing α-MEM medium supplemented with 25 mM HEPES, and then transported to the laboratory in a styrofoam container at 4 °C (within 10 minutes after removal).

2.2.2. Collection of ovarian tissue

In the laboratory, *in vitro* ovarian biopsies were performed on the fully intact ovaries (as previously described, but without ultrasound guidance). Biopsies were