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Effect of holding medium, temperature and time on structural integrity of equine ovarian follicles during the non-breeding season

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Abstract

The objective was to evaluate the efficiency of phosphate-buffered saline (PBS) and Minimum Essential Medium (MEM) during the transport of equine preantral and antral follicles at various temperatures and incubation interval. Equine ovaries (n = 10) from an abattoir were cut into 19 fragments; one was immediately fixed in Bouin's solution (control) and the other fragments were placed in PBS or MEM solution at 4, 20, or 39 °C for 4, 12, or 24 h. After the respective incubation periods, all fragments were fixed in Bouin's solution for 24 h and then submitted to standard histologic analysis. In total, 2567 ovarian follicles were analyzed, including 1752 primordial, 764 primary, 34 secondary and seven antral follicles. Relative to the control group, the transport of equine ovarian fragments in both solutions significantly reduced the percentage of morphologically normal follicles with increasing time and temperature. At 4 °C for 4 h, considering primordial and developing follicles, PBS had a higher ($P < 0.05$) rate (98.9%) of morphologically normal follicles than MEM, 48.7%. At 39 °C for 12 h, all follicles in both solutions were degenerated. Regarding the stage of follicular development, primordial follicles were less ($P < 0.05$) affected by preservation than primary and secondary follicles in all media, times and temperatures tested, except at 4 °C for 12 h in PBS, in which the primary and secondary follicles were less ($P < 0.05$) affected. Overall, 43% of antral follicles were morphologically normal when maintained in MEM at 4 °C for 4 h. In conclusion, equine follicles were successfully preserved in ovarian fragments at 4 °C in phosphate-buffered saline for up to 4 h.

Keywords: Ovary; Ovarian follicles; Follicle transport; Mare

1. Introduction

In the last few years, there has been increased interest in assisted reproductive biotechnologies to produce foals from mares that died unexpectedly or were euthanized. In these cases, the ovaries can be collected after death and processed to recover viable oocytes [1–3] for *in vitro* maturation and fertilization. Preantral follicles represent 90% of the follicular population and constitute a potential source of gametes, with the possibility of yielding thousands of oocytes for assisted reproductive biotechnologies [4]. However, a limiting factor for

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the successful use of these oocytes is the condition of the follicles after removal from the ovaries and transportation of the ovaries to the laboratory. Another important consideration is the non-breeding season, when reproductive activity is limited [5,6].

Many researchers have analyzed ovarian tissue preservation protocols for short intervals to compare combinations of temperatures and media for several species [7–19]. The type of medium previously used for *in vitro* studies has an important influence on survival and growth of follicles. For example, Minimal Essential Medium (MEM) has been used for culturing bovine ovarian tissue [20] and isolating preantral follicles [21]. Furthermore, phosphate-buffered saline (PBS) is often used for transportation of embryos and oocytes in several species [22–29].

Equine preantral follicles were isolated from ovaries using collagenase [30] or collagenase and desoxyribonucleases [31]. After isolation, individual follicles can be submitted to *in vitro* culture. Another biological model is preantral follicles within ovarian tissue, but there are no reports of the viability of these follicles after various intervals of transport, temperatures, and media. Therefore, development of protocols for preservation of equine ovarian tissue is crucial to ensure oocyte quality and, consequently, the success of reproductive biotechnologies.

The purpose of this study was to investigate the effect of time, temperature and holding medium on the structural integrity of equine follicles from ovaries obtained during the non-breeding season.

2. Materials and methods

2.1. Collection of ovaries

Ovaries (n = 10) without any CL or dominant follicle (derived from 10 mares in seasonal anestrus) were collected at an abattoir in Parana State, Brazil. This abattoir was approximately 150 km (90 min) away from the Laboratory of Animal Reproduction at Londrina State University. The ovaries were dissected with a scalpel blade and all adipose and connective tissues were removed. The ovaries were then cut sagittally and the cortical portion (more internal) was divided into small fragments (approximately 3 × 3 × 1 mm). All fragments were then washed in 70% alcohol, followed by a wash in 0.9% saline solution [32].

2.2. Media

The media tested for *in situ* transportation of equine preantral and antral follicles were Minimum Essential Medium (MEM; GIBCO, Grand Island, NY, USA, Cat. Number 41500-034) and phosphate-buffered saline solution (PBS; Sigma, St Louis, MO, USA).

2.3. Experimental protocol

Each ovary was divided into 19 fragments; one fragment was selected randomly and immediately fixed in Bouin's solution for 24 h for the control (Control/time zero -T1). The remaining 18 fragments were randomly distributed into 15 mL tubes containing 10 mL of MEM or PBS at 4, 20, or 39 °C, and stored for 4, 12, or 24 h (T2-19), as shown (Fig. 1). Temperatures were main-

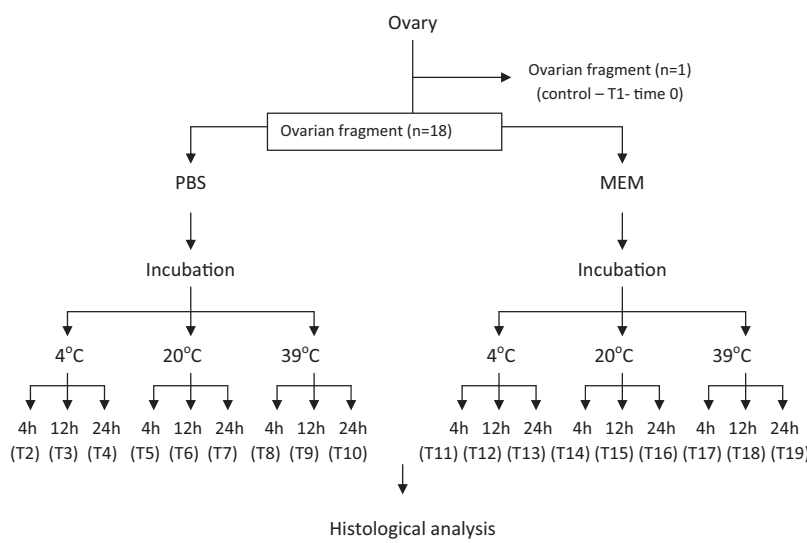

Fig. 1. Experimental protocol for *in situ* preservation of equine ovarian follicles.