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Insulin improves *in vitro* survival of equine preantral follicles enclosed in ovarian tissue and reduces reactive oxygen species production after culture

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ABSTRACT

This study investigated the effect of insulin concentration on the *in vitro* culture of equine preantral follicles enclosed in ovarian tissue. Ovarian tissue samples were immediately fixed (noncultured control) or cultured for 1 or 7 days in α-MEM$^+$ supplemented with 0 ng/mL, 10 ng/mL, or 10 μg/mL insulin. Ovarian tissues were processed and analyzed by classical histology. Culture medium samples were collected after 1 and 7 days of culture for steroid and reactive oxygen species (ROS) analyses. The percentage of morphologically normal follicles was greater ($P < 0.001$) in insulin-treated groups after 1 day of culture; likewise, more ($P < 0.02$) normal follicles were observed after 7 days of culture in medium supplemented with 10-ng/mL insulin. Furthermore, an increase ($P < 0.01$) in developing (transition, primary, and secondary) follicles between Days 1 and 7 of culture was observed only with the 10-ng/mL insulin treatment. ROS production after 1 or 7 days of culture was lower ($P < 0.0001$) in medium with 10-ng/mL insulin than the other treatments. Ovarian tissues containing preantral follicles were able to produce estradiol and progesterone after 1 and 7 days of culture; however, treatments did not differ in steroid production. In conclusion, the use of a physiological concentration (10 ng/mL) of insulin rather than the previously reported concentration (10 μg/mL) for *in vitro* culture of equine preantral follicles improved follicular survival and growth and lowered oxidative stress. Results from this study shed light on new perspectives for producing an appropriate medium to improve equine preantral follicle *in vitro* survival and growth.

1. Introduction

The progress of assisted reproductive biotechnologies in horses has been slower than in other domestic animals, mainly because of some technical barriers not present in other species and the deficient acceptance in many breed registries [1]. However, the high economic value of individual animals coupled with changing registry attitudes has resulted in a resurgence of interest and advances on horse-assisted reproductive techniques in recent years [1,2]. Studies using equine oocytes have been done with limited numbers of oocytes because of the failure of mares to respond to superovulatory regimes, and the scarce availability of horse abattoirs to collect ovaries for research projects. In this regard, the use of matured equine oocytes

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from *in vitro*–cultured preantral follicles (the main oocyte reserve) will potentially contribute to the preservation of genetic material in horses [3] and an increase in the reproductive efficiency of genetically superior animals [4].

Contrary to what has been observed in mice, in which offsprings have been produced from *in vitro* cultured preantral follicles [5,6], in farm domestic animals only the production of a few variable number of matured oocytes and embryos has been reported (rat: [7]; pig: [8]; buffalo: [9]; ewe: [10]; goat: [11]). In horses, there are only a few studies evaluating the *in vitro* culture of preantral follicles [12–14]. Recently, Haag et al. [14] used *in vitro* culture of ovarian biopsies in medium α-MEM$^+$ containing 10 μg/mL of insulin and reported that after 7 days of culture, 27% of preantral follicles were morphologically normal.

Insulin plays an important role in the regulation of ovarian function in several species. The presence of the insulin receptor has been identified in different cell types of the ovary [15]. This hormone maintains the viability and growth of ovarian follicles in humans [16], stimulates the production of steroids in ovarian interstitial cells of rats [17], and inhibits gene expressions that induce apoptosis under conditions of oxidative stress in mice [18]. However, the concentration of insulin used in the medium to culture preantral follicles presents a large variation among species as well as research groups (mice: 0.2 and 0.5 μg/mL [19]; dogs: 10 μg/mL [20]; goats: 10 ng/mL [21] or 10 μg/mL [22,23]; sheep: 10 μg/mL [24,25]; cattle: 10 ng/mL [26]; and horses: 10 μg/mL [14]). In general, in most studies, including those on horses, a supraphysiological concentration of insulin has routinely been added to a basic culture medium through the use of a commercial product called ITS (10-μg/mL insulin, 5.5-μg/mL transferrin, and 5-ng/mL sodium selenite). In this context, we hypothesized that the use of lower concentrations of insulin similar to physiological concentrations found in the horse plasma [27] would improve the survival and development of equine preantral follicles.

In addition, the effect of insulin on steroid (estradiol and progesterone) and reactive oxygen species (ROS) production after *in vitro* culture by equine preantral follicles enclosed in ovarian tissue has never been investigated up to now. ROS production is a valuable tool to evaluate *in vitro* oocyte quality [28,29], and the balance between the production and degradation of ROS is an indicative of oxidative stress control [30,31].

The aim of this study was to identify what concentration of insulin (10 ng/mL, physiological or 10 μg/mL, supraphysiological) is the most suitable for the *in vitro* culture of equine preantral follicles enclosed in ovarian tissue. To accomplish this goal, the following end points were evaluated: follicular survival, activation of primordial follicles, follicular and oocyte growth, and estradiol, progesterone, and ROS production.

2. Materials and methods

2.1. Chemicals

Unless otherwise noted, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals and ovaries

The research protocol (12637266–7) was approved by the Ethics and Animal Use Committee of State University of Ceará (UECE), Fortaleza, CE. The ovaries were obtained from euthanized mares (n = 5) positive for Equine Infectious Anemia (EIA). The mares were crossbreed, aged 3 to 11 years, and with body condition scores between 4 and 6 (1, emaciated to 9, obese; [32]).

Immediately after euthanasia, the ovaries were collected and washed in 70% alcohol, followed by two washes in minimum essential medium (MEM) supplemented with 25-mM HEPES. The ovaries were placed into tubes containing 150 mL of MEM-HEPES supplemented with 100-μg/mL penicillin and 100-μg/mL streptomycin and transported to the laboratory at 4 °C [33] within 4 hours. In the laboratory, both ovaries of each animal were stripped of surrounding fat tissue and ligaments. Subsequently, 56 ovarian tissue samples (approximate size, 3 × 3 × 1 mm) were obtained from each pair of ovaries under sterile conditions using a scalpel blade. Eight ovarian tissue samples were distributed for each treatment per day in five replicates.

2.3. Culture of preantral follicles and experimental design

Ovarian tissue was placed in 24–well culture plate containing 1 mL of culture media. Culture was performed at 39 °C in a humidified atmosphere with 5% CO_2 in air. Fresh medium was prepared immediately before use and incubated for at least 1 hour. The basic culture medium consisted of α-MEM (pH, 7.2–7.4) supplemented with 5.5-μg/mL transferrin, 5.0-ng/mL sodium selenite, 2-mM glutamine, 2-mM hypoxanthine, 1.25-mg/mL bovine serum albumin (BSA), and 100-μg/mL penicillin, and 100-μg/mL streptomycin, which was called α-MEM$^+$.

To test the effect of insulin in cultures of preantral follicles, the basic medium was supplemented with different concentrations of insulin, generating the following groups: 0-ng/mL insulin, 10-ng/mL insulin, and 10-μg/mL insulin. The ovarian tissue pieces were then either fixed for histologic analyses (fresh noncultured control group) or placed in culture for 1 or 7 days. Five replicates of each treatment were performed. The culture medium was replaced every other day, and before each replacement, 1 mL of medium was collected at Days 1 (24 hours) and 7 of culture and stored at −80 °C until hormonal and ROS analyses.

2.4. Morphologic evaluation and follicle development

Follicular morphology and development of preantral follicles were assessed *in situ* (ovarian tissues) before and after *in vitro* culture of ovarian tissue. Once harvested, ovarian tissue to be submitted to histologic analysis was fixed in paraformaldehyde solution at 4 °C for 12 hours and then kept in 70% alcohol. Ovarian tissues were dehydrated by use of a graded series of alcohol, embedded in paraffin wax, and cut into serial sections of 7 μm. Samples were stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin. Histology slides were analyzed using light microscopy (Nikon, Tokyo, Japan) at 400× magnification.