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REPORT

In vitro culture supplementation of EGF for improving the survival of equine preantral follicles

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Abstract

Folliculogenesis is a process of development and maturation of the ovarian follicles, being essential for the maintenance of fertility. In in vivo conditions, 99.9% of the follicles of an ovary do not ovulate and undergo atresia. In order to minimize this loss and to clarify the existing mechanisms, a technique was developed that allows for the in vitro follicular development. The objective of this study was to evaluate the effects of different epidermal growth factor (EGF) concentrations on the in vitro culturing of equine preantral follicles. Ovaries ($n = 10$) were collected from a local slaughterhouse of mares in seasonal anestrus, washed with 70% alcohol and PBS, and transported. The inner portion of the ovary was divided into 11 fragments of approximately 3 × 3 × 1 mm. A fragment of each ovary was immediately fixed in Bouin (control group). The remaining 10 fragments were individually cultured for 2 and 6 d. The medium was supplemented with different concentrations of EGF (0, 10, 50, 100, and 200 ng/mL). After cultivation, the fragments were processed and classified according to the developmental stage and morphology. In total, 1065 slides containing 6105 tissue sections were evaluated. Within 2 d of culture, there was a higher proportion of intact follicles at the EGF concentrations of 0 and 100 ng/mL ($p > 0.05$). After 6 d of culture, only the EGF concentration of 100 ng/mL demonstrated a difference when compared to the other treatments (0, 10, 50 and 200 ng/mL of EGF, $p > 0.05$). There was follicular development after 2 d at all EGF concentrations. Thus, we suggest that EGF promotes follicular survival in equines at a concentration of 100 ng/mL in in vitro cultures of ovarian fragments for 2 d. In addition, we suggest that EGF promotes follicular survival in equines at a concentration of 100 ng/mL in situ cultivation.

Keywords Ovaries · Mare · Ovarian follicles · Epidermal growth factor · In situ cultivation

Introduction

The in vitro culture of preantral follicles has been an increasingly attractive reproductive tool in recent years. The application of this model has enabled investigations of factors related to the development and follicular atresia. Once optimized, this system has also enabled the examination of in vitro ovulation and embryo production from oocytes found in preantral follicles. Continuous follicular growth is controlled both by hormones (gonadotrophic and somatotrophic) and growth factors that act, directly or indirectly, in an autocrine and/or paracrine manner (Figueiredo et al. 2008).

Thus, a better identification and understanding of the different substances involved in the promotion of follicular development and in the course of atresia are important aspects to support the development of a more efficient culture system. Thus, examination of follicular activation in vitro would provide further insight towards understanding the development of a large number of preantral follicles (Demeestere et al. 2005).

In addition to growth factors and hormones, hypoxanthine, glutamine, pyruvate, and insulin, transferrin and selenium are also used to increase follicular growth and inhibit atresia (ITS) in vitro. Of these factors, epidermal growth factor (EGF) has demonstrated effects on follicular survival and growth (Silva

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et al. 2010; Celestino et al. 2011). EGF plays an important role in ovarian folliculogenesis and regulates several processes, including activation of primordial follicles, proliferation of granulosa cells, reduction of atresia rate, and maintenance of follicular viability (Demeestere et al. 2005; Silva et al. 2010; Aguiar et al. 2017). In bovines, this factor promotes an increase in follicular diameter; however, in sows, it inhibits the apoptosis of granulosa cells and results in the formation of the follicular antrum (Mao et al. 2004). In caprines, Celestino et al. (2011) have demonstrated that EGF is an important regulator of ovarian physiology, regulating several processes, including follicular activation. Thus, we hypothesized that the addition of EGF into in situ culture medium demonstrates effects on the survival and integrity of preantral equine follicles.

Material and Methods

Ovarian collection

Ovaries ($n = 10$) were collected from ten mares in seasonal anestrus with unknown history, which were obtained from a slaughterhouse located approximately 40 km from the laboratory. At the slaughterhouse, the ovaries were washed with 70% alcohol, followed by a wash with PBS. Each ovary was carefully dissected with a scalpel, and the adipose and connective tissues were removed. The ovaries were sectioned along the sagittal plane, and those that contained corpus luteum or antral follicles in abundance were discarded. A portion of the parenchyma (internal) of the ten selected ovaries was sectioned into 11 fragments of approximately $3 \times 3 \times 1$ mm. A fragment of each ovary was randomly selected and immediately fixed in Bouin for histological analysis (control group; day 0). Using a protocol described by Gomes et al. (2015), we immediately placed all other fragments in PBS supplemented with penicillin (200 IU/mL) and streptomycin (200 mg/mL); the fragments were then incubated at 4°C for 1 h.

Follicular in vitro culture and classification

In the laboratory, the ovarian fragments of each animal were cultured in 24-well culture plates containing base medium (1 mL/well). The base medium (referred to as MEM^+ hereafter) consisted of minimum essential medium (MEM, pH 7.2–7.4) supplemented with penicillin (200 IU/mL), streptomycin (200 mg/mL), BSA (1.25 mg/mL), hypoxanthine (6.25 mg/mL), transferrin (6.25 mg/mL), and glutamine (2 mM), pyruvate (0.23 mM), and insulin (6.25 mg/mL).

Different concentrations of EGF (MEM^+ or 0, 10, 50, 100, or 200 ng/mL) were added to the minimal essential medium. The in vitro culture was performed at 39°C in 5% CO_2 atmosphere in air for 2 or 6 d, and medium replacement was performed every 2 d according to Gomes et al. (2015). The culture medium (MEM, product number, M 7278), EGF, and other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, EUA).

At the end of the culture period, the ovarian fragments were examined by classical histology. All samples were fixed in Bouin for 24 h, dehydrated in ethanol, xylol-diaphanized, paraffin embedded, and serially cut into 5-μm sections for assembly of slides with albumin. The staining was performed with periodic acid Schiff and Hematoxylin.

Follicles were classified according to the following stages of development: (1) primordial follicles (the oocyte is surrounded by a layer of flattened granulosa cells) or (2) developing follicles composed of primary follicles (the oocyte is surrounded by a single layer of cuboid granulosa cells) and secondary follicles (the oocyte is surrounded by more than one complete layer of granulosa cube cells). To evaluate the onset of growth of primordial follicles at different stages of development, the number of primordial and growing follicles was determined in the control group and in each in vitro culture treatment group.

The follicular classification was also classified as intact or degenerate (abnormal) follicles according to oocyte morphology and granulosa cells. Thus, the rate of follicular degeneration was determined in the control group and in each cultivated group. The follicles were classified as morphologically intact if the follicles had intact oocytes and the granulosa cells were arranged in layers without pyknotic nuclei. Degenerate follicles were defined as follicles abnormal with signs of degeneration. Degenerate follicles were those that presented an oocyte with a pyknotic nucleus and/or an oocyte surrounded by disorganized granulosa cells, which were detached from the membrane and/or present retracted cytoplasm (Gomes et al. 2015).

Statistical analysis

The proportions of primordial and developing follicles for each treatment (Control, MEM^+, EGF 10, EGF 50, EGF 100, and EGF 200) for 2 d of culture were compared. Each variable was analyzed for normality of distribution using the Kolmogorov-Smirnov test. The nonparametric Fisher's exact test was used to compare the percentages of primordial and developing follicles between the control, MEM, and different EGF concentrations. The integrity and follicular development data in 2 or 6 d of in vitro culture were compared using Student's t test. The level of significance to reject the null hypothesis was $p \leq 0.05$. All statistical analysis was performed using the Minitab® 16.1.1 statistical program.

Results

In this experiment, 1065 slides were evaluated, containing 6105 histological sections of 110 ovarian fragments obtained from

ten mares, unknown background. Of the 1109 follicles evaluated in all treatment (control, MEM, 10EGF, 50EGF, 100EGF, and 200EGF) for 2 or 6 d, of which the 37.8% (419/1109) were primordial follicles (Fig. 1a), and 62.2% (690/1109) were in development (Fig. 1b). Regarding integrity, 64.1% (711/1109) of the follicles were morphologically intact.

After 2 d of culture supplemented with 100 ng/mL (42.5%; 57/134) of EGF resulted in a greater rate of intact follicles compared to the other treatments (MEM, 27/90; 10EGF, 5/57; 50EGF, 14/82, and 200 ng/mL of EGF, 22/72; $p < 0.05$; Fig. 2). Thus, there was a greater efficiency in maintaining the morphological integrity of cultured follicles when compared to control (64.8%, 232/358; $p > 0.05$; Fig. 2). In the 6 d of culture, only the concentration of 100 ng/mL of EGF also was highlighted ($p < 0.05$; Fig. 2). When evaluating the integration between days of culture, we can observe that all the treatments were effective in maintaining the follicular integrity, except the concentration of 200 ng/mL of EGF (Fig. 2). Thus, in general, the number of whole follicles increased numerically during the in vitro culture.

In assessing follicular development during in vitro culture for 2 or 6 d, all treatments presented similar results ($p > 0.05$; Fig. 3). Therefore, it is important to emphasize that all treatments were effective in promoting the development of the primordial follicles when compared to the cultivated control (MEM; Fig. 3). When evaluating the treatments according to the days of cultivation, it was possible to observe that the different concentrations of EGF and the cultivated control (MEM) maintained the proportions of developing follicles at 2 and 6 d of cultivation.

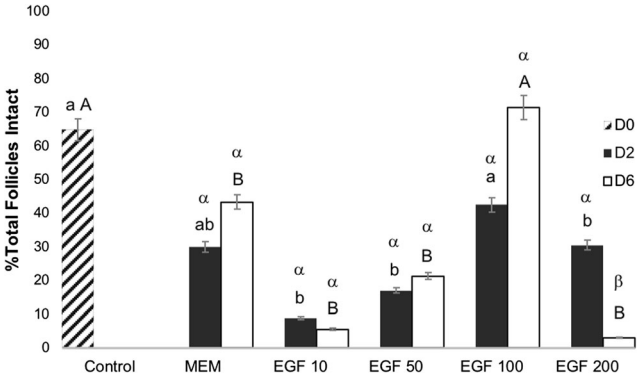

Figure 2. Percentage of whole (primordial + developmental) intact follicles in equine tissue cultured for 2 d (D2) and 6 d (D6) in minimal essential medium MEM$^+$ or supplemented with 10, 50, 100, and 200 ng/mL EGF. Values followed by *lowercase letters* (a, b, c) differ statistically between treatments (control, MEM$^+$, EGF 10, EGF 50, EGF 100, and EGF 200, $p < 0.05$) in 2 d. Values followed by *uppercase letters* (A, B, C) differ statistically between treatments (control, MEM$^+$, EGF 10, EGF 50, EGF 100, and EGF 200, $p < 0.05$) in 6 d. Values followed by *uppercase letters* (α, β) differ statistically between growing period (2 and 6 d)

Discussion

Therefore, in this study, the addition of 100 ng/mL of EGF for 2 and 6 d to in vitro equine culture medium demonstrated beneficial effects on the morphological integrity of preantral follicles. This is the first investigation of the effect of EGF on the in vitro culture of preantral follicles of in situ ovaries obtained from a slaughterhouse.

In vitro culture with the addition of EGF has been examined in swine, bovine, caprine, and ovine species. In these species, EGF is believed to be related to the regulation of ovarian physiology, including the activation, differentiation, and proliferation of granulosa cells, in addition to inhibiting follicular apoptosis (Markström et al. 2002; Celestino et al. 2009).

EGF in the culture medium of preantral porcine follicles at the concentration of 10 ng/mL has been shown to inhibit the apoptosis of granulosa cells and increase the formation of antrum (Mao et al. 2004). In the case of goats, EGF (50 ng/mL) has been shown to stimulate oocyte viability (Zhou and Zhang 2005) and, at the 100 ng/mL concentration, enhance the oocyte growth of primary follicles (Silva et al. 2004).

Similar to results observed in caprines, studies with ovine ovarian follicles supplemented with 50 ng/mL of EGF to the culture medium resulted in the formation of the antral cavity, high rates of follicular growth, and an increase in the proportion of morphologically intact follicles (Da Paz Santos et al. 2014). In addition, culture with MEM or TCM-199 in combination with EGF (10 ng/mL) has been shown to increase the diameter of the oocyte during follicular growth (Andrade et al. 2014).

Our findings are quite close to the data recently described by Aguiar et al. (2017). These authors reported 50 ng/mL of EGF

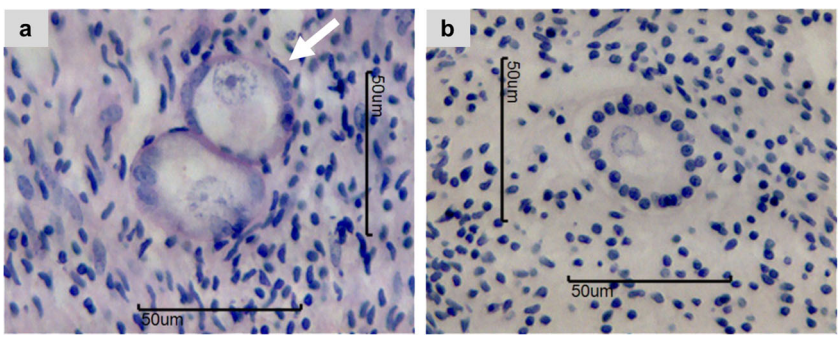

Figure 1. Morphological aspects of preantral follicles. (*a*) Normal primordial follicle (*arrow*) and (*b*) normal primary follicle. Sections were stained with periodic acid-Schiff (PAS) stain and hematoxylin.

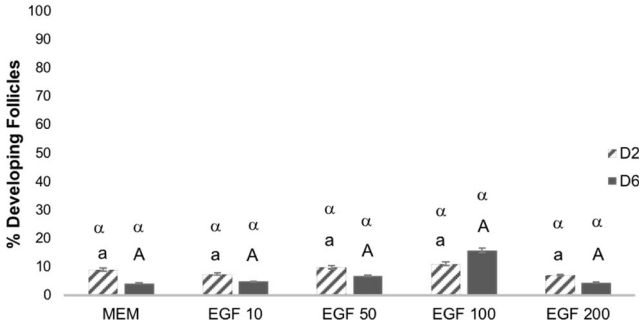

Figure 3. Percentage of developing follicles with preserved morphological integrity observed in equine tissues cultured for 2 d (D2) and 6 d (D6) in minimal essential medium (MEM^+) or MEM^+ supplemented with 10, 50, 100, and 200 ng/mL EGF. Values followed by lowercase letters (a, b, c) differ statistically between treatments (control, MEM^+, EGF 10, EGF 50, EGF 100, and EGF 200, $p < 0.05$) in 2 d. Values followed by uppercase letters (A, B, C) differ statistically between treatments (control, MEM^+, EGF 10, EGF 50, EGF 100, and EGF 200, $p < 0.05$) in 6 d. Values followed by uppercase letters (α, β) differ statistically between growing period (2 and 6 d)

as the most appropriate to improve the in vitro culture of equine follicles. We identified the concentration of 100 ng/mL as the best one. This little difference may probably be related to the source of the ovaries. Aguiar et al. (2017) obtained the equine follicles from mares submitted to euthanasia which allowed a quick access to the ovaries. In our case, the gonads came from the slaughterhouse and the work conditions are not so organized. We believe that our samples were subjected to a higher level of challenge in comparison to the follicles used by Aguiar et al. (2017). Because of that, a higher concentration of EGF (100 ng/mL) in our conditions was necessary to provide an appropriate environment to the follicular development. Moreover, we used the double ($n = 10$) of animals than those authors, which is an interesting condition to provide accuracy in our data.

Despite the clear effect of EGF on maintaining the morphological integrity of primordial equine follicles, it is interesting to consider the positive influence of EGF during the growth of the early stages of the preantral follicles. When evaluating the rate of follicular development, all treatments presented similar results. Importantly, all treatments were efficient in promoting the development of primordial follicles.

The findings of the present study did not demonstrate a dose-dependent growth of preantral follicles. Therefore, the findings are consistent with those obtained by Andrade et al. (2005), in which the concentration of 100 ng/mL epidermal growth factor promoted the in situ follicular activations of ovine primordial follicles, cultivated for 5 d. Differently, Silva et al. (2004) demonstrated that the concentration of 100 ng/mL of EGF did not provide the activation of the primordial follicles, but it demonstrated beneficial effects in primary goat follicles. However, a concentration of 10 ng/mL did not have significant beneficial effects, which is inconsistent with previous findings obtained for swine and ovine species. Considering our system involves the in situ culture (follicles included in the ovarian fragment), it is possible that there is some paracrine interaction between EGF and other ovarian components.

The growing period may also vary according to the species. Some studies have shown that cultivation can be carried out for a short period (approximately 7 d, Saraiva et al. 2010; Duarte et al. 2013; Pessoa et al. 2014; Serafim et al. 2015), or for longer periods pronged (28 d; Gutierrez et al. 2000). The length of the culture period of follicles may be considered according to experimental design for each hypothesis.

Another factor to be considered during the in vitro cultivation of ovarian follicles is the composition of the culture medium, such as the concentrations of minerals, vitamins, nucleosides, and amino acids. Due to some peculiarities of in vitro development and follicular activation, the culture medium is the first source of nutrients that come into contact with the follicles. Therefore, α-MEM should be considered the medium of choice for preantral follicle culture, due to its ability to maintain integrity and development (Araújo et al. 2014; Jimenez et al. 2016).

The technique of in vitro culture of preantral follicles in equines presents important impediments due to the restriction in obtaining ovaries from slaughterhouses, which makes it difficult to access this material for research. Thus, information regarding equine ovarian follicles is limited, a situation that is distinct from the species of production. Recent studies have used ovarian biopsy in vivo to obtain small ovarian fragments (Haag et al. 2013) as well as ovaries obtained from the slaughterhouse (Gomes et al. 2015; Max et al. 2017; Gonzalez et al. 2017). Such resources have proven to be efficient for the study of folliculogenesis in equines.

In this study, there was a quantitative variation of preantral follicles found in each treatment. This finding has also been reported by Driancourt et al. (1982) and recently by Gonzalez et al. (2017), in which they reported significant differences in the preantral follicular population in saddle ponies and mares, respectively.

In the study by Gomes et al. (2015), in which the culture of preantral follicles included fragments of equine ovary, there was no quantitative homogeneity of follicles in the evaluated fragments. The follicular population in equines (35,590: Driancourt et al. 1982) is significantly lower than that of bovines (130,000: Erickson 1966), making it difficult to obtain similar quantities between the two populations.

Conclusion

In vitro culture of preantral follicles in equines is progressing and requires further study on antioxidants, hormones, and growth factors added to the environment. Our results with EGF in vitro culture contribute to the identification of an

optimal medium for studies examining follicular development in these species. In conclusion, EGF is important for ovarian folliculogenesis because it is involved in follicular survival. In vitro culture of preantral equine ovarian follicles for 2 and 6 d in MEM supplemented with 100 ng/mL EGF maintained follicular integrity.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

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