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Growth and Differentiation Factor-9 Supplementation Affects Viability and Morphology of Preantral Follicles in Equine Ovarian Fragments During Short-term *in vitro* Culture

Polyana Carolina Marino[1]
https://orcid.org/0000-0002-0829-9225

Camila Bizarro-Silva[1*]
https://orcid.org/0000-0003-0230-924X

Isabela Búfalo[1]
https://orcid.org/0000-0003-1832-1534

Camila Oliveira Rosa[1]
https://orcid.org/0000-0002-2721-0652

Gustavo Romero Gonçalves[1]
https://orcid.org/0000-0003-1467-1406

Livia Aires Lisboa[1]
http://orcid.org/0000-0001-9161-2982

Marcelo Marcondes Seneda[1]
https://orcid.org/0000-0002-5097-5119

[1]University of Londrina, Laboratory of Animal Reproduction, Paraná, Brazil.

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*****Correspondence: camilabizarros@gmail.com; Tel.: +55-43-33715622 (C.B.S.); Laboratory Animal Reproduction, DCV, CCA, UEL, Londrina, PR 86051-990, Cx. Postal: 10.011, Brazil.

HIGHLIGHTS

- We evaluated GDF-9 on the *in vitro* culture of equine follicles.
- *Concentrations of 0, 50, 100 and 200 ng/mL the GDF-9 were tested for for 2 or 6 days.*
- *Based on histology the morphology and the integrity of the follicles were evaluated.*
- *GDF-9 presented a dynamic effect on the equine follicles cultured in vitro.*

Abstract: This study aimed to evaluate different concentrations of growth and differentiation factor-9 (GDF-9) on the development and maintenance of equine preantral follicle morphology during short-term *in vitro* culture. Ovaries (n=5) from five mares were collected from a local slaughterhouse and transported to the laboratory, where nine fragments (5x5x1mm) were procured from each ovary. One fragment from each was immediately fixed and submitted for histological analysis (control group; D0). The other eight fragments were cultured *in situ* for two (D2) or six (D6) days in MEM+ or MEM+ supplemented with GDF-9 at different concentrations (i.e., 50, 100 and 200 ng/mL the GDF-9). After culturing with different concentrations of GDF-9 for 2 or 6 days, the fragments were processed for histological analysis. After two days of cultivation, we observed an increase in the percentage of developing follicles for 0 (MEM+), 50, 100 and 200 ng/mL GDF-9 compared to control (D0; $P<0.05$). When we evaluated all treatments that preserved follicular integrity, the GDF-9 concentration of 100 ng/mL presented results superior to those of the other cultures ($P<0.05$). While, at six days of culture, the concentration of 200 ng/mL of GDF-9 appeared to be more efficient in providing development compared to MEM+ ($P<0.05$). The percentage of morphologically intact follicles in the 6 days culture samples treated with 50 ng/mL of GDF-9 indicated that this concentration was effective in maintaining the integrity of the follicle ($P<0.05$). We conclude, therefore, that graduated GDF-9 addition to the medium ensure follicular development and is sufficient maintain the architecture.

Keywords: Assisted Reproduction; GDF-9; Mare.

INTRODUCTION

The ability to grow immature oocytes *in vitro* and then recover preantral follicles dramatically increases the availability of fertilizable oocytes for assisted reproduction techniques. Regarding the limited efficacy of assisted reproductive technology in mares, follicular loss is a significant naturally occurring obstacle. Approximately 99% of follicles present in the preantral reserve pool are destined for follicular atresia, in which the majority of the follicular population does not complete the ovulation process [1,2].

In vitro culture is a biological technique that allows preantral follicular maturation *in vitro* by the use of an ideal culture medium composed of several substances that modulate follicular dynamics [3,4]. Some of these substances, such as antibiotics, buffers, nutritional substrates, protein sources, antioxidants, hormones and growth factors, are added to favor cell nutrition and maintain the integrity of the cells to avoid degenerative processes.

Growth factor supplementation of the medium may contribute to follicular growth and maturation. In equine species, few studies have focused on the *in vitro* culture of preantral follicles with the addition of growth factors, especially growth and differentiation factor-9 (GDF-9). This growth factor assists in the proliferation of granulosa and theca cells, contributes to the growth of primary follicles and promotes the maintenance of follicular viability [5,6]. Furthermore, GDF-9 promoted the survival and continuation of follicular development to the secondary stage in goats [7,8]. GDF-9 is a protein secreted by the oocyte and is considered a potent regulator of ovarian activity, stimulating the proliferation and maintenance of follicular cells. The knockout of GDF-9 can cause infertility in female transgenic and block the development of preantral follicles [9,10]. Its inclusion in culture media has proven to be effective in promoting the development of primordial follicles in goats, rodents, and humans [5,11-12]. In contrast, the information of this substance for the equine follicles is scarce.

The development of an ideal preantral follicle culture system can potentially provide large numbers of oocytes [2,13]. Thus, great interest has been shown in its application for animal breeding biotechnology for equine species, in addition to accelerating genetic gains, preantral follicle culture could lead to improved birth rates for animals with subfertility [14].

Despite the great interest in this technique, it is difficult to obtain enough material for the development of this research in horses. Thus, it is suggested the use of ovaries from mares

that are sacrificed or from imminent death and by surgical methods such as biopsies and ovariectomies [2,13]. Another alternative would be to obtain these ovaries in slaughterhouses, which are scarce all over the world [13]. Based on this information, the objective of the present study was to evaluate the effects of different GDF-9 concentrations on the *in vitro* culture of preantral follicles in the equine species.

MATERIAL AND METHODS

Collection and transport of ovaries

Ovaries were collected from undefined breed mares (n=5) in seasonal anestrus and without signs of cyclicity (i.e., absence of corpus luteum and follicles with diameters greater than 10 mm), of unknown breed, age, and body condition score, from the local slaughterhouse (latitude 23º33'03" S e longitude 51°27'39" W). Ovarian transport was performed according to Gomes et al. (13), briefly, in a thermal container containing modified PBS solution (Cultilab, Campinas, SP, Brazil) with penicillin (200 IU/mL) and streptomycin (200 mg/mL) at 4°C; the approximate time to reach the laboratory was 40 minutes.

Ovarian processing

In the laboratory, the ovaries were washed with 70% alcohol and modified PBS solution at 4°C. Using a scalpel and scissors, the ligaments and connective tissue were removed, and then a sagittal cut in the greater curvature of the ovary was performed to yield two hemi-ovaries. Ovarian fragments measuring 5x5x1 mm were obtained from 5 animals (n=9 fragments from each) through sections of the parenchyma in the region equidistant from the major and minor curvatures of the ovary. The fragments were then washed with PBS solution, and one of them was fixed in Bouin as the experimental control (D=0), while the other fragments were sent for *in vitro* culture of the preantral follicles.

In vitro culture preantral follicles

The ovarian fragments (n=8) were individually cultured in 24-well culture plates containing 1 mL of base medium (MEM+) or MEM+ supplemented with different concentrations of GDF-9 (50, 100 and 200 ng/mL - Figure 1). MEM+ was composed of MEM (Gibco BRL, Rockville, MD, USA – osmolarity 300 mOsm/L, pH 7,2, 47 mL), with added penicillin (200 IU/mL), streptomycin (200 mg/mL), bovine serum albumin (1.25 mg/mL), insulin (6.25 mg/mL), transferrin (6.25 mg/mL), selenium (6.25 mg/mL), 0.23 mM pyruvate, 2 mM glutamine, and 2 mM hypoxantine. The fragment-containing plates were routed to the oven at 39°C and exposed to ambient air with 5% CO2 and saturated humidity for 2 (D2) or 6 (D6) days. The culture medium was exchanged every 2 days. After completion of the culture, the fragments were fixed in Bouin and processed for classical histology to evaluate the growth and morphology of the preantral follicles.

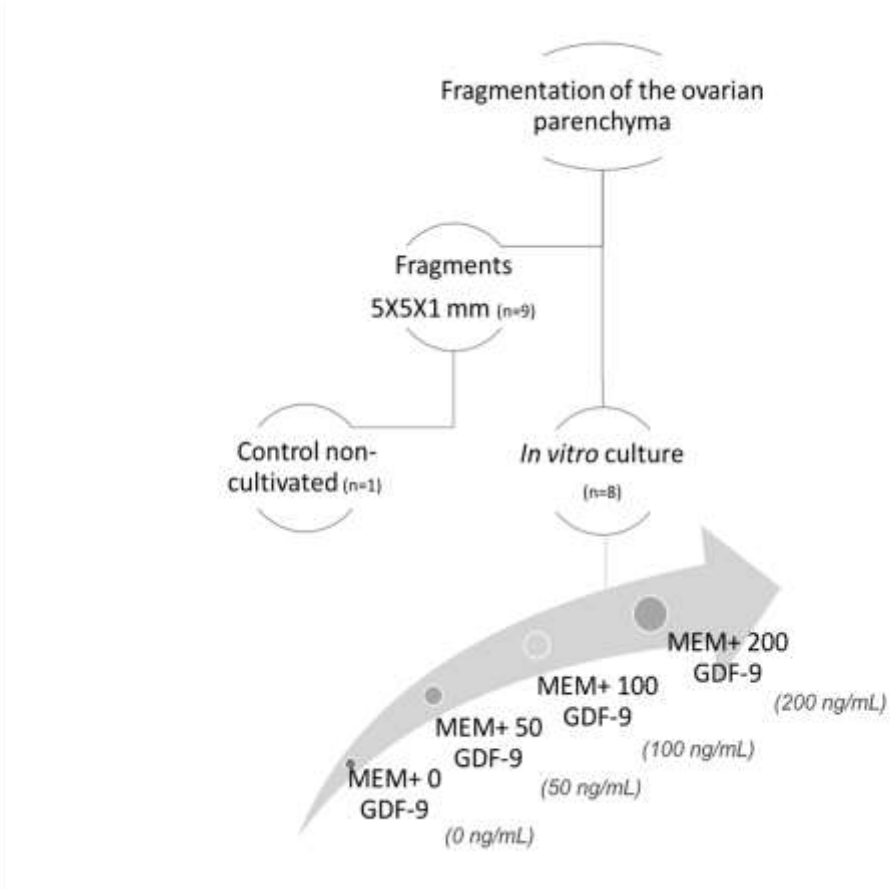

Figure 1. Experimental protocol for *in vitro* culture of equine preantral follicles at variable GDF-9 concentrations.

Histological processing

The ovarian fragments (control, D2 and D6) were fixed in Bouin for 24 hours and then maintained in 70% alcohol until the beginning of histological processing. When destined for histology, the fragments were dehydrated in ethanol, diaphanized in xylol, embedded in paraffin blocks, serially cut at 5 μm intervals using a rotary microtome (Leica®, Wetzlar, Germany), fixed to histological slides and stained using the Periodic acid- Schiff (PAS) and hematoxylin. To avoid counting the same follicle more than once, only the sections in which the oocyte nucleus was observed were considered.

Follicular Classification

Regarding follicular development, preantral follicles were classified as primordial and developing (primary and secondary). The primordial follicles were those that displayed a single layer of granulosa cells, with a pavement-like appearance, surrounding the oocyte. The developing follicles were considered primary follicles when they had a layer of cuboidal granulosa cells surrounding the oocyte and considered secondary follicles when they had two or more layers of (cuboid) granulosa cells [13,15].

As for follicular morphology, the follicles were classified as intact or degenerate. Briefly, follicles classified as degenerate had a pyknotic nucleus, granulosa cell disorganization, cytoplasmic retraction, chromatin condensation or total destruction of follicular components, whereas normal follicles exhibited structural integrity [16].

Statistical analysis

Statistical analysis was performed by the Action 3.1 version of R 3.0.2 software (Campinas, SP, Brazil). The data were initially submitted to tests for normality of residuals (Shapiro-Wilk) and homogeneity of variance (Bartlett). The mean number of morphologically intact preantral follicles as well as the primordial and developing follicles obtained in both the control samples and in samples with each concentration of GDF-9 supplementation, grown for 2 or 6 days, were submitted to ANOVA and Tukey's test. Findings were considered significant at P≤0.05.

RESULTS

In this experiment, 810 slides were evaluated, containing 2,430 histological sections of 45 ovarian fragments that were obtained from five mares. Of the 271 follicles evaluated, 51.7% were primordial follicles, 48.3% were developing, and 70.1% were intact. The control group had 69.2% primordial follicles and, of these, 82.2% were considered intact (Figure 2). The distribution of the primordial and developing follicles is shown in Table 1, whereas the morphologically normal follicles are represented in Figure 3.

Table 1. Percentage of primordial and developing follicles evaluated during a 2 or 6 days *in vitro* culture of equine ovarian fragments treated with minimal supplemented essential medium (MEM+) or MEM+ supplemented with variable GDF-9 concentrations (50, 100, 200 ng/mL).

Treatment/	**Primordial Follicles**	**Follicles in Development**	**Total Follicles**
Cultivation period	**% (N)**	**% (N)**	**N**
D0			
Control	69.2 (74)	30.8 (33) [c]	107[#]
D2			
MEM+	36.8 (7)	63.2 (12) [b]	19[ab]
GDF-9 50	43.9 (18)	56.1 (23) [b]	41[ab]
GDF-9 100	57.1 (4)	42.9 (3) [#]	7[b]
GDF-9 200	42.9 (24)	57.1 (32) [b]	56[a]
D6			
MEM+	52.4 (11)	47.6 (10) [bc]	21[ab]
GDF-9 50	33.3 (1)	66.7 (2) [#]	3[b]
GDF-9 100	0	100.0 (2) [#]	2 [b]
GDF-9 200	6.7 (1)	93.3 (14) [a]	15[ab]

a, b, c and within the same column differ statistically (p <0.05) between treatments (control, MEM+, GDF-9 50, GDF-9 100 and GDF-9 200).
Values not compared by statistical tests.

For two-day cultivation, specimens treated with different concentrations of GDF-9 and MEM+ maintained higher percentages of developing follicles relative to uncultivated control specimens (P>0.05; Table 1). When evaluating the percentage of developing follicles, there was no difference between the GDF-9 concentration treatments; however, the treated cultures showed that they supported the additional development of the cultivated follicles compared to the non-cultivated controls (D0). When we evaluated follicular integrity, the GDF-9 concentration of 100 ng/mL gave superior results as compared to the other two-day cultures (Figure 3).

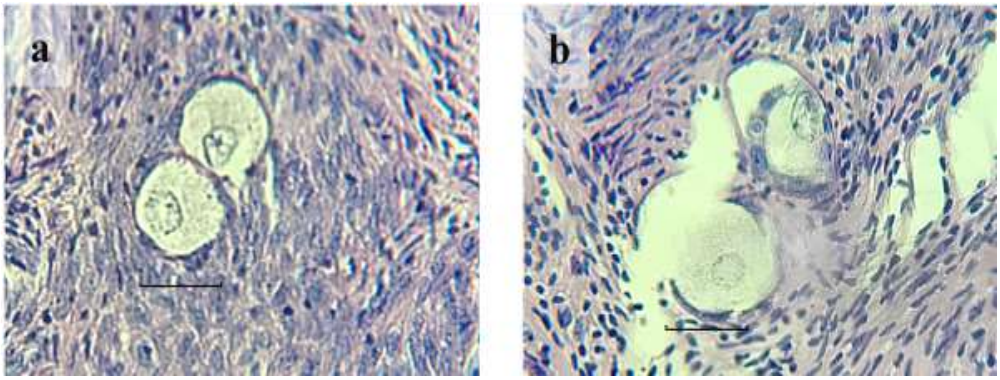

Figure 2. Morphological aspects of preantral follicles grown *in vitro*. (a) Normal primordial follicles and (b) Abnormal primordial follicles. The sections were stained with periodic acid-Schiff (PAS) and hematoxylin.

When we evaluated the results at 6 days of culture, we observed a representative increase in the percentage of developing follicles present only at the GDF-9 concentration of 200 ng/mL, relative to the MEM+ control (P<0.05; Table 1). The percentage of morphologically intact follicles obtained in the 6-day culture indicated that treatment with 50 ng/mL GDF-9 was efficacious in maintaining follicular integrity (P<0.05; Figure 3).

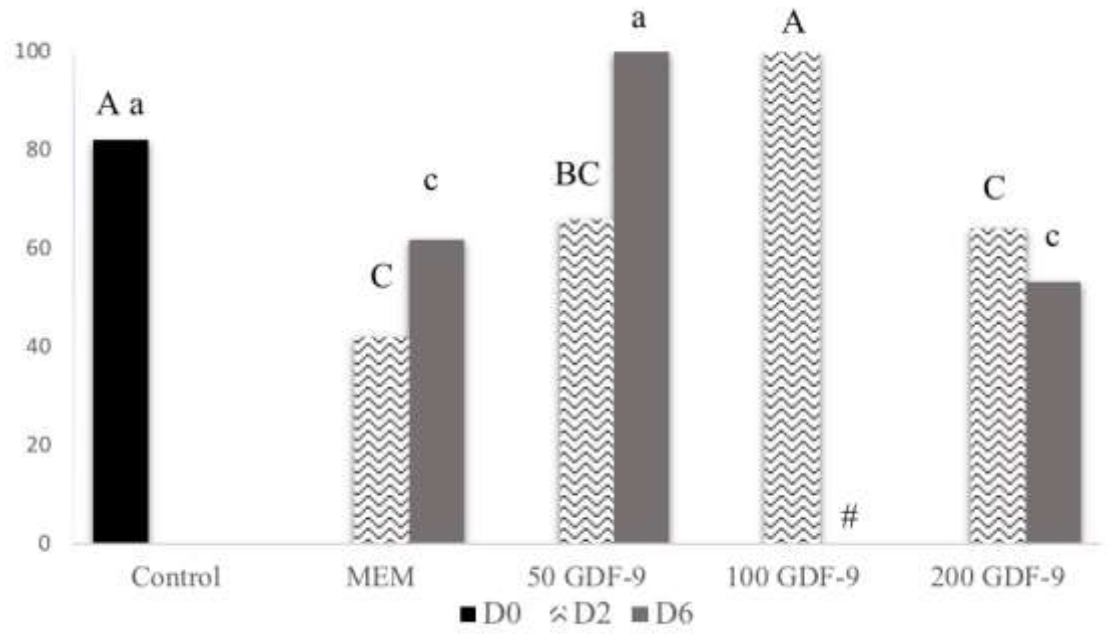

Figure 3. Percentage of total intact follicles in 2 or 6 days (D2 or D6) *in vitro* culture of equine ovarian fragments in supplemented minimal essential medium (MEM+) or MEM+ supplemented with variable GDF-9 concentrations (50, 100 and 200 ng/mL). Values followed by uppercase letters (A, B) differ statistically (p <0.05) between treatments (control, MEM+, GDF-9 50, GDF-9 100 and GDF-9 200) at 2 days of culture. Values followed by lowercase letters (a, b) differed statistically (p <0.05) between treatments (control, MEM+, GDF-9 50, GDF-9 100 and GDF-9 200) at 6 days of culture. Values followed by a # were not compared by statistical test.

DISCUSSION

The present study demonstrated for the first time that the addition of GDF-9 to an *in vitro* culture of equine preantral follicles has the capacity to promote follicular survival as well as its activation and development.

When evaluating supplementation with different concentrations of GDF-9 for *in vitro* cultures of ovarian fragments (*in situ*), we observed (i.e., detected histologically) that on the second day, all culture concentrations of GDF-9 (50, 100 and 200 ng/mL) supported the development of preantral follicles. On the other hand, the six-day cultures revealed that the highest number of developing follicles occurred when cultivated at a GDF-9 concentration of 200 ng/mL. Similarly, *in situ* (7 days) goat follicle culture studies identified that a GDF-9 concentration of 200 ng/mL was essential for primordial follicle activation and progression to more advanced stages of development [7].

GDF-9 plays an important role in growth and follicular development. This growth hormone is part of the superfamily of "growth factors β" (TGFβ; 17), which are necessary and vital for follicular activation and growth. Although the factors and mechanisms that involve folliculogenesis are not well elucidated, it is possible to affirm that GDF-9 plays an important role in early folliculogenesis, follicular differentiation and steroid hormone synthesis [18,19]. GDF-9 is so essential that in an experiment with GDF-9-depleted rats and sheep, primordial follicles remained at this stage of development, and these animals became sterile [8,20]. Based on these findings and our results, the effect of GDF-9 on *in vitro* follicular development is confirmed.

It was not possible to observe significant differences between treatments at the second day of cultivation; however, all treatments were developmentally responsive as compared to the uncultivated control (D0). This is because GDF-9 promotes granulosa cell proliferation, cumulus expansion, production of steroid hormones, activation and follicular growth [21, 22].

In this study, it is also presumed that the concentration of 100 ng/mL of GDF-9 at 2 days of culture achieved a satisfactory result in terms of the maintenance of follicular integrity when compared to the other treatments. In contrast, at 6 days of culture, the adequate concentration to maintain follicular integrity was 50 ng/mL. Similarly, it was found that the concentration of 100 ng/mL of GDF-9 was required for follicular survival in a culture of follicles isolated from goat ovaries [5]. Furthermore, *in vitro in situ* cultures of human preantral follicles demonstrated that GDF-9 promoted follicular viability for 14 days as well as the progression of primordial and primary follicles to the secondary stage [11].

As in other studies with preantral follicle cultures, it was difficult to find a high density of preantral follicles compared to other species [bovine: 4, ovine: 23, caprine: 24], even after the removal of internal fragments of the equine ovary. This finding was also reported by Driancourt et al. [25] and in recent investigations by Gonzalez et al. [26], Gomes et al. [27] and Alves et al. [28], who found little quantitative homogeneity of follicles in their evaluated fragments.

CONCLUSION

Thus, we can conclude that the addition of GDF-9 to the medium, in a graduated way, guarantees improved follicular development as well as the satisfactory maintenance of follicular architecture. Thus, this study corroborates a wider and innovative use of GDF-9 in culture media and opens new horizons for the investigation of other substances and factors that may benefit follicular development. In turn, equine follicular development can be further optimized.

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Conflicts of Interest: The authors declare no conflict of interest.

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