http://dx.doi.org/10.1590/1678-4162-10602

Arq. Bras. Med. Vet. Zootec., v.71, n.5, p.1445-1452, 2019

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Effect of somatotropin on survival and diameter of bovine preantral follicles

[*Efeito da somatotropina na sobrevivência e no diâmetro de folículos pré-antrais de bovinos*]

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ABSTRACT

The aim of this study was to evaluate the effect of Recombinant bovine somatotropin (rbST) on survival and diameter of bovine preantral ovarian follicles (PAOF) cultured *in vitro*. Ovaries were collected from adult cows and fragments of ovarian cortex were immediately fixed (non-cultured control) or cultured *in vitro* in α-MEM$^+$ alone or containing 10, 50, 100 or 1,000ng/mL rbST. The fragments were processed for Classical Histology and Transmission Electron Microscopy. After one and seven days of culture, the percentage of normal follicles in the non-cultured control was superior ($P< 0.05$) to the follicles cultured in α-MEM$^+$ alone or with different rbST concentrations. The oocyte and follicular mean diameter did not increase during the culture for one and seven days, both in media containing rbST and in the medium without this hormone. The only medium in which there was no reduction in follicular diameter with the time of culture was the medium without rbST. Ultrastructural damage in PAOF cultured *in vitro* was found. It is concluded that the use of rbST at different concentrations in *in situ* culture of bovine preantral follicles has no beneficial effects on survival and growth of bovine PAOF.

Keywords: bovine, *in vitro* culture, somatotropin, preantral follicles

RESUMO

O objetivo deste trabalho foi avaliar o efeito da somatotropina recombinante bovina (rbST) sobre a sobrevivência e o diâmetro de folículos ovarianos pré-antrais (FOPA) bovinos cultivados in vitro*. Ovários foram coletados de vacas adultas e fragmentos do córtex ovariano foram imediatamente fixados (controle não cultivado) ou cultivados* in vitro *em α-MEM$^+$ sozinho ou contendo 10, 50, 100 ou 1.000ng/mL de rbST. Os fragmentos foram processados para histologia clássica e microscopia eletrônica de transmissão. Após um e sete dias de cultivo, o percentual de folículos normais no controle não cultivado foi superior ($P<0,05$) aos cultivados em α-MEM$^+$ sozinho ou acrescido de diferentes concentrações de rbST. Os diâmetros médios oocitário e folicular não aumentaram durante o cultivo por um e sete dias, tanto nos meios contendo rbST, como no meio sem esse hormônio (α-MEM$^+$). O único meio em que não houve redução no diâmetro folicular com o tempo de cultivo foi o sem rbST. Verificaram-se ainda danos ultraestruturais em FOPA cultivados* in vitro*. Conclui-se que o uso de rbST em diferentes concentrações no cultivo* in situ *de folículos pré-antrais bovinos não tem efeitos benéficos na sobrevivência e no crescimento de FOPA bovinos.*

Palavras-chave: bovinos, cultivo in vitro*, somatotropina, folículos pré-antrais*

INTRODUCTION

The growing development of Brazilian livestock has led to an incessant search for the production and reproduction of species with economic interest. For this purpose, the development and application of reproduction biotechniques, such as the Manipulation of Ovocytes Enclosed in Preantral Ovarian Follicles (MOEPF), is fundamental (Araújo *et al.*, 2014).

Recebido em 15 de março de 2018
Aceito em 25 de janeiro de 2019
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Of all the follicular population present in the mammalian ovary, 90% correspond to preantral follicles (PAOF) (Liu *et al.*, 2001). Considering that more than 99% of these suffer a natural physiological process of follicular atresia (Matsuda *et al*., 2012), the importance of MOEPF is emphasized, which main objective is to recover them, so that they are cultured *in vitro* until they reach maturation. Currently, several authors have used different *in situ* culture systems to study the development of preantral follicles in rodents and ruminants (Bruno *et al.*, 2008; Costa *et al.*, 2014).

Despite the excellent results related to the production of mature oocyte from PAOFs cultured *in vitro* achieved so far in mice, the repeatability of these results in other species is low, including production animals (Magalhães *et al.*, 2011). In cattle, the best result reported for this species so far was the formation of the antral cavity after in vitro culture of advanced secondary follicles (Gutierrez *et al.*, 2000). In the attempt to develop an efficient culture system, different substances (hormones and growth factors) have been tested in the *in vitro* culture of PAOF (Silva *et al.*, 2018). Among the hormones that have been the target of research in the area of animal reproduction is Somatotropin (ST), also known as Growth Hormone (GH). It is a peptide composed of 192 amino acids, synthesized by cells located in the adenohypophysis, which participates in several physiological and metabolic processes of cattle. This peptide acts through the synthesis of Insulin-like Growth Factor 1 (IGF-1) and IGF Binding Proteins (IGFBP) (Gong *et al.*, 1997).

Receptors for rbST and IGF-1 have been found in immature oocytes, as well as in bovine granulosa and *cumulus* cells (Lonergan *et al.*, 2000). Also, Eckery *et al.* (1997) demonstrated that mRNA for the GH receptor is abundant in the oocyte and in PAOF granulosa cells of sheep. This hormone has influence on the mechanisms related to follicular growth and maturation (Iga *et al.*, 1998), as well as on the regulation of the physiological processes of the animals. *In vivo*, the administration at different rbST concentrations (250mg and 500mg) in Holstein cows increased the percentage of viable embryos (Neves *et al.*, 2005).

Despite such studies, the effect of ST on survival and growth of bovine PAOF is not yet known. Thus, the aim of this work was to evaluate the effect of ST on the survival and diameter of bovine preantral follicles cultured *in vitro*.

MATERIAL AND METHODS

The culture medium and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, MO), unless otherwise indicated. This study was approved by the Ethics Committee on the Use of Animals of the Federal University of Viçosa (CEUA/UFV, process number 08/2013). Pairs of ovaries (n= 5) from bovine females were collected aseptically at local abattoirs. Immediately after collection, they were washed in 70% alcohol and then in Minimum Essential Medium (MEM), supplemented with 100μg/mL Penicillin and 100μg/mL Streptomycin. Subsequently, they were transported to the Animal Reproduction Laboratory (DVT/UFV) in MEM at 4ºC for approximately one hour (Chaves *et al.*, 2008).

In the laboratory, each ovarian pair was placed in a Petri dish containing α-MEM$^+$, on which fragments of approximately 3x3mm (1mm thick) were obtained using tweezers and scalpel blades under sterile conditions. A fragment from each ovary pair was randomly selected and immediately fixed for analysis by Classical Histology (CH) and Transmission Electron Microscopy (TEM), constituting the non-cultured control. The other fragments were individually cultured in 1ml medium for one or seven days, in a culture dish at 38°C, in an atmosphere of 5% CO_2. It is worth mentioning that each ovarian pair corresponded to a repetition, and therefore five repetitions were performed.

The base medium, called α-MEM$^+$ (pH 7.2 – 7.4), was supplemented with ITS (10μg/mL Insulin, 5.5μg/mL Transferrin and 5ng/mL Selenium), 2mM Glutamine, 2mM Hypoxanthine and 1.25mg/mL Bovine Serum Albumin (BSA). The culture only with the base medium was called cultured control, and the other treatments consisted of the culture with α-MEM$^+$, added of different Recombinant bovine somatotropin (rbST) concentrations (10, 50, 100 and 1000ng/mL).

The culture media were stabilized for one hour before use, being completely renewed every two days. After the period of one and seven days of culture, the fragments were destined for CH and TEM.

For histological analysis, the follicles were fixed in Carnoy for four hours. Once fixed, they were dehydrated at increasing concentrations of ethanol and then diaphonized in xylol. The fragments were then embedded in paraffin blocks and serially sectioned to the thickness of 7μm. Each section was placed on a slide and stained by the Periodic Acid Schiff technique and Hematoxylin. All sections were examined under optical microscope (Olympus), at 400x magnification.

In relation to follicular survival, the follicles were classified as normal or degenerated. PAOFs were considered morphologically normal when they presented granulosa cells organized around the oocyte, whereas it was spherical or slightly elongated, with nucleus without evidence of pyknosis (Bruno *et al.,* 2008). Degenerated follicles were characterized according to granulosa cell disorganization and to the presence of cytoplasmic retraction areas and nuclear pyknosis. The mean oocyte and follicular diameters were measured with the aid of an ocular micrometer, and the measurement was performed on follicles classified as normal in the histological analysis.

Ultrastructural analysis on PAOF from the group that presented the best results regarding oocyte and follicular survival and growth after *in vitro* culture was also performed. For TEM procedures, small fragments ($1mm^3$) of ovarian tissues were fixed for four hours at room temperature in Karnovsky (2.5% glutaraldehyde and 4% formaldehyde in 0.1M Cacodylate buffer, pH 7.2). Afterwards, the samples were washed twice in 0.1M Cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated in alcohol and embedded in Epon 812 resin (Basso & Esper, 2002).

Initially, semithin sections (0.5μm) were performed with glass knives in ultramicrotome. These were stained with toluidine blue, placed on a slide/coverslip and examined under light microscopy, selecting the best semithin sections for the ultrathin sections (60-70nm). The ultrathin sections were then obtained with diamond knife and collected on (200 mesh) copper grids, stained with uranyl acetate and lead citrate, according to the protocols of Watson (1958) and Reynolds (1963). Subsequently, the ultrathin sections were examined in transmission electron microscope as the integrity and density of the cytoplasmic organelles of the oocyte and granulosa cells, the degree of cytoplasmic vacuolization and the integrity of cell membranes.

The variables were submitted to Normality (Lilliefors) and Homoscedasticity (Cochran) tests, followed by analysis of variance at a probability of 5%. In case of significance, the most appropriate test of comparison between means was performed, avoiding type I and II statistical errors. When they did not satisfy the requirements of normality and homoscedasticity, even after the appropriate changes, data were submitted to Wilcoxon or Kruskal-Wallis non-parametric test, depending on the degree of freedom for treatments (Sistema…, 1999).

RESULTS AND DISCUSSION

The number of normal follicles observed in the present study in the non-cultured control was similar to the number described by Luna *et al*. (2011) for the bovine species (93.6%). However, the percentage of normal follicles in the treatments cultured in the different media was inferior ($P< 0.05$) compared to the non-cultured control (Table 1).

There was no difference ($P< 0.05$) in the percentage of normal follicles among cultured treatments and when compared to the different culture times. These results demonstrate that rbST, at the concentrations used, was not able to maintain follicular survival. Bruno *et al*. (2008) observed this same condition, when culturing PAOF of goats in different media.

Table 1. Mean percentage of morphologically normal preantral follicles in non-cultured and in vitro cultured bovine ovarian tissue for one and seven days utilizing different rbST concentration medium

Treatment	Day 1 (%)	Day 7 (%)
Control	92.2±19.3[a]	
α-MEM$^+$	71.7±11.4[b]	72.8±18.7[b]
α-MEM$^+$ + 10ng/mL	81.7±12.7[b]	78.3±20.7[b]
α-MEM$^+$ + 50ng/mL	65.0±13.0[b]	66.1±17.0[b]
α-MEM$^+$ + 100ng/mL	74.4±14.0[b]	83.9±20.6[b]
α-MEM$^+$ + 1,000ng/mL	79.4±12.5[b]	76.7±20.2[b]

[a,b] Values with different superscripts indicate difference (P< 0.05) by Duncan test. α-MEM$^+$: Minimum Essential Medium.

The mechanisms by which rbST hormone promotes beneficial effects on follicular survival have not yet been fully elucidated. Some authors suggest a direct effect on the oocyte and granulosa cells (Lucy, 2000), while others report that it induces an increase in plasma and ovarian concentrations of IGF-1 (Buratini *et al.*, 2000). In this context, studies indicate that IGF-1 is capable of suppressing DNA fragmentation by apoptosis in oocytes of rats cultured *in vitro*, acting as an anti-apoptotic factor. However, the present study corroborates with Costa *et al.* (2014), who reported that IGF-1 was not able to maintain the viability of PAOF of goats cultured *in vitro* for seven days.

According to Gutierrez *et al.* (1997), this growth factor is also responsible for accentuating the effects of FSH by increasing its receptors on the granulosa cells. This action mechanism may justify why rbST did not improve the rate of viable follicles in the present study, when compared to the culture in medium without this hormone, with a higher rate of atretic PAOF during the culture.

In the present study, the main alteration observed in follicles classified as degenerated was the presence of pyknotic nuclei (Figure 1). Nevertheless, the normal follicle rates observed are considered high and corroborate with the results of other authors, who reported a total survival of 83.5% of bovine PAOF after culture isolated for four days, using only a similar base culture medium, which was also supplemented with insulin, transferrin, selenium (ITS), glutamine and hypoxanthine (Machado *et al.*, 2006).

The follicular survival rate was maintained (P> 0.05) after the culture progression from the first to the seventh day (Table 1). This demonstrates that culture time did not interfere with the survival of these follicles. This result is satisfactory, since other authors have observed a reduction in the number of surviving follicles over the time of *in vitro* culture, using 50 and 100ng/mL IGF-1 (Costa *et al.*, 2014). However, the stability of follicular survival cannot be attributed due to the presence of rbST, since the number of normal follicles when cultured with different concentrations of the hormone was similar (P> 0.05) to that found in the follicles cultured only with the base medium (α-MEM$^+$).

The composition of the base medium is an important aspect to be considered for *in vitro* culture of PAOF. The culture medium used in the present study is one of the richest α-MEM$^+$ formulas, consisting of 21 essential amino acids, B complex vitamins, vitamins C and D and inorganic salts. Insulin, transferrin and selenium were also added to this medium. According to Liu *et al.* (2001), insulin allows a greater use of the medium energy sources. Selenium has the function of activating enzymes that participate in the process of elimination of free radicals produced by cells (Demeestere *et al.*, 2004). According to Silva *et al.* (2009), the addition of these substances to α-MEM$^+$ contributes to the maintenance of follicular survival in the culture. This was observed in the present experiment, in view of the survival rates obtained. Thus, in the present study, the α-MEM$^+$ culture medium (even without the addition of rbST) was able to sustain follicular survival, as well as to maintain the number of viable follicles after seven days of culture.

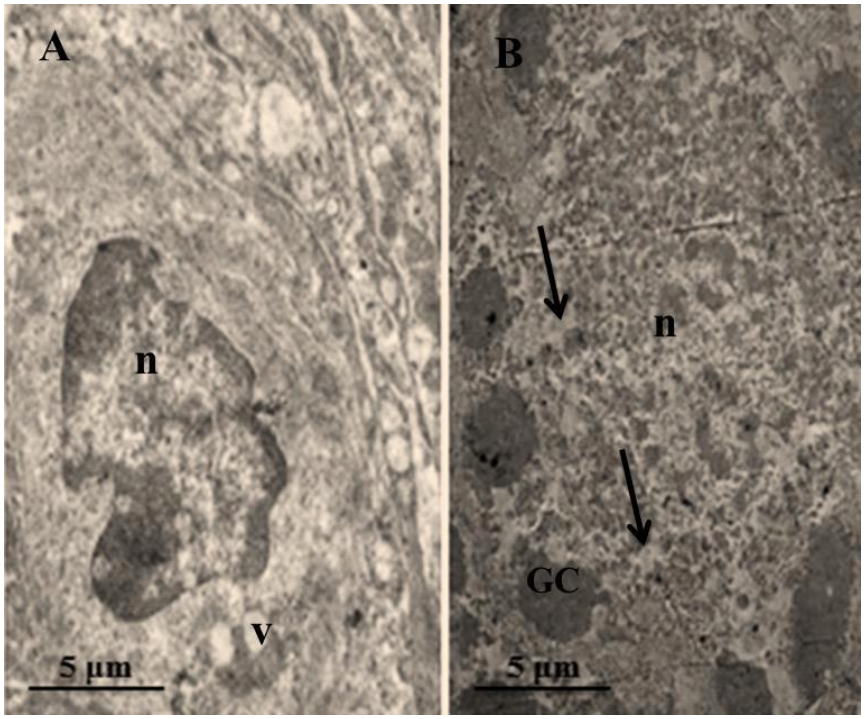

Figure 1. Ultrastructure of bovine preantral follicles non-cultured (A) and cultured for seven days (B) in medium containing 1,000ng/mL rbST. The intact oocyte nucleus is observed presenting several areas of chromatin condensation (A), characteristic of the non-cultured control. In figure B intense vacuolization in the cytoplasm is observed, indicative of degeneration. GC: granulosa cells; v: vesicles; n: oocyte nucleus; arrows: vacuoles. 3,000x magnification.

It was also found that the mean diameter of the oocytes reduced (P< 0.05) after the culture for one day in the medium without rbST, as well as in those containing 10, 50 or 100ng/mL of the hormone when compared to the non-cultured control. However, this mean diameter remained unchanged when tissues were cultured in medium containing 1,000ng/mL rbST. After seven days of culture, there was a reduction in this diameter in oocytes of all treatments (Table 2). Comparing the oocyte diameter in function of the culture time, a reduction of this diameter (P< 0.05) was observed in all cultures carried out in the media containing the hormone, except when the culture was performed in the media without its (α-MEM$^+$) presence.

Table 2. Mean diameter (micrometers) of bovine oocytes and ovarian follicles non-cultured and in vitro cultured for one and seven days, in medium without the hormone (α-MEM$^+$) and containing different rbST concentrations

Treatment	Oocyte diameter		Follicular diameter	
	Day 1	Day 7	Day 1	Day 7
Control	25.5±3.2^{a}		35.6±5.2^{a}	
α-MEM$^+$	19.8±4.6$^{b, A}$	21.3±15.8$^{b, A}$	32.6±8.7$^{a, A}$	28.7± 6.4$^{b, A}$
α-MEM$^+$ + 10ng/mL	21.2±4.1$^{b, A}$	17.6±4.8$^{b, B}$	34.8±7.1$^{a, A}$	27.2±5.6$^{b, B}$
α-MEM$^+$ + 50ng/mL	21.5±5.0$^{b, A}$	19.0±5.6$^{b, B}$	36.7±7.7$^{a, A}$	33.1±13.1$^{a, B}$
α-MEM$^+$ + 100ng/mL	21.5±4.3$^{b, A}$	19.7±3.9$^{b, B}$	39.8±15.8$^{a, A}$	30.4±5.9$^{a, B}$
α-MEM$^+$ + 1,000ng/mL	24.6±4.0$^{a, A}$	20.3±5.8$^{b, B}$	40.6±12.7$^{a, A}$	35.2±10.8$^{a, B}$

^{A,B} Values with different uppercase superscripts indicate difference in the same line (P< 0.05) by Wilcoxon non-parametric test. Oocyte and follicular diameter: ^{a,b} Values with different lowercase superscripts indicate difference in the same column (P< 0.05) by Duncan and Kruskal-Wallis tests, respectively. α-MEM$^+$: Minimum Essential Medium.

In cattle, it was shown that growth hormone acts through IGF-1 (Iga *et al.*, 1998). According to Silva *et al.* (2009), growth hormone increases the development of small antral follicles at the gonadotropin dependent stages and stimulates oocyte maturation, while IGF increases proliferation of granulosa cells, steroidogenesis and oocyte growth in the majority of mammal species. However, in the present experiment, no positive effect of rbST in oocyte growth was found. After one day of culture, the mean diameter of follicles cultured in α-MEM$^+$ and in all concentrations of rbST were similar (P> 0.05) to the verified in the follicles of the non-cultured control (Table 2).

However, after seven days of *in vitro* culture, there was a reduction in follicular diameter in the α-MEM$^+$ culture alone and in the medium with 10ng/mL rbST when compared to the non-cultured control and the other treatments. These results corroborate with those found by Kikuchi *et al.* (2001), who observed an increase in follicular diameter after four days of PAOF culture at the highest concentrations of GH (1mIU/mL). Also, Magalhães *et al.* (2011) verified an increase in follicular diameter induced by GH (10 and 50ng/mL), culturing PAOF of goats for 18 days. The same effect was observed in PAOF of rats *in vitro* (Kikuchi *et al.*, 2001), as well as in PAOF of mice, on which this hormone has been observed to stimulate the production of estradiol, the secretion of inhibin and the proliferation of granulosa and theca cells (Kobayashi *et al.*, 2000). In cattle, this hormone associated with insulin increases progesterone synthesis and proliferation of granulosa cells, after culture for four days (Langhout *et al.*, 1991).

For a more accurate analysis of the follicular survival, besides the histological evaluation, an ultrastructural analysis was performed. This analysis is considered relevant because it reveals crucial information about the quality of follicles and oocytes and can detect damage occurred in cell membranes and organelles after *in vitro* culture (Jimenez *et al.*, 2016). The TEM of the present study was performed on PAOF, after *in vitro* culture for one and seven days. The findings revealed damage in the karyotheca and cytoplasmic membrane, increase of cytoplasmic vacuolization and low density of organelles distributed in the cytoplasm (Figure 1).

Similar results were described by Bruno *et al.* (2008), after 7 days of cultivation of FOPA in medium supplemented with estrus goat serum. The authors reported increased cytoplasmic vacuolization and detachment of granulosa cells. According to Silva *et al.* (2000), these vacuoles are possibly composed of altered organelles that were phagocytosed but remained in the cells, characterizing one of the first signs of follicular degeneration. Similar findings were also described by Jimenez *et al.* (2016), where follicles cultured in TCM-199þ were characterized by a high number of vacuoles scattered throughout the cytoplasm, showing a very low density of organelles, as well as irregular or fragmented nuclear and cytoplasmic membranes.

These results found in the ultrastructural analyzes demonstrate that bovine PAOF cultured under the conditions of the present experiment did not prevent ultrastructural degenerative lesions.

CONCLUSIONS

The use of rbST at different concentrations in the *in-situ* culture of bovine preantral follicles has no beneficial effects on the survival and growth of bovine PAOF. However, other studies should be performed using rbST at different concentrations to find out the relationship of this hormone with PAOF and how it acts in this cell.

ACKNWLEDMENTS

To FAPEMIG for the financial support. To CAPES for providing the scholarship to the master's student. To the Nucleus of Microscopy and Microanalysis of the Federal University of Viçosa (UFV) for the support in the processing and ultrastructural analysis.

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