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Effect of indole acetic acid on the *in vitro* activation and survival of bovine (*Bos indicus*) preantral follicles

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Abstract

The objective of this study was to evaluate the development of preantral follicles from the Nelore breed of *Bos indicus* after *in vitro* culture of ovarian cortices in different concentrations of indole acetic acid (IAA). In addition, this study investigated the possible association of mammalian genes with IAA activity in bovine preantral follicles using an *in silico* approach. Ovaries (n = 8) from Nelore heifers were collected, and the ovarian cortex was divided into 14 fragments. From these samples, two fragments were immediately fixed in Carnoy solution (control), and the other fragments were cultured individually for two or six days in Minimum Essential Medium (MEM) with 0, 10, 40, 100, 500 or 1000 ng/ml of IAA. Follicles were classified as primordial or as developing (primary and secondary) follicles. The *in silico* approach to search for auxin-responsive candidate genes was performed with bioinformatics tools, such as GenBank and SWISS-PROT database, Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Clustal W program. Compared to the control culture, the percentage of primordial follicles was reduced ($P < 0.05$) and the percentage of developing follicles was increased ($P < 0.05$) after 2 or 6 days of culture in all media tested. Furthermore, culture of the ovarian cortex for 6 days reduced the percentage of healthy, morphologically normal follicles when compared with the control ($P < 0.05$). In contrast, cultures supplemented with 10 ng/ml of IAA were the only samples that had similar ($P > 0.05$) percentages relative to the control group. Finally, we found a mammalian gene that was homologous to the plant gene, ROOTY, which may be involved in the oocyte response to IAA. We conclude that the 10 ng/ml concentration of IAA promoted follicular survival and activation of Nelore bovine preantral follicles after 6 days of culture *in vitro*.

Keywords: culture, follicles, in silico, indole acetic acid, preantral.

Introduction

Most techniques used to produce embryos *in vitro* depend on oocytes aspirated from antral follicles, which are present in low numbers in the mammalian ovary. However, thousands of oocytes are enclosed in ovarian preantral follicles and could potentially be matured *in vitro* for embryo production, thereby optimizing the oocyte potential of valuable animals (Chaves *et al.*, 2008). During a female's reproductive life a low percentage of follicles (approximately 0.1%) reach the preovulatory stage (Clark *et al.*, 2004), and the remaining follicles are lost through atresia (McGee and Hsueh, 2000).

Several studies have been conducted to evaluate the initiation of follicle growth by culturing the ovarian cortex *in vitro* in media supplemented with different hormones and growth factors (see the following examples for bovine: Shimizu *et al.*, 2008; caprine: Rosseto *et al.*, 2009; rat: Lu *et al.*, 2009; and human: Abir *et al.*, 2009). Furthermore, much attention has been paid to the presence of growth factors, their receptors and hormone receptors within the various preantral follicle compartments. These studies have contributed to elucidating the mechanisms underlying the activation of primordial follicles as well as additional follicle growth and differentiation (van den Hurk and Zhao, 2005). Overall, there are many oocyte- and granulosa-derived factors (and theca-derived factors in later follicle stages), as well as other factors and hormones that may be involved in the regulation of follicle development.

Indole-3-acetic acid (IAA) is an important plant hormone that controls processes such as growth, movement and tropism, which lead to cellular expansion and division (Becker and Hedrich, 2002). In animal cells, IAA has been used to preserve goat preantral follicles for up to 24 h at 4ºC (Ferreira *et al.*, 2001). In *in vitro* cultures of sheep ovarian preantral follicles a concentration of 40 ng/ml of IAA has been shown to induce follicular activation (Andrade *et al.*, 2005), while 10 ng/ml increases follicular survival *in vitro* (Costa *et al.*, 2010). In addition, IAA acts as an antioxidant by inhibiting peroxidation (Cano *et al.,* 2003), which may be important for follicular survival *in vitro*. However, the effects of IAA on *in vitro* cultures of bovine ovarian preantral follicles remain unknown.

One of the hot interests of current quantitative genetics is systematically exploring an exact genetic architecture of the number, distribution and interaction

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of loci affecting the variations of biomedically, economically, and evolutionarily important complex and quantitative traits (Zhu and Zhao, 2007). There are two approaches for genetic dissections of complex and quantitative traits, i.e., genome-wide scanning and candidate gene approach. Candidate genes are generally the genes with known biological function directly or indirectly regulating the developmental processes of the investigated traits, which could be confirmed by evaluating the effects of the causative gene variants in an association analysis. Candidate gene approach has been ubiquitously applied for gene-disease research, genetic association studies, biomarker and drug target selection in many organisms from animals to humans (Tabor *et al*., 2002). Comparative genomics strategy makes the utility of cross-species approach to identify and characterize the effect of putative candidates (Young, 2001; Ewart-Toland and Balmain, 2004).

The aims of the present study were to investigate the effects of different concentrations of IAA on the survival, activation and growth of bovine primordial follicles and to use an *in silico* approach (Katoh, 2002) to find candidate mammalian genes that may be associated with the effects of IAA on the *in vitro* activation and development of preantral follicles.

Materials and Methods

Source of ovaries

Ovaries (n = 8) from 4 adult, non-pregnant, healthy Nelore heifers were obtained from a local abattoir. All abattoir-derived ovaries were washed once in 70% alcohol and twice in 0.9% saline solution. The pairs of ovaries were transported to the laboratory in saline solution at 33°C, within 1 hour of resection. The pH of saline solution was adjusted to 7.2 to 7.4.

Experimental procedure

At the laboratory, both ovaries from each animal were stripped of the surrounding fat tissue and ligaments and cut in half. Subsequently, the medulla, large antral follicles and corpora lutea were removed. Following this, the ovarian cortex was divided into 14 fragments of approximately 3 x 3 mm (1-mm thick). Two fragments were immediately fixed for classic histological studies (non-cultured controls – Day 0). The other fragments (n = 12) from the ovarian cortex were individually cultured *in vitro* in 1 ml of culture medium for 2 or 6 days at 39°C with 5% CO2 using a 24-well culture dish. The control medium was MEM (Sigma, St Louis, MO, USA) supplemented with penicillin (200 UI/ml; Sigma, St Louis, MO, USA), streptomycin (200 mg/ml; Vetec, Rio de Janeiro, RJ, Brazil), BSA (1.25 mg/ml; Sigma, St Louis, MO, USA), ITS (insulin, 6.25 μg/ml; transferrin, 6.25 μg/ml and selenium, 6.25 ng/ml; Invitrogen, Carlsbad, CA, USA), pyruvate (0.23 mM; Invitrogen, Carlsbad, CA, USA), glutamine (2mM; Invitrogen, Carlsbad, CA, USA) and hypoxanthine (2 mM; Sigma, St Louis, MO, USA). As indicated in Fig. 1, this control medium (MEM+) was supplemented with different concentrations of IAA (10, 40, 100, 500 or 1000 ng/ml). Every 2 days, the culture medium was replaced by fresh medium. The media were compared using ovarian fragments from the same animal, and each treatment was repeated four times. The duration of culture was based on the time-course of primordial follicle activation in other species (Braw-Tal and Yossefi, 1997; Fortune *et al*., 1998). The concentration of IAA was based on preliminary studies culturing ovine preantral follicles (Andrade *et al.,* 2005).

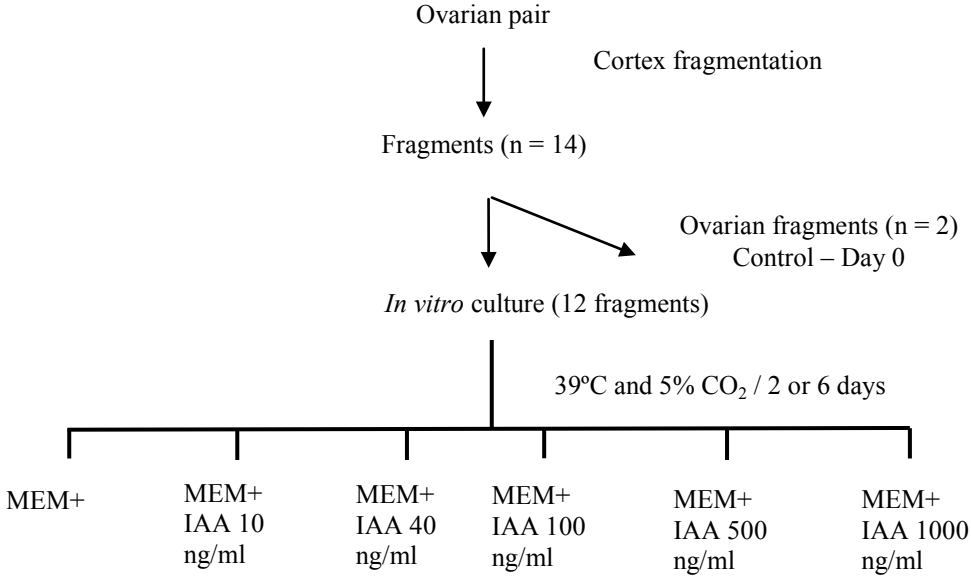

Figure1. Experimental protocol for *in vitro* culture of bovine preantral follicles in different concentrations of IAA.

After being cultured for either 2 or 6 days in each media, pieces of the ovarian cortex were fixed in Carnoy solution for 12 hours (Matos *et al*., 2007). After fixation, the tissues were sequentially dehydrated in increasing concentrations of ethanol, clarified with xylene, and embedded in paraffin wax. Serial sections (5 µm) were cut, and every fifth section was mounted on a glass slide and stained using a standard hematoxylin-eosin protocol (Tolosa *et al.*, 2003). The sections were examined by light microscopy (Zeiss, Germany) under 40X magnification.

Histological analysis and assessment of in vitro follicle activation

The preantral follicles were classified according to the stage of development as described by Hulshof *et al.* (1994). Follicles were considered as either primordial (with one layer of flattened granulosa cells surrounding the oocyte) or developing follicles. Developing follicles possessed one or multiple layers of cuboidal granulosa cells surrounding the oocyte and were characterized as primary or secondary, respectively. Primordial and developing follicles were further classified as either intact or degenerate. They were considered as intact when a morphologically normal oocyte with a non-pyknotic nucleus was surrounded by granulosa cells organized in discrete layers. In contrast, they were considered as degenerate when the oocyte was shrunken with a pyknotic nucleus and surrounded by disorganized granulosa cells detached from the basement membrane (Silva *et al.,* 2002). All of these criteria were carefully defined, and the accuracy of this analysis was confirmed by another independent researcher. In total, 120 follicles were studied per culture medium and per culture duration (30 follicles per replicate with four replicates = 120 follicles). When evaluating the rates of follicle activation and survival, only intact follicles were considered, and the percentage of primordial and developing follicles was calculated on Day 0 (control) and after 2 or 6 days of culture in each treatment.

In silico approach to search for auxin-responsive candidate genes in Bos sp. and Ovis sp.

An initial search of the scientific literature, which mainly consisted of papers that used large-scale expression analyses, was performed to find auxin-responsive genes in plants. Genes with a direct response to an auxin stimulus in plants were selected, and their protein sequences were retrieved from the *Arabidopsis thaliana* genome deposited in the GenBank database using the gene names as keywords. The sequence datasets were stored in FASTA file format. The putative function of each gene was obtained from the genome annotation databank from *A. thaliana* as well as from the corresponding entries from the SWISS-PROT database.

The selected plant amino acid sequences were used to perform a TBlastn alignment (Gertz *et al*., 2006), whereby the query amino-acid sequence was compared with the genome sequences, expressed sequence tags (ESTs), or cDNAs translated in all 6 frames. This search was performed using default stringency criteria against *Ovis* sp. and *Bos* sp. EST databases to identify probable orthologous genes. If no orthologs were identified from this specific search, protein sequence banks from mammals in general were tested.

The functions of the selected mammalian genes were verified by SWISS-PROT (Bairoch and Apweiler, 2000), and the metabolic pathways in which these gene products were involved were defined based on the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa and Goto, 2000). The selected sequences from *Arabidopsis thaliana, Bos taurus* and *Ovis aries* were aligned using the Clustal W program (Thompson *et al.*, 1994).

Statistical analyses

When evaluating follicular activation and survival, only healthy follicles were considered. The efficiency for the induction of activation was evaluated by the measured increase in the percentage of follicles undergoing development as well as the reduction in the percentage of primordial follicles. In this analysis, we compared follicles from the control (Day 0) group with follicles from all treatments on Days 2 and 6 of culture. Follicle viability was calculated as the percentage of all preantral follicles that were morphologically normal. This comparison was carried out among the control (Day 0) samples and all treatments (on Days 2 and 6 of culture).

An arcsine transformation was performed prior to analysis. Repeated measures analysis of variance was used to evaluate the percentages of primordial and developing follicles and the percentage of viable follicles within the non-cultured ovarian cortex and after culturing for 2 or 6 days. Pair-wise comparisons were conducted using Tukey's procedure. All analyses were done with the Statistical Analysis System (SAS Institute, Cary, NC, USA) and $P < 0.05$ was considered as significant.

Results

Primordial follicle activation

In regard to follicle activation, the non-cultured ovarian cortex (Day 0) contained mostly primordial follicles (85.8%) and a small percentage of developing follicles (14.2%). However, by Days 2 and 6 of culture, the percentage of primordial follicles was reduced, concomitant with the increased percentages of developing follicles in all media tested ($P < 0.05$).

Comparisons among the treatments for the same culture period were performed (Fig. 2 and 3). Only the ovarian cortex cultured for 6 days in media supplemented with 500 and 1000 ng/ml of IAA had higher percentages of primordial follicles and lower percentages of developing follicles when compared with the other treatments ($P < 0.05$ for each).

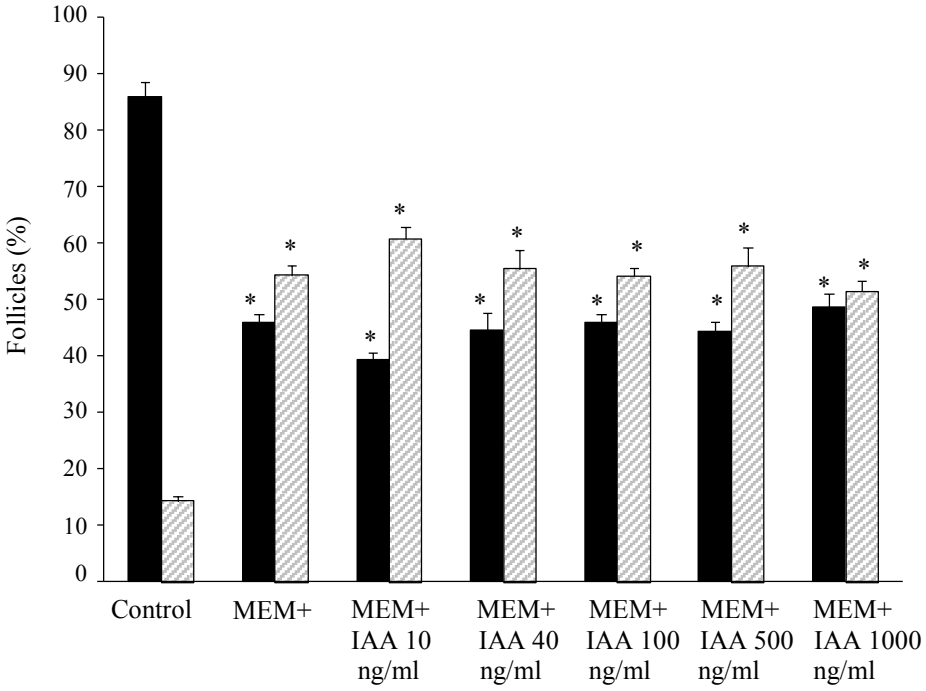

Figure 2. Mean (± SEM) percentages of normal preantral bovine follicles that were at primordial (■) and developing (▨) stages in non-cultured ovarian tissue (Control) and in ovarian tissue after *in vitro* culture for 2 days in various treatments. For both primordial- and developing-stage follicles there were differences ($P < 0.05$ for each) between the Control group and all other treatments. However, no significant difference was observed among all the other treatments (excluding the Control group). *Different ($P < 0.05$) from Control group.

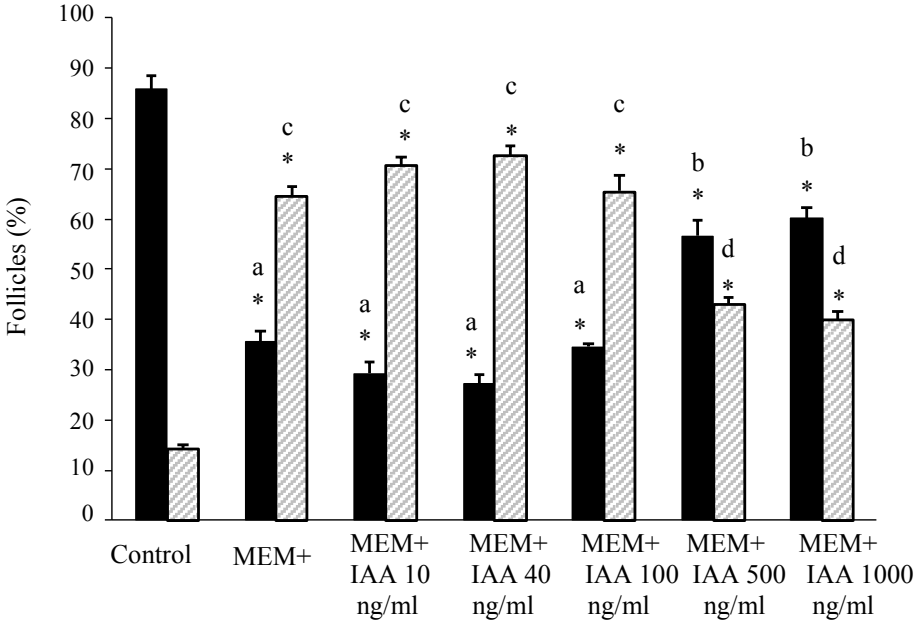

Figure 3. Mean (± SEM) percentages of normal preantral bovine follicles that were at primordial (■) and developing (▨) stages in non-cultured ovarian tissue (Control) and in ovarian tissue after *in vitro* culture for 6 days in various treatments. For both primordial- and developing-stage follicles there were differences ($P < 0.05$ for each) between the Control group and all other treatments. *Different ($P < 0.05$) from Control group. [ab]For primordial-stage follicles, treatments (excluding the control) without a common superscript differ ($P < 0.05$). [cd]For developing-stage follicles, treatments (excluding the control) without a common superscript differ ($P < 0.05$).

Follicular survival

Normal preantral follicles exhibited a healthy spherical or oval oocyte with a large central or eccentrically located nucleus. Granulosa cells, well organized in layers, without pyknotic nucleus were observed surrounding the oocyte (Fig. 4A; Control group). Degenerated follicles exhibited a retracted oocyte and swollen granulosa cells detached from basement membrane (Fig. 4B; group treated with 1000 ng/ml of IAA). It is important to note that neither pyknotic bodies in the granulosa cells nor rupture of the basement membrane were observed in degenerated follicles.

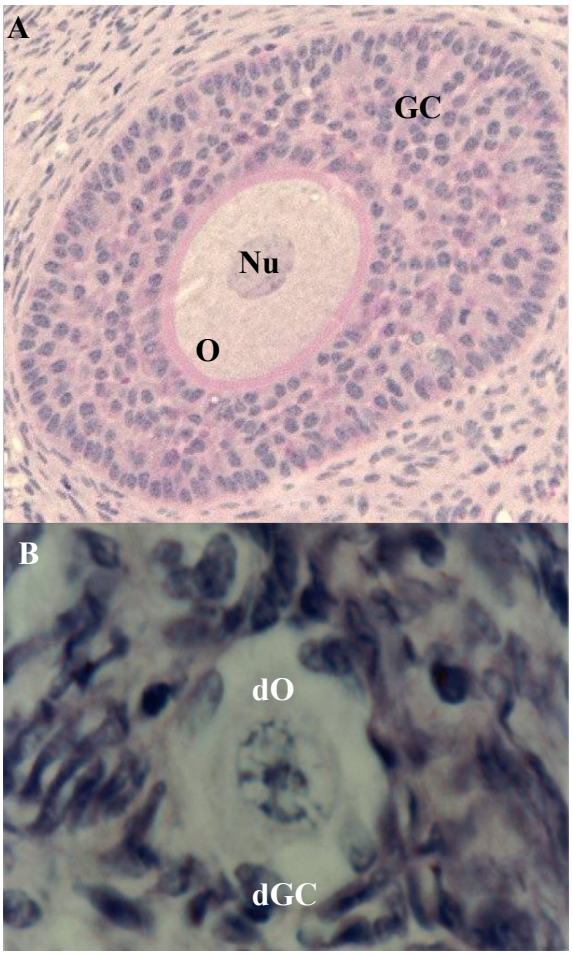

Figure 4. Histological section of ovarian fragment after staining with Periodic Acid Schiffhematoxylin, showing (A) normal preantral follicle; (B) Degenerated follicles. O: oocyte; Nu: oocyte nucleus; GC: granulosa cells; dO: degenerated oocyte, and dGC: degenerated granulosa cells.

Data for follicular survival are shown in Fig. 5 and 6. On Day 2, the percentage of morphologically normal primordial follicles was significantly reduced only in cultures supplemented with 500 and 1000 ng/ml of IAA compared to the Control group; conversely, the percentage of developing follicles decreased in all treatments tested, except when it was added 10 ng/ml of IAA to the media, in comparison the Control group (Fig. 5). On Day 6, the percentage of normal primordial and developing follicles in cultures supplemented with 10 ng/ml of IAA were not significantly different from the percentage in the Control group; yet, in all of the other treatments, the percentage of morphologically normal follicles was significantly lower than that of the Control group (Fig. 6). Regarding the effect of culture duration, the percentage of normal primordial and developing follicles decreased ($P < 0.05$) from 2 to 6 days in four of the cultures, including the MEM+ (control medium) and cultures supplemented with 100, 500 and 1000 ng/ml of IAA.

Search for auxin-responsive candidate genes in Bos *sp. and* Ovis *sp.*

The gene search strategy returned 10 plant genes that seemed extremely responsive to IAA. From these 10 genes, the gene ROOTY (*Arabidopsis*

thaliana) had the best homology scores with *Ovis aries* (E-value $2e^{-39}$ and Identity: 40%) and *Bos taurus* (E-value $1e^{-57}$ Identity: 37%) tyrosine aminotransferase genes (EC: 2.6.1.5).

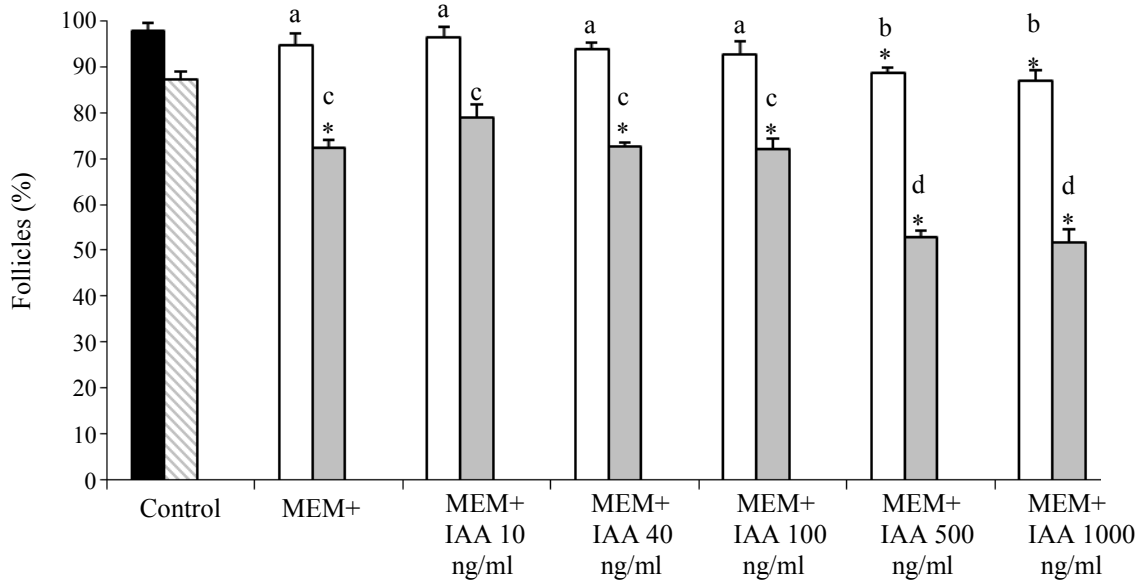

Figure 5. Mean (± SEM) percentages of preantral bovine follicles that were normal in non-cultured ovarian tissue [Control – primordial (■) and developing (▨)] and in tissue after culture for 2 days [primordial (□) and developing (▭)] in various treatments. *Different ($P < 0.05$) from Control group. [ab]For primordial-stage follicles, treatments (excluding the control) without a common superscript differ ($P < 0.05$). [cd]For developing-stage follicles, treatments (excluding the control) without a common superscript differ ($P < 0.05$).

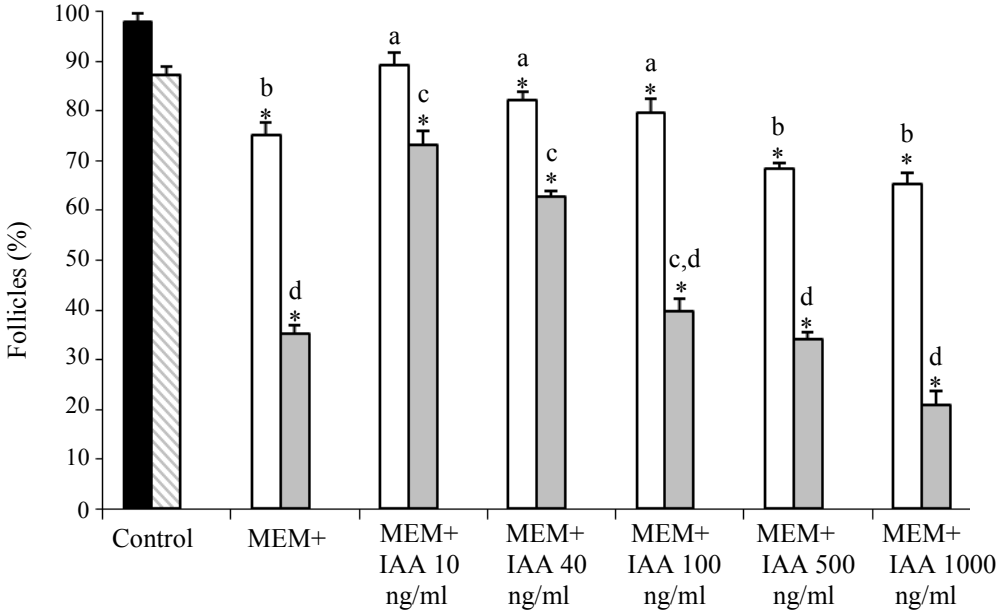

Figure 6. Mean (± SEM) percentages of preantral bovine follicles that were normal in non-cultured ovarian tissue [Control – primordial (■) and developing (▨)] and in tissue after culture for 6 days [primordial (□) and developing (▭)] in various treatments. *Different ($P < 0.05$) from Control group. [ab]For primordial-stage follicles, treatments (excluding the control) without a common superscript differ ($P < 0.05$). [cd]For developing-stage follicles, treatments (excluding the control) without a common superscript differ ($P < 0.05$).

Discussion

To our knowledge, this is the first work to describe the *in vitro* culture of follicles from *Bos indicus* females exposed to different doses of IAA. After only 2 days in culture, there was a sharp decrease in the percentage of primordial follicles and a concomitant increase in the percentage of developing follicles, thereby indicating the activation of primordial follicles (*i.e.*, when primordial follicles leave the resting pool and enter into the growth pool). Similar results have been obtained in studies with bovine (Braw-Tal and Yossefi, 1997; Fortune *et al.*, 1998) and baboon (Fortune *et al.*, 1998) preantral follicles enclosed in ovarian sections. Therefore, we hypothesize that the release of stimulatory factors or a cessation in the production of inhibitory factors by stromal, granulosa or pre-thecal cells within the culture of ovarian cortical tissue triggered the activation of these bovine primordial follicles *in vitro*. In support of this hypothesis, previous studies have demonstrated that anti-Mullerian hormone and IGF-1 can impair, but not block, the activation of primordial follicles *in vitro* (Durlinger *et al.*, 1999; Fortune *et al.*, 2004). The main source of these hormones is the granulosa cells of more advanced follicles. Thus, low concentrations of such inhibitory factors in the cultured cortical pieces or the presence of possible stimulatory factors in the culture media, such as insulin (Kezele *et al.*, 2002), may explain the observed primordial follicle activation *in vitro*. Moreover, there are locally produced growth factors that stimulate primordial follicle activation, such as BMP-7, Kit ligand, GDF-9 and FGF-2 (Fortune, 2003; van den Hurk and Zhao, 2005).

Culture conditions have a profound influence on follicle activation and viability. Regarding the activation, the addition of IAA had variable effects on preantral follicles. In the present study, follicular activation (i.e., the transition from primordial to developing follicles) was observed after 2 and 6 days of *in vitro* culture in all treatments supplemented or not with IAA. The addition of IAA did not cause an additional effect on activation when compared to control and MEM+ alone, different from that reported in other studies with sheep (Andrade *et al*., 2005), which is probably due to species-specific differences. Follicular activation in MEM+ presumably occurred because this medium is enhanced with nutrients such as amino acids and carbohydrates (van Wezel and Rodgers, 1996). Follicular activation has been obtained in previous studies in goats (Martins *et al*., 2010), cows (Braw-Tal and Yossefi, 1997) and baboons (Fortune *et al*., 1998), in which the number of primordial follicles was dramatically reduced, with a concomitant increase in the number of developing follicles after *in vitro* culture of ovarian tissue.

In the present study, the highest percentage of viable follicles occurred when IAA was used in the concentration of 10 ng/ml; this is in agreement with the work of Costa *et al*. (2010) that showed when an *in vitro* culture of ovine preantral follicles was incubated with IAA (10 ng/ml), the follicular viability was maintained in a similar manner to the control group during the 5-day culture. Notably, the molecular mechanisms underlying the action of auxin in plants have been explained by Barbier-Brygoo (1995). Auxins bind to a soluble auxin-binding protein, and the formed complex associates with a transmembrane receptor, causing, directly or indirectly, a variety of cellular responses (Toniolli *et al.*, 1996). The cellular effects of auxin stimulation include increased cell wall plasticity and cell water uptake, changes in cell permeability and respiratory patterns, and nucleic acid metabolism (Santner and Estelle, 2009). However, the molecular mechanisms underlying the actions of auxin in animal cells have not been elucidated.

It is important to emphasize that we observed a difference in susceptibility to degeneration among primordial and developing follicles (ie., primary and secondary follicles). On days 2 (except for treatment with the addition of 10 ng/ml of IAA) and 6 of *in vitro* culture, the percentage of developing follicles was significantly different from the Control group in all treatments, whereas the viability of primordial follicles remained similar to the condition *in vivo* (control) in four of six treatments of Day 2 and in one treatment of Day 6. The little morphological evidence of biosynthetic activity in the granulosa cells of primordial follicles can make these cells less sensitive to degeneration (Hirshfield, 1991). After the oocyte activation, organelle multiplication and an increase of the uptake of nutrient occurs. Some of these oocytes die after activation due to an inadequate environment to continue their normal development (Rüsse, 1983). Besides that, an increase of the number of granulosa cells in secondary follicles could make these cells more sensitive to atretic processes (Hirsfield, 1988).

Increasing the IAA concentration resulted in a progressive reduction in the percentage of normal follicles. However, little is known about the deleterious effects of IAA. Although IAA is known for its antioxidant activity (Cano *et al.*, 2003), this agent may also act as a pro-oxidant agent by accelerating lipid peroxidation (Candeias *et al.*, 1995) when it is oxidized by heme-containing peroxidases (Kim *et al.*, 2004), which are common enzymes present in the serum. We suggest that at concentrations of 40, 100, 500 and 1000 ng/ml, there was a corresponding increase in the amount of IAA oxidation, thereby increasing the level of reactive oxygen species (ROS), which resulted in a progressive reduction of follicular viability. Oxidative stress has been related to apoptosis in many cell types (Chandra *et al.*, 2000), including ovarian follicles (Tsai-Turton and Luderer, 2006; Zhang *et al.*, 2006). Additionally, the administration of IAA to pregnant mice induces p53-mediated apoptosis in the embryo's

neuroepithelium, decreases the formation of neurons and leads to microencephaly in the fetus (Furukawa *et al.*, 2007).

The results obtained in the present study using an *in silico* approach showed that the plant gene ROOTY seemed to play an important role in IAA metabolism and that ROOTY mRNA was found when plant tissues were stimulated with IAA. Prather *et al*. (2005), from the *DNA Core Facility (Bovine Project)*, have described the expression levels of the tyrosine aminotransferase gene (an ortholog of ROOTY) in the following bovine samples: germinal vesicle-stage oocytes, *in vitro* derived embryos (2-cell, morula, blastocyst and nuclear transfer blastocyst), *in vivo* blastocysts and conceptuses, corpora lutea, ovarian follicles, oviducts, endometrium, and placenta/embryo from day 35 bovine conceptuses. Even though the role of ROOTY in the control of auxin homeostasis in plants has yet to be elucidated (Barlier *et al.*, 2000), the presence of its orthologous transcript in bovine oocytes and embryos indicates that it participates in general mammalian metabolism.

We conclude that the 10 ng/ml concentration of IAA promoted follicular survival and activation of Nelore bovine preantral follicles after 6 days of culture *in vitro*. The determination of the molecular basis of *in vitro* oocyte development is still complicated due to the small amount of available RNA at early oocyte stages. The identification of candidate genes that can affect oocyte development may assist in the discovery of molecular markers, such as SNP's, which should be useful in choosing the best oocyte donors and may contribute to the genetic quality of livestock improvement programs.

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