Anim. Reprod., v.3, n.4, p.415-422, Oct./Dec. 2006

Histological and ultrastructural features of caprine preantral follicles after *in vitro* culture in the presence or absence of indole-3-acetic acid

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Abstract

The aim of the present study was to evaluate the effects of indole-3-acetic acid (IAA) on survival, activation, and growth of caprine preantral follicles using histological and ultrastructural criteria. Pieces of caprine ovarian cortex were cultured for 1 or 5 days in Minimum Essential Medium (MEM - control medium) supplemented with different concentrations of IAA (10, 20, 40, or 100 ng/ml). Small fragments from non-cultured ovarian tissue and from those cultured for 1 or 5 days in a specific medium were processed for classical histology and ultrastructural analysis. Follicle and oocyte growth were evaluated before and after 1 or 5 days of culture in the various media tested. The results showed a greater percentage of histologically-normal follicles in MEM alone or MEM supplemented with IAA (20 ng/ml) than other treatments. Indole-3-acetic acid added at a concentration of 20 or 40 ng/ml increased the proportion of primordial follicles that entered the growth phase after 5 days. In the presence of 10 or 20 ng/ml IAA, the follicles increased in diameter after 5 days of culture. Ultrastructural studies, however, did not confirm maintenance of the morphological integrity of caprine follicles cultured for 1 or 5 days in MEM supplemented with 20 ng/ml of IAA as was demonstrated by histological studies but in contrast showed distinct degenerative changes in both oocytes and granulosa cells. In conclusion, ultrastructural integrity of caprine preantral follicles can be successfully maintained after *in vitro* culture for 5 days in MEM without addition of IAA. In addition, ultrastructural analysis is necessary to judge the morphology of cultured preantral follicles since IAA negatively affects the ultrastructural composition of *in vitro* activated follicles, which was not detected by classical histology.

Keywords: caprine, preantral follicles, culture, indol-3-acetic acid, ultrastructure.

Introduction

Several studies have evaluated the initiation of follicle growth by culturing ovarian cortex *in vitro* in media supplemented with different hormones and growth factors (bovine: Wandji *et al*., 1996; Cushman *et al*., 2002; caprine: Silva *et al*., 2004; baboon: Fortune *et al*., 1998; murine: Parrot and Skinner, 1999; and human: Hovatta *et al*., 1997; Wright *et al*., 1999; Hreinsson *et al*., 2002). Furthermore, attention has been paid to the presence of growth factors, their receptors, and hormone receptors in the various preantral follicle compartments to understand the mechanisms underlying the activation of primordial follicles and further follicle growth and differentiation (reviewed in Fortune, 2003; van den Hurk and Zhao, 2005). There are many oocyte-, granulosa, and in later follicle stages also theca-derived factors or other factors and hormones that may be involved in the regulation of follicle development. However, it is unknown if indole-3-acetic acid (IAA) could regulate preantral follicle activation and growth.

The auxin IAA was discovered in plants (Went and Thimann, 1937) as a hormone that controls processes like growth, movements, or tropism that lead to cellular expansion and division (Becker and Hedrich, 2002) and is, for example, an important ingredient of coconut water (Toniolli *et al*., 1996). In animals, IAA is obtained from a diet rich in vegetable stems or by synthesis from tryptophan (Gordon *et al*., 1972; Mills *et al*., 1991), and it is present in cerebrospinal fluid (Hu and Dryhurst, 1997), blood (Martinez *et al*., 1983), and in several organs like the lungs, kidneys, liver, and brain (Tusell *et al*., 1984). Indole-3-acetic acid from coconut water was shown to be effective in promoting spermatozoa survival after *in vitro* preservation (Toniolli *et al*., 1996). In addition, IAA was used successfully to preserve caprine preantral follicles during transportation (Ferreira *et al*., 2001). Used at a concentration of 40 ng/ml, IAA prevented cultured ovine preantral follicles from degeneration, but only when it was administered to the culture medium in a mixture with FSH or EGF (Andrade *et al*., 2005). The latter data were based on histological studies only. However, several authors have emphasized the importance of TEM after *in vitro* culture of preantral follicles, since it gives close insight into the ultrastructural characteristics of follicles, thus allowing a better evaluation of their quality (van den Hurk *et al*.,

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Received: November 1, 2006
Accepted: May10, 2007

1998; Salehnia *et al*., 2002).

The aim of the present study was to investigate whether IAA benefits the survival, activation, and further growth of *in vitro* cultured caprine preantral follicles. Both histological and ultrastructural studies were performed to investigate and compare the morphology of non-cultured follicles to those cultured for 1 or 5 days in the absence or presence of different concentrations (10, 20, 40, or 100 ng/ml) of IAA. In addition, follicle and oocyte diameters were evaluated before and after 1 or 5 days of culture in the various media tested.

Materials and Methods

Experimental protocol

Ovaries (n = 10) from 5 adult, non-pregnant, mixed-breed goats were collected at a local slaughterhouse (Fortaleza, Brazil). The animals were cyclic and in good body condition. The ovaries were washed and transported in 0.9% saline solution to the laboratory in thermo flasks with water at 20ºC. Both ovaries from each animal were stripped of surrounding fat tissue and ligaments and cut in half, after which the medulla, large antral follicles, and corpora lutea were removed. The ovarian cortex was divided into 11 fragments (3 x 3 mm area; 1 mm thick). A small part (1 mm^3) of one fragment was taken away randomly and subsequently fixed for ultrastructural examination while the remainder of the fragment was fixed for classic histological studies (non-cultured controls). The other fragments of ovarian cortex were individually *in vitro* cultured in 24-well culture plates with 1 ml of culture medium for 1 or 5 days at 39°C with 5% CO_2 in air. The basic control medium was MEM (Cultilab, Rio de Janeiro, Brazil) supplemented with ITS (insulin 6.25 μg/ml, transferrin 6.25 μg/ml, and selenium 6.25 ng/ml), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/ml BSA, 100 μg/ml penicillin, 100 μg/ml streptomycin (Vetec, Rio de Janeiro, Brazil), and 0.25 μg/ml fungizone. This control medium (MEM) was supplemented with different concentrations of IAA (10, 20, 40, or 100 ng/ml; Vetec, Rio de Janeiro, Brazil). All chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Every 2 days, the culture medium was replaced by the same volume of fresh medium. Each treatment was repeated five times, thus using the ovaries of 5 different animals.

Histological analysis and assessment of in vitro follicle growth

To evaluate the morphology of the caprine follicles, a small part (1 mm^3) from each non-cultured ovarian tissue fragment and those that were cultured for 1 or 5 days was randomly chosen and removed for TEM studies while the remainder of the fragments were fixed in Carnoy for 12 hours for light-microscopy (LM) studies. Briefly, after fixation, for LM analysis, the tissue fragments were dehydrated in a graded series of ethanol solutions, clarified with xylene, and embedded in paraffin wax. For each piece of ovarian cortex, 7 μm sections were mounted on slides, stained with periodic acid Schiff and hematoxylin (PAS staining system, Sigma, Inc., St. Louis, MO, USA), and examined by LM (Zeiss, Germany) at 100x and 400x magnification.

Preantral follicles were classified as primordial (one layer of flattened granulosa cells around the oocyte) or growing follicles (intermediate - one layer of flattened to cuboidal granulosa cells around the oocyte; primary - single layer of cuboidal granulosa cells around the oocyte; or secondary - oocyte surrounded by two or more layers of cuboidal granulosa cells; as described by Silva *et al*., 2004). These follicles were classified individually as histologically-normal intact when an intact oocyte was present (i.e., an oocyte without a pyknotic nucleus, surrounded by granulosa cells, which were well organized in one or more layers and did not have a pyknotic nucleus). Degenerated follicles were defined as those with a retracted oocyte that had a pyknotic nucleus and/or was enveloped by disorganized granulosa cells, which have detached from the basement membrane. From each medium and each culture period, approximately 150 follicles were randomly evaluated.

To evaluate follicular activation and growth, only intact follicles with a visible oocyte nucleus were recorded, and the proportion of primordial and growing follicles were calculated at Day 0 (before culture - controls) and after 1 or 5 days of culture in the various media tested. Major and minor axes of each oocyte and follicle were measured under a microscope with an ocular micrometer. The means of the minor and major axes were reported as oocyte and follicle diameters, respectively. These values were used to assess the effect of the hormonal treatment on follicular growth.

Ultrastructural analysis

For ultrastructural analysis, 11 small pieces of ovarian cortex were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate buffer at a pH of 7.2. After washing the ovarian pieces with sodium cacodylate buffer, they were post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer. Subsequently, the samples were dehydrated in a graded series of acetone and embedded in Spurr´s epoxy resin. First, semi-thin sections (3 μm) were cut with an ultramicrotome (Reichert Supernova, German) for LM studies and stained with toluidine blue. Subsequently, follicles classified as histologically normal in semi-thin toluidin blue stained sections were submitted to ultrastructural analysis. For that purpose, thin sections (70 nm) were cut and then contrasted with uranyl acetate and lead citrate, and examined using a Jeol 1011 (Jeol, Tokyo, Japan) transmission electron microscope operating at 80 kV.

Statistical analyses

The percentages of histologically-normal follicles as well as primordial and growing follicles in non-cultured and cultured tissue were compared by the Chi-square test (StatView for Windows, SAS Institute Inc., Cary, NC, USA). Mean diameters of oocytes and follicles obtained after the various treatments and after the different culture periods, were analyzed by ANOVA and Fisher's test (StatView for Windows,). Differences were considered significant at $P < 0.05$.

Results

Effect of media and culture periods on the percentage of histologically-normal follicles

The effects of IAA supplementation on follicle survival after 1 or 5 days of culture are shown in Table 1. There was a decrease ($P < 0.05$) in the percentage of histologically-normal follicles (Fig. 1) after 1 and 5 days of culture compared to non-cultured follicles. However, no considerable effect of IAA concentration was observed after 1 day of culture. With the increase of the culture period from 1 to 5 days, a decrease ($P < 0.05$) in the percentage of histologically-normal follicles was observed in medium containing 10 ng/ml IAA. After 5 days culture, the addition of 10, 40, or 100 ng/ml IAA significantly reduced the percentage of histologically-normal follicles when compared with MEM alone or MEM supplemented with 20 ng/ml IAA.

Table 1. Percentages of histologically-normal follicles[†] in non-cultured and cultured tissues for 1 or 5 days in MEM supplemented with various concentrations of indole-3-acetic acid (IAA).

Non-cultured (Day 0; %)	87.3	
Cultured	Day 1 (%)	Day 5 (%)
MEM	64.1* ^{A,a}	63.4* ^{A,a}
IAA - 10 ng/ml	66.7* ^{A,a}	54.7* ^{B,b}
IAA - 20 ng/ml	64.1* ^{A,a}	64.0* ^{A,a}
IAA - 40 ng/ml	56.0* ^{A,a}	51.1* ^{A,b}
IAA - 100 ng/ml	59.8* ^{A,a}	59.5* ^{A,b}

*Different from non-cultured ovarian cortex tissue ($P < 0.05$; control/Day 0).

^{A,B}Different letters in the same row denote differences between culture periods within the same medium ($P < 0.05$).

^{a,b}Different letters in the same column denote differences among treatments within the same period ($P < 0.05$).

[†] n = 150 follicles/treatment.

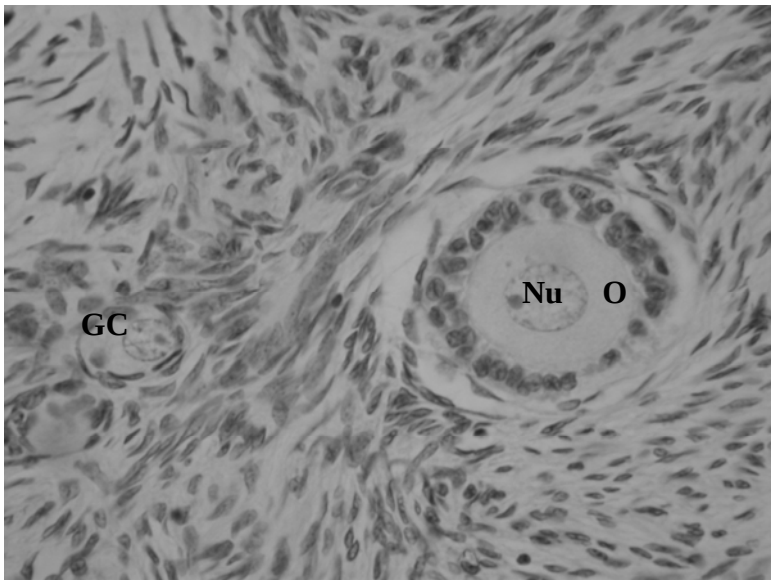

Figure 1. Histological section of a non-cultured ovarian tissue fragment, showing normal primordial and primary follicles. (Periodic acid Schiff-hematoxylin staining (O: oocyte; NU: oocyte nucleus; GC: granulosa cells; 400x).

Primordial follicle activation and growth during in vitro culture

The percentages of primordial and growing follicles in ovarian cortical tissue before and after 1 and 5 days of culture are shown in Table 2. The percentages of primordial and growing follicles in non-cultured cortex tissues were 81.7% and 18.3%, respectively. Compared to non-cultured and 1-day cultured tissues, all 5-day cultured tissues showed a reduction ($P < 0.05$) in the proportion of primordial follicles and an increased proportion of growing follicles ($P < 0.05$).

Table 2. Percentages of primordial and growing follicles† (intermediate, primary, and secondary) in non-cultured and cultured tissues for 1 or 5 days in MEM supplemented with various concentrations of indole-3-acetic acid (IAA).

	Primordial Follicles (%)		Growing Follicles (%)	
Non-cultured (Day 0)	81.7		18.3	
Cultured	Day 1	Day 5	Day 1	Day 5
MEM	70.3 ^{A,a}	43.4 ^{*B,a}	29.7 ^{A,a}	56.6 ^{*B,a}
IAA - 10 ng/ml	71.4 ^{A,a}	46.3 ^{*B,a}	28.6 ^{A,a}	53.7 ^{*B,a}
IAA - 20 ng/ml	80.9 ^{A,a}	42.5 ^{*B,a,b}	19.1 ^{A,a}	57.5 ^{*B,a,b}
IAA - 40 ng/ml	75.0 ^{A,a}	27.4 ^{*B,b}	25.0 ^{A,a}	72.6 ^{*B,b}
IAA - 100 ng/ml	69.2 ^{A,a}	44.3 ^{*B,a}	30.8 ^{A,a}	55.7 ^{*B,a}

*Different from non-cultured ovarian cortex tissue ($P < 0.05$; control/Day 0).
^{A,B}Different letters in the same row denote differences between culture periods within the same medium and type of follicles ($P < 0.05$).
^{a,b}Different letters in the same column denote differences among treatments within the same period ($P < 0.05$).
† n = 150 follicles/treatment.

When compared to the 5-day cultured tissues in control MEM, the addition of 40 ng/ml of IAA to MEM increased ($P < 0.05$) the percentage of growing follicles. No significant effect of IAA on growing follicles was observed after 1 day of culture. Compared to non-cultured tissue, a significant increase in oocyte diameter was observed when tissues were cultured for 5 days in a medium with 10 ng/ml IAA (Table 3). Likewise, follicle diameter significantly increased when tissues were cultured for 5 days in the presence of 10, 20, or 40 ng/ml IAA while both oocyte and follicle diameters increased ($P < 0.05$) in media containing 10 or 20 ng/ml IAA.

Table 3. Oocyte and follicle† diameters (mean ± S.D.) in non-cultured or cultured tissues for 1 or 5 days in MEM supplemented with various concentrations of indole-3-acetic acid (IAA).

	Oocyte diameter (μm)		Follicle diameter (μm)	
Non-cultured (Day 0)	40.7 ± 2.2		52.2 ± 2.2	
Cultured	Day 1	Day 5	Day 1	Day 5
MEM	39.7 ± 1.2 ^{A,a}	41.9 ± 1.0 ^{A,a}	53.9 ± 2.0 ^{A,a}	57.8 ± 1.5 ^{A,a}
IAA - 10 ng/ml	43.6 ± 1.5 ^{A,a}	52.9 ± 2.4* ^{B,b}	57.6 ± 1.8 ^{A,a}	79.8 ± 5.4* ^{B,b}
IAA - 20 ng/ml	43.5 ± 1.1 ^{A,a}	47.5 ± 2.9 ^{B,a,b}	47.0 ± 1.4 ^{A,a}	74.9 ± 5.9* ^{B,b}
IAA - 40 ng/ml	39.7 ± 1.7 ^{A,a}	41.9 ± 2.2 ^{A,a}	55.7 ± 1.9 ^{A,a}	67.5 ± 6.2* ^{B,a}
IAA - 100 ng/ml	43.0 ± 1.1 ^{A,a}	43.9 ± 2.4 ^{A,a}	57.9 ± 1.3 ^{A,a}	59.8 ± 5.3 ^{A,a}

*Different from non-cultured ovarian cortex tissue ($P < 0.05$; control/Day 0).
^{A,B}Different letters in the same row denote differences between culture periods within the same medium oocyte and follicle diameters ($P < 0.05$).
^{a,b}Different letters in the same column denote differences among treatments within the same period ($P < 0.05$).
† n = 150 follicles/treatment.

Ultrastructural analysis of preantral follicles cultured in vitro with 20 ng/ml IAA

Culture with 20 ng/ml IAA yielded the best results for follicle activation and growth after 5 days of culture. Regardless of the stage of follicular development, the ultrastructure of histologically-normal follicles from the control group and from those cultured in MEM alone for 5 days appeared similar. These follicles exhibited sparse vesicles spread throughout the ooplasm. Moreover, the homogeneous cytoplasm contained numerous rounded mitochondria with peripheral cristae and continuous mitochondrial membranes although there were occasional elongated forms with parallel cristae. Golgi complexes were rarely observed. Both smooth and rough endoplasmic reticulum were present, either as isolated aggregations or as complex associations with mitochondria and vesicles (Fig. 2A). The oocyte nucleus had uncondensed chromatin and the nucleolus could generally be observed. In all developmental stages, granulosa cells were small with a greater nuclear-to-cytoplasm ratio as compared to somatic cell structures. The nuclei were irregularly shaped with loose chromatin in the center and small aggregates of condensed chromatin in the periphery. Well-developed rough endoplasmic reticulum and mitochondria with well-developed lamellar cristae were the most evident organelles observed in granulosa cells. Abundant gap junctions were observed between granulosa cells as well as between granulosa cells and the oocyte (Fig. 2B).

When cultured in IAA for one day, the histology of follicles seemed to be well preserved in semi-thin sections stained with toluidin blue. However, TEM studies revealed some discrete changes in their ultrastructure, which are indicative of initial degeneration. Such follicles generally had an oocyte with large numbers of vesicles spread throughout the ooplasm. Cytoplasmic organelles were more randomly observed. In addition, initial signs of endoplasmic reticulum proliferation and damage to mitochondrial membranes and cristae were observed. The oocyte nucleus appeared misshapen, retracted, and had a wavy membrane (Fig. 2C). Granulosa cells appeared swollen with a low density of cytoplasmic organelles. Furthermore, granulosa cells showed less contact with each other and exhibited fewer gap junctions.

Although the histological images of follicles cultured in 20 ng/ml IAA for 1 or 5 days were comparable, they showed remarkable differences at the ultrastructural level. Besides the progression of the changes described above, the follicles cultured for 5 days presented a substantial irregularity in follicle, oocyte, and nuclear outlines. As degeneration progressed, the ooplasm in these follicles was extremely vacuolated with the vacuoles often fused, thus producing a larger vacuolated area. In some oocytes, the nuclear membrane was broken. Furthermore, some granulosa cells were fragmented or had disappeared, leaving an empty space in the follicular granulosa. Frequently, the connection between the oocyte and granulosa cells had disappeared, and organelles were no longer identifiable (Fig. 2D).

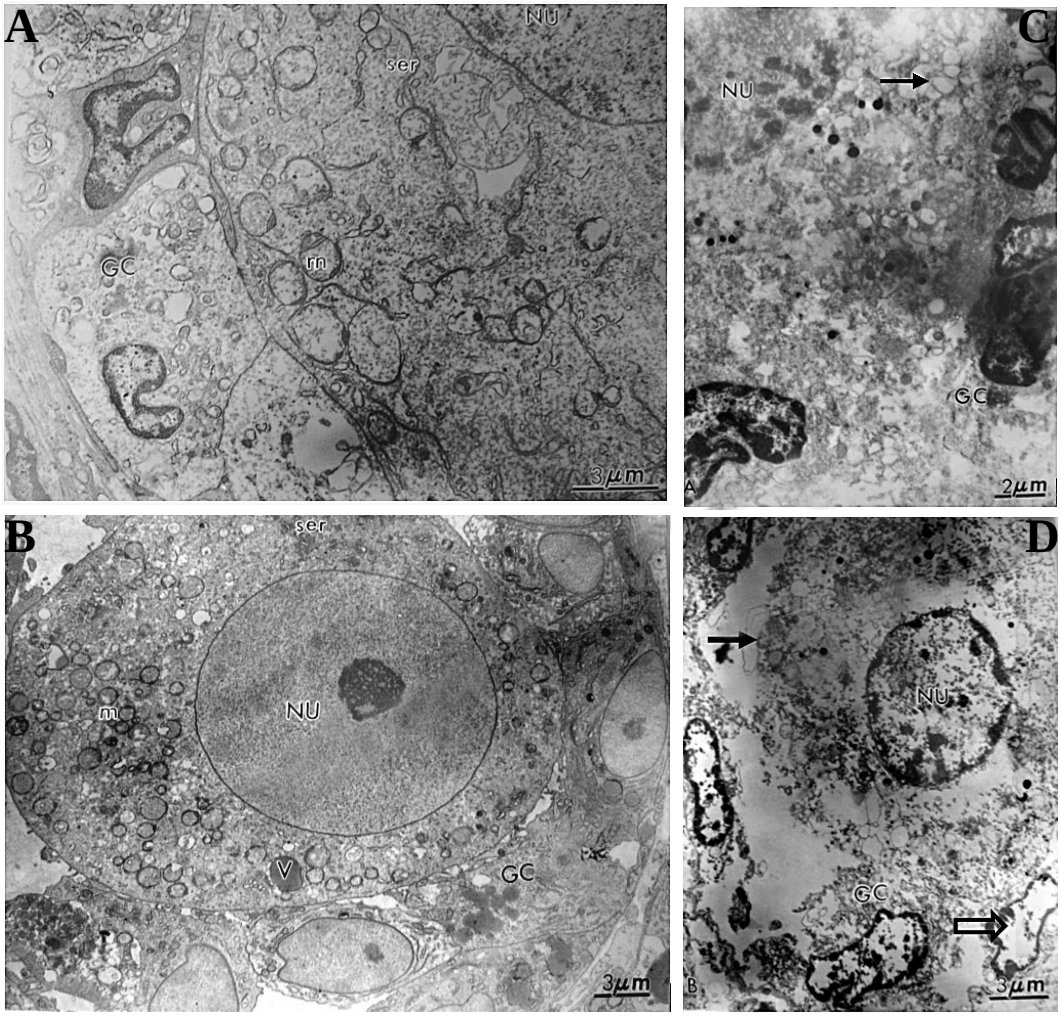

Figure 2. Electron micrographs of caprine preantral follicles before (A; 5200x) and after culture in MEM for 5 days (B; cultured control; 3240x), in indole-3-acetic acid (IAA; 20 ng/ml) for one day (C; 5600x) or 5 days (D; 3240x). In non-cultured follicles and in follicles cultured in MEM, note the homogeneous cytoplasm with numerous rounded mitochondria (2A and B). Note the extreme vacuolization and the great holes present in the cytoplasm, indicative of degeneration (2C; solid arrow). Note the empty space in degenerated granulosa cells after *in vitro* culture with IAA (2D; open arrow; NU: oocyte nucleus; GC: granulosa cells; m: mitochondria; ser: smooth endoplasmic reticulum; v: vesicles).

Discussion

The present study described ultrastructural changes in caprine preantral follicles after *in vitro* culture for 1 or 5 days. It furthermore reported a dose-dependent *in vitro* effect of IAA on the activation of primordial follicles and preantral follicle growth. After 5 days of culture, IAA at a concentration of 20 ng/ml appeared to maintain preantral follicle survival, based on histological parameters, and stimulated both primordial follicle activation and preantral follicle growth whereas the follicular ultrastructure was negatively affected. In addition, a lower concentration of IAA (10 ng/ml) was not effective in maintaining follicle survival after 5 days of culture. There are only few studies that dealt with the effects of IAA in animals. Cardoso *et al*. (2002) demonstrated that canine spermatozoa could be well preserved in presence of 20 ng/ml IAA, which appeared effective in our studies. However, 40 ng/ml IAA was shown to be effective in promoting porcine spermatozoa survival after *in vitro* preservation (Toniolli *et al*., 1996). In females, the effects of IAA were previously described on caprine preantral follicle preservation (Ferreira *et al*., 2001) and *in vitro* cultured ovine preantral follicles (Andrade *et al*., 2005). In these studies, positive effects were obtained after LM evaluation (classical histology) of follicles only. Andrade *et al*. (2005) showed that, in combination with EGF or FSH, IAA at a concentration of 40 ng/ml prevented the degeneration of preantral follicles. However, several authors have emphasized the importance of TEM studies of follicles after *in vitro* culture, since it gives close insight into the ultrastructural characteristics of follicles, which could help to avoid overly-optimistic opinions about the quality of cultured follicles based on LM investigations (van den Hurk *et al*., 1998; Salehnia *et al*., 2002; Matos *et al*. 2004). Our current findings thus confirm the latter view.

The present TEM studies revealed obvious differences in ultrastructural quality of follicles cultured in MEM for 5 days when compared to those cultured in medium supplemented with IAA (20 ng/ml) for 1 or 5 days. Although they had comparable histological images, follicles cultured in MEM maintained their ultrastructural integrity. Those cultured with IAA showed various signs of initial degeneration after one day of culture and exhibited more clear degenerative features like extreme ooplasm vacuolization after 5 days of culture. Cytoplasmic vacuoles are characteristic signs of degeneration in oocytes (Silva *et al*., 2000), granulosa (Hay *et al*., 1976), and cumulus cells (Assey *et al*., 1994) during degeneration and may represent endoplasmic reticulum swelling (Tassel and Kennedy, 1980) or altered mitochondrial structure (Fuku *et al*., 1995). In caprine preantral follicles, mitochondria showing extensive swelling and disappearance of their cristae and endoplasmic reticulum with increased volume were previously indicated as the first signs of degeneration (Silva *et al*., 2001). In plants, the auxin IAA increased cell wall plasticity, cell water uptake, and changed cellular permeability, respiratory patterns, and acid nucleic metabolism (Galston and Purves, 1960). However, the molecular mechanisms of auxin action in animal cells are not clear.

Melo *et al*. (2004) indicated that the induction of reactive oxygen species (ROS) such as H_2O_2 and O_2^-. When cellular anti-oxidative defense is limited, these ROS may cause oxidation of key molecules that normally release proteases, lipases, and nucleases from mitochondria (Fiers *et al*., 1999). In our study, IAA might have increased the production of toxic ROS, thus causing the observed higher rate of degeneration.

After 5 days of culture, addition of 20 ng/ml IAA to the medium resulted in a significant increase in activation rate of primordial follicles and oocyte and follicle diameters. Our TEM findings suggest that these LM-observed phenomena are due to follicular necrosis, which is characterized by swelling of the cell as a result of increased cellular vacuolization (Barros *et al*., 2001). Based on findings of Andrade *et al*. (2005), IAA might activate early folliculogenesis after its possible binding to certain growth factors present in the ovarian tissue. On the other hand, interaction between IAA and local growth factors could have stimulated primordial follicle activation.

When compared with one day of culture, oocyte and follicle diameters had increased after 5 days of culture in the presence of 10 or 20 ng/ml IAA. Similar results were obtained with pea culture in which IAA at a low concentration (5 μM) stimulated growth of pea stem segments while little growth occurred in the absence of IAA (Moore *et al*., 1983). More studies are necessary to determine the molecular mechanism of IAA action on the growth of caprine preantral follicles. It is of interest to note here that hydroxyl-acetic indols are synthesized by the pineal gland and influence reproduction by effecting on gonadal steroidogenesis (Öcal-Irez *et al*., 1989). Indole-3-acetic acid thus may function similarly to pineal indols in affecting folliculogenesis. However, like the molecular mechanisms of IAA action on the activation and growth of caprine preantral follicles, the way in which IAA and pineal indols can influence these follicles has to be further investigated.

In conclusion, the ultrastructural integrity of caprine preantral follicles can be successfully maintained after *in vitro* culture for 5 days in MEM without addition of IAA. In addition, this study showed that IAA is not effective in maintaining the ultrastructural morphology of follicles during *in vitro* culture. The findings prove that TEM studies are indispensable to evaluate the morphological status of follicles and for investigating preantral follicle development, since the positive effects of compounds on follicle activation and growth observed after routine histological studies could be the result of a negative

effect caused by induced degeneration, which can only be visualized ultrastructurally. Further studies will be needed to elucidate the mechanism through which IAA and pineal indols act on ovarian tissue.

Acknowledgments

This work was supported by CNPq, FINEP and Fundação Cearense de Apoio à Pesquisa (FUNCAP). MHT Matos is a recipient of a grant from CNPq (Brazil). The authors thank Dr. Vicente José de Figueiredo Freitas, PhD (LFCR) for logistical support.

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