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Morphological-metric, ultrastructural and immunohistochemical effects of gossypol on cultured granulosa cells and oocytes of ewes using MOEPF

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ABSTRACT

Manipulation of oocytes enclosed in preantral follicles (MOEPF) allows for analyzing follicular development and use of this biotechnology in the pre-analysis of the beneficial or toxic effects of bio-products on granulosa cells and oocytes at different developmental stages. In this study, there was evaluation of the effects of gossypol by culturing granulosa cells and oocytes in ewe ovarian tissues. Ovarian tissues were cultured with gossypol at 37 °C, in humidified air and 5% CO_2. Variables that were evaluated were morphology, morphometry, ultrastructure and abundance of estradiol receptor α (α-ER). There were no differences in developmental characteristics when there was treatment with any of the gossypol doses that were evaluated. Immunostaining indicated that when the gossypol dose increases, the abundance of α-ER also increases in the cytoplasm, nucleus, and granulosa cells. Findings with the ultrastructural analysis indicated that for granulosa cells there was fewer cells and greater disorganization and a lack of structural integrity of follicular cell layers as a result of all gossypol treatments. The culture of oocytes in preantral ovarian follicles in presence of gossypol did not affect the morphological-metric structure at the doses evaluated. The findings with evaluation of ultrastructural and immunohistochemical structures indicated granulosa cells and α-ER were affected by the treatments with gossypol indicating there were effects of this compound on ovarian function in sheep. This study indicated there is a toxic action of gossypol when using the biotechnology, MOEPF. Thus, gossypol negatively affects granulosa cell development and structural integrity of preantral follicles in sheep.

1. Introduction

Gossypol is a yellow polyphenolic pigment in cotton plants (Gossypium spp.) and is regarded as a toxic agent that affects animal

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reproduction (Randel et al., 1992). The toxic effects of gossypol on animal reproduction have already been reported in male cattle (Randel et al., 1992; Hassan et al., 2004); rats (Deoras et al., 1997; El-Sharaky et al., 2010; Fonseca et al., 2013); goats (East et al., 1994; Nunes et al., 2010); sheep (Guedes and Soto-Blanco, 2010; Braga et al., 2012; Paim et al., 2016) and some other animals. The anti-fertility effect depends on the dose and reproductive stage when gossypol intake occurs through inhibition of spermatogenesis and decreases, in sperm motility, and viability, as well as induction of morphological damage to sperm (Yuan and Shi, 2000), mitochondrial lesions and damage to the germinal epithelium (Randel et al., 1992).

In females, gossypol affects the estrous cycle in cows (Villaseñor et al., 2008; Gadelha et al., 2011, 2014) and rodents (Lin et al., 1985). Gossypol also affects steroidogenesis, nuclear maturation and follicular size (Randel et al., 1996) as well as proliferation and steroidogenic activity in granulosa cells of pigs (Basini et al., 2009). Embryo toxic effects of gossypol have also been observed using *in vitro* assays and lead to the damaging of cattle embryos (Hernandez-Cerón et al., 2005). Randel et al., (1996) reported that, *in vivo* effects of gossypol did not affect most physiological processes, such as ovulation, fertilization of eggs, and the number of transferable and degenerate embryos in donor Brangus Heifers used for embryo production in embryo transfer programs. Dabrowski et al. (2000) reported that the anti-fertility effect of gossypol is related to the efficiency in which this polyphenolic compound crosses the gonadal circulation barrier which varies depending on permeability of reproductive organs, age of the animal and metabolic rate of each species.

With use of the MOEPF procedure, there was maintenance of viability, morphology and ultrastructure of granulosa cells, theca cells and oocytes in ovarian tissues during the preantral developmental stage in culture (Jimenez et al., 2016a). There is use of this biotechnology in the pharmaceutical industry for preclinical analysis of the effect of products (beneficial or toxic) on granulosa cells and oocytes at different developmental stages. The MOEPF, therefore, is an *in vitro* model that can also be used to assess follicular viability *in vivo* (Figueiredo and Silva, 2010).

The negative effects of gossypol on reproduction in males are well known; however, in females, most studies are with cattle and thus studies are required for a greater understanding of the effects of gossypol on reproduction in ewes. In the present study, the hypothesis was that gossypol in the *in vitro* culture of ovarian tissues of ewes affects the morphology, ultrastructure and abundance of estradiol receptors in follicles during the preantral developmental stages. Thus, the objective of the present study was to evaluate the effect of gossypol using MOEPF in granulosa cells and oocytes in ovarian tissue during the preantral developmental stages in ewes.

2. Materials and methods

2.1. Origin, collection and transport of ovaries

The experiment was conducted at the Laboratory of Cellular and Molecular Biology and Laboratory of Animal Nutrition at the Center for Nuclear Energy in Agriculture (CENA), University of São Paulo, Campus Luiz de Queiroz, Piracicaba, São Paulo-Brazil. It was conducted in compliance with the standards of the ethics committee for the use of animals in experimentation CENA/USP (Protocol No. 008/2015).

After slaughter, pairs of ovaries ($n = 5$) were collected from Santa Inês ewes that were approximately 1-year old and in different stages of the estrous cycle. The ovaries were washed in 70% alcohol and subsequently in Minimum Essential Medium Eagle with HEPES and sodium bicarbonate (MEM-M7278, Sigma-Aldrich, Brazil) and were placed in a solution as previously described by Jimenez et al., (2016aJimenez et al., 2016b that contained 25 μg/mL pyruvic acid, 75 μg/mL penicillin and 50 μg/mL streptomycin, referred to as transport medium.

Using sterile conditions, all ovary tissues and ligaments were separated in the laboratory. The ovarian cortex was divided into fine cortical tissue of approximately 9 mm^3. The tissues ($n = 45$) were individually cultured in a 24-well plate containing MEM (M7278, Sigma-Aldrich, Brazil), supplemented with 3 mM glutamine; ITS (10 μg/mL of insulin, 5.5 μg/mL of transferrin and 5 ng/ml of selenium), 0.1% bovine serum albumin (BSA); 0.1 mg/mL penicillin; and 0.1 mg/mL streptomycin, called MEM^+. The tissues were cultured in MEM^+ with different concentrations of gossypol acetic acid (G4382, Sigma-Aldrich, Brazil) (0, 5, 10, 20 μg/mL). The tissues were cultured for 24 and 96 h, at 37 °C, 5% CO_2 humidified air, and with total media replacement every 24 h. The tissues were analyzed at 0, 24 and 96 h for morphological, morphometric analyses (classical histology), ultrastructural (transmission electron microscopy) and abundance of α-ER (immunohistochemical).

2.2. Histological processing

After 4 h of fixation in Carnoy, the ovarian tissues were dehydrated with increasing alcoholic solutions (70%, 80%, 90% and absolute), diaphanized in xylol and paraffin embedded at 60 °C. Sections 5-μm thickness were obtained from the paraffin blocks, mounted on slides and stained with Periodic Acid-Shiff (PAS)-hematoxylin. The slides were evaluated using an optical microscope (Leica MLD7000, Wetzlar, Germany) coupled to a camera (Leica DFC310 FX, Wetzlar, Germany) using a magnification of 400 × .

For histological description, preantral follicles were classified as primordial and developing (primary and secondary). Primordial follicles were characterized by the presence of spherical or ovoid oocytes, completely surrounded by a simple granular layer or cells that had started to take on a cuboidal shape. In primary follicles, a single layer of cubic-shaped granulosa cells was observed around the oocyte and in secondary follicles, the oocyte was surrounded by two or more layers of cubic-shaped granulosa cells (Basso and Esper, 2002; Jimenez et al., 2016c). The follicles were classified morphologically as normal (containing an intact oocyte and granulosa cells well organized in layers, without a pyknotic nucleus) and degenerate (oocyte with a pyknotic nucleus, cytoplasmic shrinkage or disorganized granulosa cells) (Jimenez et al., 2016c, 2018). For the morphometric analysis, histological fields were

obtained using an optical microscope (400X; Leica MLD7000, Wetzlar, Germany), and five normal follicles were observed by repetition. The diameters were calculated using an Image J/Fiji 1.46 program and each vertical and horizontal region of the follicle and oocyte was analyzed (Jimenez et al., 2018).

2.3. Transmission electron microscopy analysis

Tissues of approximately 1 mm^3 were fixed in Karnovsky solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2) for 4 h at room temperature. The tissues were then stored in a refrigerator in 0.1 M cacodylate buffer until the analysis. Subsequently, three washes were performed in the 0.05 M sodium cacodylate buffer, pH 7.2. Post-fixation procedures were performed in 1% osmium tetroxide in 0.1 M cacodylate buffer pH 7.2, for 2 h at 4 °C. Ovarian tissues were stored overnight in 0.5% uranyl acetate at 4 °C. Dehydration was then conducted with increasing solutions of acetone (30%, 50%, 70% and 90% for 15 min and three times with 100% acetone for 10 min). Tissues were subsequently infiltrated with Resin Epon-812. Semi-thin sections (three μm) and ultrafine sections (70 nm) were obtained using ultramicrotomy (Leica Ultracult UCT, Wetzlar, Germany) and harvested in copper grids (300 μm mesh), stained with uranyl acetate and lead citrate and examined using transmission electron microscopy (Jeol ge 1011, Akishima, Tokyo, Japan). Characteristics were evaluated of the nucleus, oocyte, granulosa cells, mitochondria, endoplasmic reticulum, Golgi apparatus, cytoplasmic and basal membrane, pellucida zone, transport or secretory vesicles, lysosomes and peroxisomes in different follicular electro-micrographs ($n = 5$) (Jimenez et al., 2018).

2.4. Immunohistochemical analysis of α-ER

The presence of α-ER in sheep preantral follicles was evaluated using the antibody Alpha/Estrogen Receptor (Tomanek et al., 1997; Juengel et al., 2006; anti-ER; dilution 1:50, C88670, LSBio®). Histological paraffin sections were deparaffinized and submitted to antigenic recovery in 10 mM sodium citrate/pH 6.0 in a water bath (steamed) for 45 min at 100 °C. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide (H_2O_2) in methanol for 10 min. To block non-specific binding, the samples were incubated in the blocker (0.1% normal goat serum in PBS, 0.01% Tween) for 2 h. Shortly after, the sections were incubated with primary anti-ER antibody (C88670, LSBioÂ®) in a humidified chamber at 4 °C overnight. Slides were then washed in PBS and incubated with secondary antibody (Vectastain ABC kit Rabbit, PK-4001) for 30 min. The reaction was assessed by the addition of diaminobenzidine (DAB-SK 4100) for 3 min. The slides were stained with Harris hematoxylin for 30 s, dehydrated in increasing concentrations of alcohols, immersed in xylene, mounted on plates and analyzed using an optical microscope (Leica MLD7000, Wetzlar, Germany). Immunoreactivity was considered positive when brown staining was detected in preantral follicles and, according to estrogen receptor tone (light, medium and strong), its cellular activity was verified in the oocyte and granulosa cells.

2.5. Statistical analyses

The percentages of normal, primordial and developing preantral follicles (primary and secondary), as well as follicular and oocyte diameters, were subjected to the analysis of variance (ANOVA) and the means were compared using the Dunnett test (control compared with treatment groups) and Tukey test (between treatments), using SAS software (version 9.1, 2004).

3. Results

Uncultured follicles had a greater percentage of normal tissue than follicles cultured with 5, 10 and 20 μg/mL of gossypol for 24 h and follicles cultured with 10 and 20 μg/mL of gossypol for 96 h, ($P < 0.5$; Table 1). Between cultured treatment groups after 24 h, follicles cultured with 0, 10 and 20 μg/mL gossypol had similar morphological characteristics as follicles not treated with gossypol, but follicles cultured in 5 μg/mL gossypol had abnormal characteristics ($P < 0.05$; Table 1). The treatment of ovarian tissues with

Table 1
Percentage (mean ± standard error) of normal, primordial and developmental preantral follicles of uncultured (control) and cultured ovarian tissues with addition of 0, 5, 10 and 20 μg / mL of gossypol for 24 and 96 h.

Control	Hours	Treatments			
		0 ug/mL	5 ug/mL	10 ug/mL	20 ug/mL
70.67 ± 4.6	24h	62.00 ± 7.0^{a}	33.33 ± 2.1^{*Bb}	40.00 ± 2.8^{*ab}	38.00 ± 11.0^{*ab}
	96h	66.00 ± 8.5^{a}	55.33 ± 7.5^{Aab}	41.33 ± 2.3^{*b}	46.66 ± 1.7^{*ab}
68.00 ± 4.6	24h	46.00 ± 8.7	28.66 ± 2.1^{*B}	30.66 ± 1.5*	36.66 ± 9,9*
	96h	49.33 ± 9.0	42.66 ± 4.9^{*A}	33.33 ± 1.4*	36.00 ± 2.0*
2.67 ± 1.8	24h	16.00 ± 4.5^{a*}	4.66 ± 2.1^{b}	9.33 ± 1.8^{ab}	1.33 ± 1.2^{Bb}
	96h	16.66 ± 4.2	12.67 ± 6.0	8.00 ± 1.2	10.66 ± 1.5^{A}

*Difference for treatment-control ($P < 0.05$); a,b Superscript lowercase letters indicate difference between treatments ($P < 0.05$); A,B Superscript uppercase letters in the same column are different when there were differing durations of culturing ($P < 0.05$), gossypol doses expressed in (μg/mL).

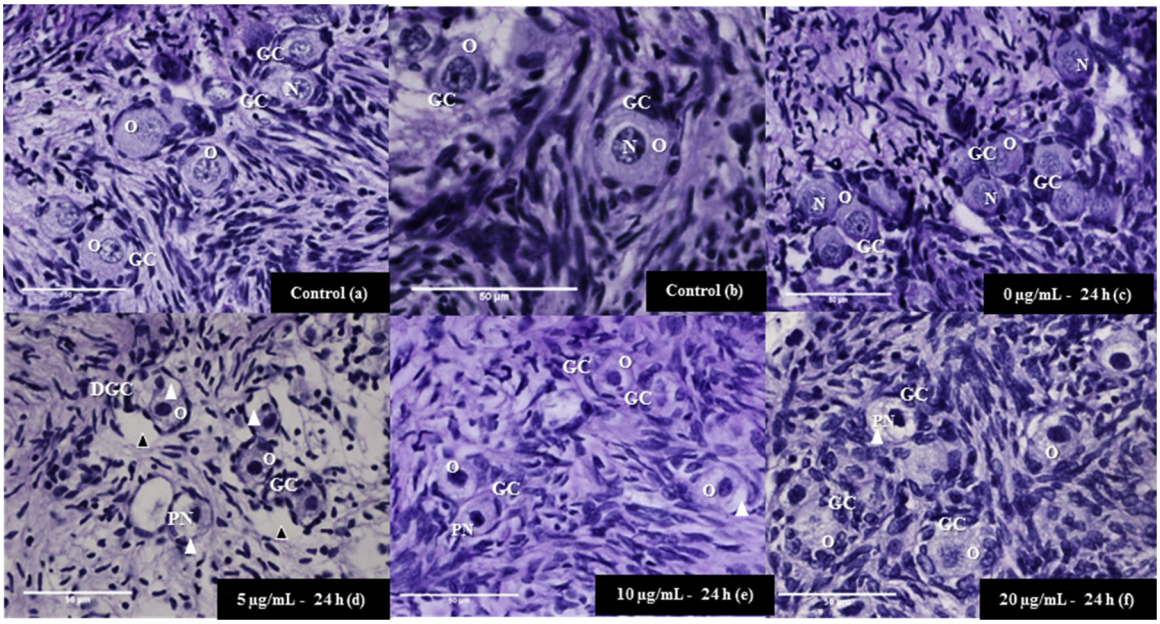

Fig. 1. Histological sections of uncultured (control, a, b) and cultured primordial follicles in MEM + with or without gossypol (0, 5, 10, 20 µg/ml); (c) 0 µg/ml; (d) 5 µg/ml; (e) 10 µg/ml and (f) 20 µg/ml of gossypol for 24 h. Shrinkage of oocytes (white arrow), retraction of granulosa cells (black arrow), disorganized granulosa cells (DGC), picnotic nucleus (PN); O = oocyte; N = nucleus; GC = granulosa cells; Magnification 400x, PAS-hematoxylin.

5 µg/mL of gossypol, however, resulted in an enhanced development of normal follicles between 24 and 96 h of culture ($P < 0.05$), while the other treatments (0, 10, and 20 µg/mL of gossypol) had no effect on follicle structure until 96 h of culture ($P > 0.05$).

Tissues cultured for 24 and 96 h in gossypol (5, 10 and 20 µg/mL) had fewer primordial follicles than uncultured control tissues ($P < 0.05$). In cultured tissues, the number of primordial follicles did not differ among treatment groups after 24 or 96 h of culture ($P > 0.05$). Ovarian tissues cultured for 24 h in absence of gossypol (0 µg/mL) had more developing follicles than uncultured control tissues ($P < 0.05$). The culturing of tissues with 20 µg/mL of gossypol was the only treatment that increased the follicular development rate between 24 and 96 h of culture ($P < 0.05$; Table 1).

The morphological evaluation of preantral follicles in uncultured tissues indicated there was a large population of normal primordial follicles and few developing follicles. There, however, were degenerate follicles with pyknotic oocytes and disorganized granulosa cells also observed (Fig. 1). Cultured tissues had a sustained follicular structural normality during culture (24–96 h). With all treatments (0, 5, 10 and 20 µg/mL of gossypol) there was a shrinkage of the oocyte and disorganization of granulosa cells (Figs. 1 and 2). In addition, in tissues with developing follicles there were few oocyte defects with oocytes being rounded with a pellucida zone when all treatments were imposed for 96 h (Fig. 3).

Follicular and oocyte measurements did not differ in uncultured tissues and those where there was culturing with 0, 5, 10 and 20 µg/mL gossypol for 24 and 96 h ($P > 0.05$; Table 2).

Using the immunohistochemical analysis of α-ER, different intensities of immunostaining (light, medium and strong brown) were detected, indicating that α-ER were present in oocytes, nuclei and granulosa cells of different follicular types, such as those in the primordial and developing stages (primary and secondary).

The α-ER in the uncultured control (Figs. 4a and 4b) stained with a light brown color when present in oocytes from primordial, primary and secondary follicles and there was variability of staining intensity in granulosa cells with there being an absence and presence of medium intensity

Staining of α–ER. In secondary follicles, greater immunostaining was observed at the boundary of granulosa cells where theca cells were beginning to form.

After 24 h of *in vitro* culture, ovarian tissues cultured with 0, 5, 10 and 20 µg/mL of gossypol had an increased intensity of α-ER (media) staining in the oocytes as compared with those in the uncultured control. The ovarian tissues that were not treated with gossypol (0 µg/mL) had granulosa cells with the same characteristics as those of the uncultured control, whereas the tissues treated with gossypol (5, 10 and 20 µg/mL gossypol) had granulosa cells with moderate immunolabeling or without immunolabeling. There, however, was an intense immunoreactive signal in oocyte nuclei (Figs. 4c-f).

After 96 h of *in vitro* culture, intensities of oocyte immunolabeling varied for the treatment groups, from light, medium and strong intensity for treatments with 0 µg/mL, 5 and 10 µg/mL and 20 µg/mL gossypol, respectively. The nuclei were immunoreactive in all treatment groups cultured in gossypol; however, the treatment with 20 µg/mL of gossypol had a greater amount of immunoreactivity in the nucleolus and a greater abundance of granulosa cells immunolabelled with α-ER in relation to treatments with 5 and 10 µg/mL of gossypol that had granulosa cells with very little immunolabeling or without immunostaining being evident (Fig. 5).

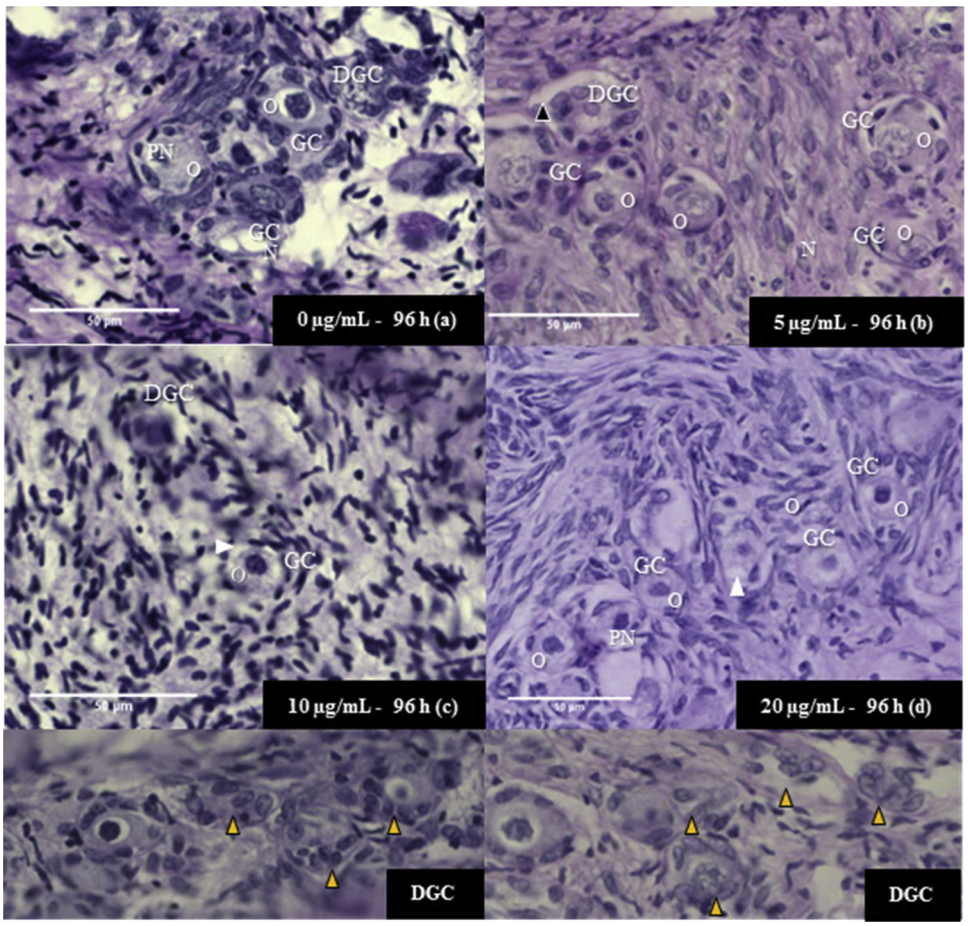

Fig. 2. Histological sections of primordial follicles cultured *in vitro* in MEM + with or without gossypol (0, 5, 10, 20 µg/ml); (a) 0 µg/ml; (b) 5 µg/ml; (c) 10 µg/ml and (d) 20 µg/ml of gossypol for 96 h; Shrinkage of oocytes (white arrow), shrinkage of granulosa cells (black arrow), disorganized granulosa cells (DGC, yellow arrow), pycnotic nucleus (PN), O = oocyte; N = nucleus; GC = granulosa cells; Magnification 400x, PAS-hematoxylin **(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)**.

The ultrastructural analysis of ovarian tissues indicated there were granulosa cells with characteristic morphological structure, cylindrical oocytes, eurochromatin and heterochromatin dispersed in the nucleus and well-demarcated nuclear and cytoplasmic membranes in the uncultured control ovarian tissues (Fig. 6). Several cytoplasmic organelles were also observed, such as mitochondria, ribosomes, Golgi apparatus and secretory vesicles. With ultrastructural ovarian tissue analyses, it was obvious treated with 0, 5, and 10 µg/mL of gossypol induced degeneration of the structures analyzed in this study. There were shrunken oocytes, pyknotic nuclei, and/or disorganized granulosa cells, fewer organelles and greater vacuolization (Figs. 6b, 6c, and 6d). Ovarian tissues cultured for 96 h with 20 µg/mL of gossypol had a greater presence of heterochromatin and normal eurochromatin, cylindrical oocytes, numbers of secretory vesicles, Golgi apparatuses, ribosomes, smooth and rough endoplasmic reticulum, transport vesicles, lipid droplets, mitochondria, peroxisomes, normal nuclear and cytoplasmic lysosomes, In ovarian tissues treated with 0, 5, 10 µg/mL of gossypol, ultra-follicular degeneration was more evident. With these treatments, there was a greater presence of shrunken oocytes, pyknotic nuclei, and/or disorganized granulosa cells, decreased numbers of organelles and marked vacuolization were observed (Fig. 6 b–d). Ovarian tissues cultured for 96 h with 20 µg/mL of gossypol had a greater presence of heterochromatin and normal eurochromatin, cylindrical oocytes, secretory vesicles, Golgi apparatuses, ribosomes, smooth and rough endoplasmic reticulum, transport vesicles, lipid droplets, mitochondria, peroxisomes, normal nuclear and cytoplasmic lysosomes. The absence and degeneration were observed in granulosa cells where there was membrane rupture, abnormal morphological structures and dark pigmentation indicating that cellular pyknosis was occurring (Fig. 6 e–i).

4. Discussion

Gossypol is a promising bioactive compound due to its broad biological activities, such as antiviral, antifungal, antimicrobial (Kovacic, 2003) and is also promising in the treatment of leukemia (Balakrishnan et al., 2008), lymphoma (Johnson et al., 2009) and as a human contraceptive (Coutinho, 2002). This phenolic compound causes temporary sterility in cattle (Hassan et al., 2004), goats

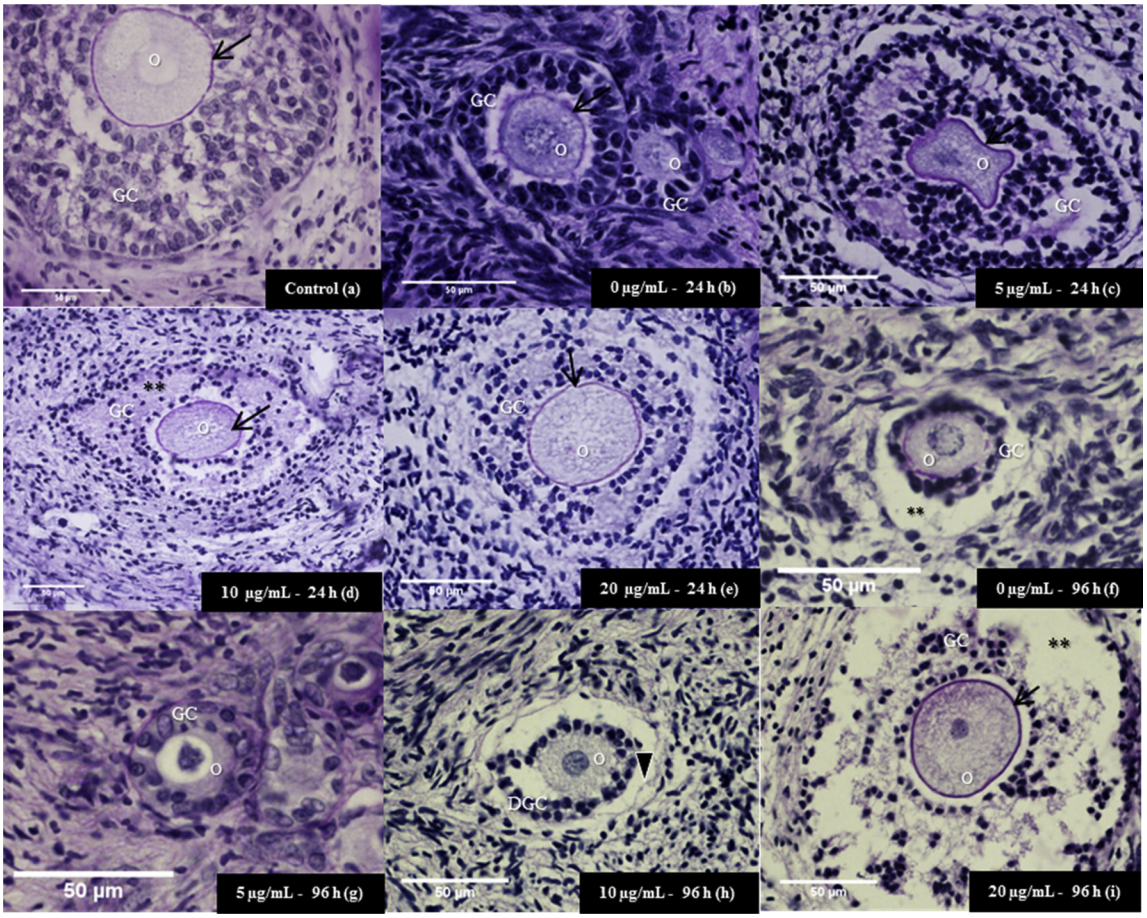

Fig. 3. Histological sections of non-cultured (control, secondary; a) and cultured developing follicles that were cultured for 24 and 96 h in MEM + with or without gossypol (0.5, 10, 20 µg/ml). (b) 0 µg/ml - 24 h; secondary, (c) 5 µg/ml - 24 h; secondary, (d); 10 µg/ml – 24 h; secondary and (e) 20 µg/ml - 24 h; secondary, (f) 0 µg/ml - 96 h; primary, (g) 5 µg/ml - 96 h; primary, (h); 10 µg/ml - 96 h; primary and (e) 20 µg/ml - 96 h; secondary, O = oocyte; GC = granulosa cells, DGC = disorganized granulosa cells, **absence of GC in the histological sectioned layer; Magnification 400x, PAS-hematoxylin.

Table 2
Follicular and oocyte diameter (µm) of uncultured (control) and ovarian tissues that were cultured in 0, 5, 10 and 20 µg/ml in gossypol for 24 and 96 h.

Hours	Treatments▲				
	Uncultured control	0 ug/mL	5 ug/mL	10 ug/mL	20 ug/mL
24h	33.08 ± 0.001	33.64 ± 0.003	40.92 ± 0.006	38.55 ± 0.006	42.12 ± 0.006
96h		29.78 ± 0.001	39.98 ± 0.008	32.57 ± 0.003	42.13 ± 0.007
24h	20.61 ± 0.001	19.81 ± 0.001	22.05 ± 0.002	21.14 ± 0.002	23.01 ± 0.002
96h		18.72 ± 0.001	21.01 ± 0.002	20.00 ± 0.001	20.13 ± 0.001

▲No difference for treatment-control ($P > 0.05$).

(Nunes et al., 2010), sheep (Paim et al., 2016) and some other animals. Ingestion of gossypol by animals has also been associated with estrous cycle disruption (Gadelha et al., 2014), steroidogenesis, nuclear maturation, follicular size (Randel et al., 1996) and oocyte and embryonic toxicity effects (Hernandez-Cerón et al., 2005) in cattle. In addition, Ueno et al. (1988) speculated that gossypol may inhibit a variety of enzymes, including adenylate cyclase, protein kinase DNA polymerase, topoisomerase, malate dehydrogenase, and enzymes involved in ion transport, metabolism nucleotides and steroidogenesis.

The present study was conducted to evaluate the effect of gossypol on granulosa cells and oocytes in ovarian tissues. There was normal follicle development sustained in the cultured ovarian tissues of ewes treated with 0, 5, 10 and 20 µg/mL of gossypol for 24–96 h. Furthermore, when follicular and oocyte morphometry assessments were performed, no differences were observed among treatments. Thus, preantral follicle development was normal in cultured tissues of ewes with inclusion of gossypol in the culture

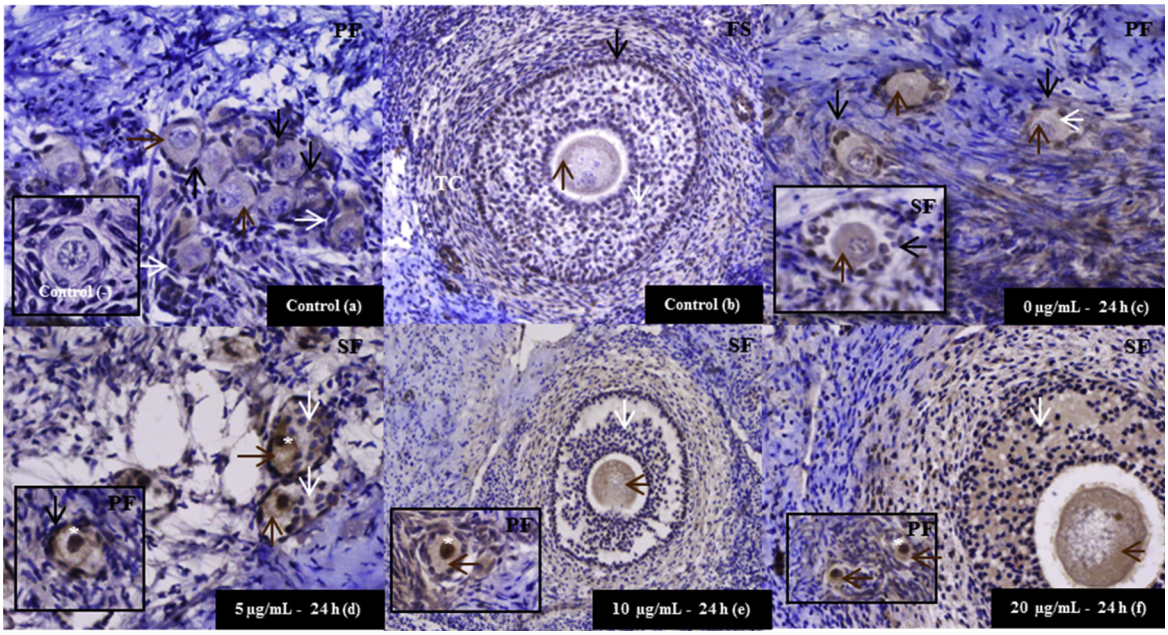

Fig. 4. Immunostaining for estradiol receptors (α-ER) in preantral follicles of ovarian tissues from ewes Non-cultured treatment (control; a) and cultured *in vitro* in MEM + with or without gossypol for 24 h (c) 0 µg/ml; (d) 5 µg/ml; (and); 10 µg/ml and (f) 20 µg/ml; Positive (black arrow) and negative (white arrow) immureaction in the granulosa cells; Positive immunoreaction in the nucleus (white asterisks); PF: primordial follicle, SF: secondary follicle.

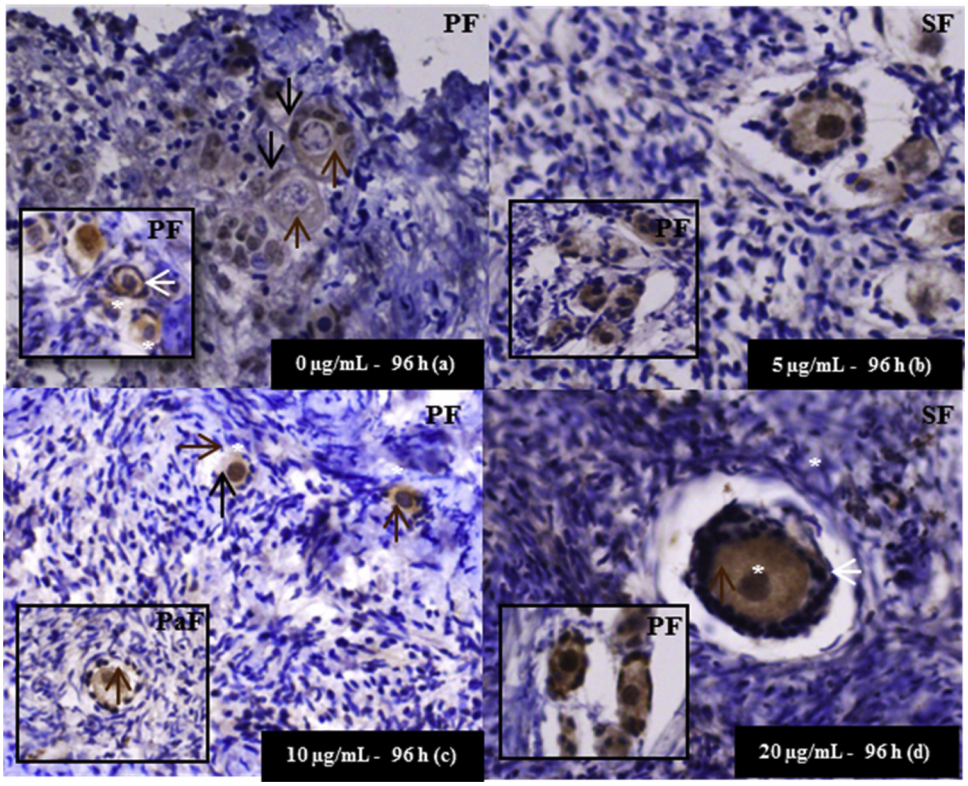

Fig. 5. Immunostaining for estradiol receptors (α-ER) in preantral follicles of ovary tissues from ewes; Non-cultured treatment (control, a, b) and cultured in MEM + with or without gossypol for 96 h (c) 0 µg/ml; (d) 5 µg/ml; (and); 10 µg/ml and (f) 20 µg/ml; Positive (black arrow) and negative (white arrow) immureaction in the granulosa cells; PF: primary follicle, SF: secondary follicle.

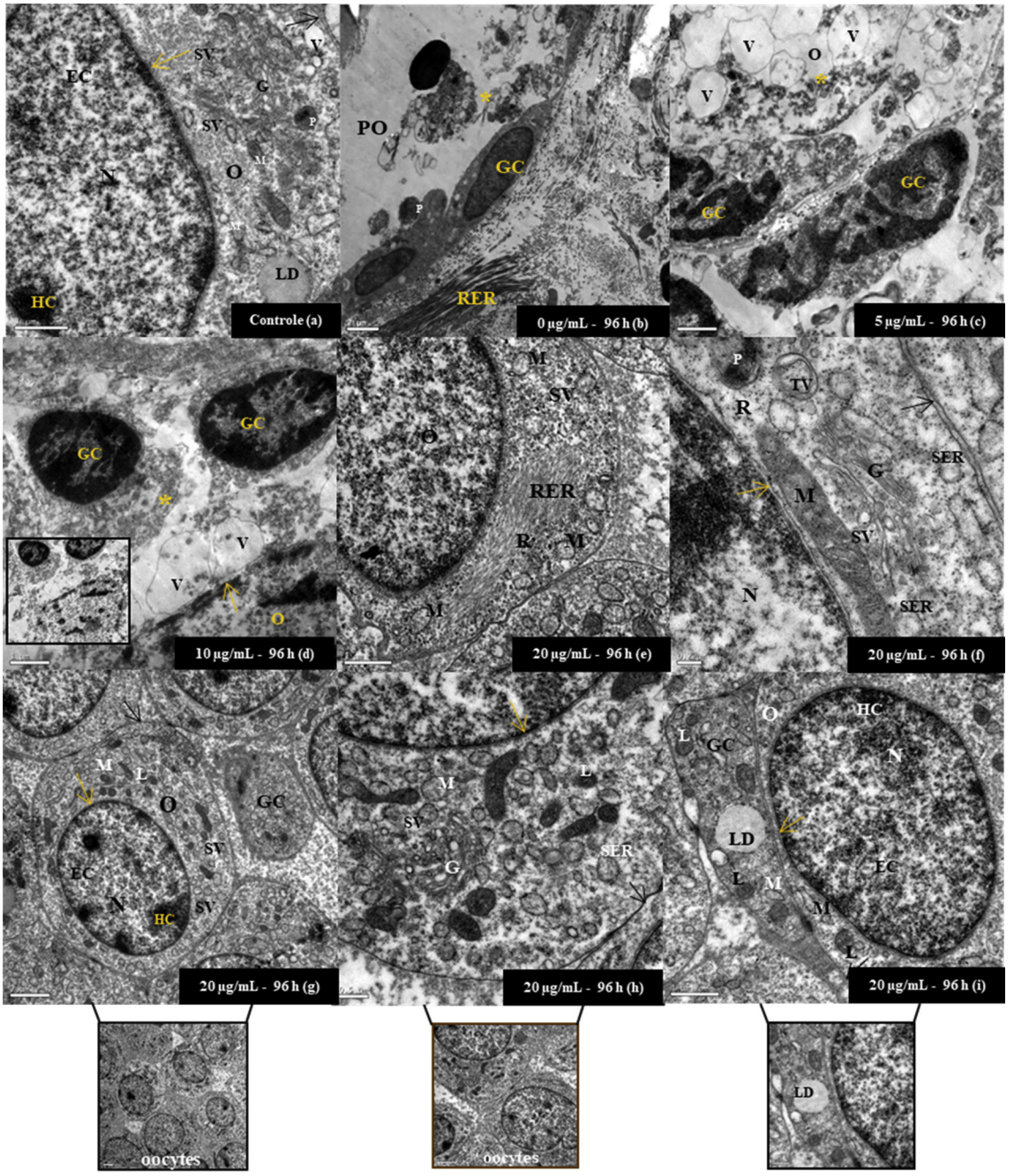

Fig. 6. Electromicography of uncultured (control; a) and preantral follicles that were cultured for 96 h in MEM + plus (b) 0 µg/ml; (c) 5 µg/ml; (d) 10 µg/ml and (e–i) 20 µg/ml of gossypol; HC = heterochromatin; EC = eurochromatin; N = nucleus; O = oocyte; PO = pycnotic oocyte; GC granulosa cells; DGC = degenerate granulosa cells; nuclear membrane (yellow arrow); cytoplasmic membrane (black arrow), cytoplasmic degeneration (asterisk); TV = transfer vesicles; SV = secretory vesicle; M = mitochondria; SER = smooth endoplasmic reticulum; RER = rough endoplasmic reticulum; LD = lipid droplet; V = vacuoles; PN = pycnotic nucleus; DC = degenerating cytoplasm, L: lysosomes, P: peroxisomes, R: Ribosomes (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

media for 96 h. This is the first study where there was development of preantral follicles in culture medium containing gossypol. Most studies using the technique (MOEPF) focused on studying follicular development o, but this biotechnology is also used to test bioproducts that influence the ovary reproductive functions of animals of zootechnical interest.

Considering the effects of gossypol on follicular degeneration, results of the present study revealed that, morphologically, the primordial follicles had defects in the oocytes and granulosa cells; however, developing follicles were affected to a greater extent by granulosa cells having little proliferation and being disorganized in structural morphology. Villaseñor et al. (2008) observed that

when one-cell embryos were cultured in medium containing gossypol, the cleavage rate did not change, but the percentages of one-cell embryos that developed for blastocysts after 8 d were markedly reduced when cultured in a medium with 10 μg/mL gossypol. It was concluded that oocytes are less susceptible to gossypol action and its primary effect is on proliferating cells.

Leblanc et al. (2002) studied cleavage rates of embryos when the spermatozoa that were used for fertilization were treated with different gossypol (0, 10 and 50 μg/mL) and the results indicated that treatments with or without gossypol did not affect cleavage rates. In the same study, there was evaluation of the *in vitro* maturation of oocytes treated with 0, 2.5, 5 and 10 μg/mL of gossypol and it was concluded that gossypol did not affect cleavage rates of embryos. In contrast, the addition of 10 μg/mL of gossypol to embryos reduced development rate of embryos into blastocysts.

In the present study, immunostaining results indicated that the larger the dose of gossypol, the greater the abundance of cytoplasmic and nuclear α-ER compared with that in uncultured control and cultured ovarian tissues where there was no gossypol treatment. In addition, immunostaining in granulosa cells was also greater in the gossypol-treated tissues as compared with the control tissues. The results indicated that the suppression of granulosa cell proliferation may be affected by gossypol in a dose-dependent fashion. Basini et al. (2009) tested different gossypol doses in culture of granulosa cells and concluded that gossypol stimulates proliferation, but it inhibits steroidogenesis, suggesting that this substance could affect fertility in pigs. Akira et al. (1994) suggested that gossypol reduces estradiol production by inhibiting aromatase induction by FSH. Similar data indicate that the production of 17β-estradiol was inhibited in granulosa cell cultures of swine when there was treatment with gossypol (Ohmura, 1996. Lin et al. (1994) provided evidence that *in vitro* culture with gossypol affects the steroidogenic pathway in granulosa cells of cattle. Thus, in general, this research indicates a similar pattern of gossypol effect on granulosa cells. Thus, regardless of the ovary follicular category, gossypol directly affected granulosa cells or subsequent development of luteal cells.

The results of the ultrastructural analysis in the present study confirmed oocyte viability was enhanced relative to gossypol dose; however, there was a decrease in granulosa cell proliferation in all gossypol treatment groups. Moh et al. (1992) reported that gossypol binds to the endoplasmic reticulum and mitochondria and, to a lesser extent, to peroxisomes and plasma membranes. Peroxisomes were in lesser abundance when there was gossypol treatments in smaller amounts in the present study. The role of these organelles in cell metabolism is still poorly understood; nevertheless, it is believed that peroxisomes participate in cell detoxification processes (Del Río and López-Huertas, 2016). The results of Moh et al. (1992) also indicate that gossypol binds covalently to microsomal proteins, possibly influencing the metabolism of sterols, steroids or fatty acids. The action of gossypol has usually been associated with membrane fluidity (Wu et al., 1991), which explains the great variability of its inhibitory effects and the greater effects on cell membranes in proliferating than in quiescent cells.

In the present study, the culture of oocytes included in preantral ovary follicles in the presence of gossypol did not affect the morphology or morphometry of primary or developing follicles at any of the doses tested. Ultrastructural and immunohistochemical evaluation, however, indicated granulosa cells and α-ER were affected indicating that gossypol affects ovarian function in ewes. The results of this study, therefore, indicate the toxic action of gossypol using a biotechnological approach (*i.e.*, MOEPF) and it is concluded that gossypol functions to damage the granulosa cells in the preantral follicles of sheep.

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Conflict of interest

There is no conflict of interest that could be compromise the impartiality of the research reported.

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