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Cottonseed (gossypol) intake during gestation and lactation does affect the ovarian population in ewes and lambs?

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ABSTRACT

The aim of this study was to evaluate if the cottonseed intake during gestation and lactation affects the ovarian population in ewes and lambs. Therefore, 39 ewes were evaluated during 10 months under two treatments: Cottonseed and soybeans. The quantification of ovarian follicular dynamics was analyzed by ultrasound and the determination of progesterone and estradiol levels was interpreted by radioimmunoassay. After weaning, ovaries of lambs (n = 10) were collected by ovariectomy and fixed for the assessment of follicular parameters as normality, classification, diameter, ultrastructure, stereology, and as well as immunoexpression of the α-estradiol receptor α (ER-α). The results showed that the cottonseed consumption altered neither the ovarian nor the hormonal follicular dynamics of Santa Inês ewes after calving and did not affect the normality, classification, diameter, stereology and follicular ultrastructure of offspring. Nevertheless, the offspring of ewes fed with cottonseed showed high ER-α immunoexpression in the ovarian structures. It is concluded that cottonseed did not affect the maternal-descendant follicular dynamics. However, lambs' ovaries had highest α-ER immunoexpression in oocytes, granulosa and theca cells and corpus luteum. This fact warns of a possible change in the future steroidogenic response of these lambs that had progenitors consuming cottonseed in their reproductive period.

1. Introduction

Cottonseed is an alternative source of protein, fiber and energy (Bellows et al., 2001). It is not used for human consumption, but is used in animal feed with a price that is advantageous in comparison with other protein sources, such as soybeans (Louvandini et al., 2019). However, cottonseed Improperly supplied can present adverse reactions in the respiratory, hepatic, circulatory, renal and reproductive systems by the presence of gossypol anti-nutritional factor (EFSA, 2018).

The reproductive damage causes serious economic loss for the breeder. Effects of anti-fertility have been reported in the presence of gossypol depending on the dose and time of exposition, inhibiting the spermatogenesis and decreasing the mortality and viability as well as provoking morphologic damage in males bovines (Hassan et al., 2004; Randel et al., 1992); rodents (Deoras et al., 1997; El-Sharaky et al., 2010; Fonseca et al., 2013); goats (East et al., 1994; Nunes et al., 2010) and ewes (Braga et al., 2012; Guedes and Soto-Blanco, 2010; Paim et al., 2019), among others.

On females, gossypol has been associated with the interference in the estrous cycle in bovines (Gadelha et al., 2014; Gadelha et al., 2011; Villaseñor et al., 2008) and rodents (Lin et al., 1985) such as in the steroidogenesis, nuclear maturation and follicular size in bovines (Gu et al., 1990; Lin et al., 1994; Randel et al., 1996).

As for the embryogenesis gossypol may reach the uterine fluids through maternal circulation, showing a higher number of degenerated bovine embryos number interfering with embryo implantation (Lin et al., 1991) and reducing birth weight of rats (Sein and Phil, 1986). In *in vitro* assays with o gossypol were observed damage secondary oocytes, and bovine embryos (Brocas et al., 1997; Hernández-Cerón et al., 2005). Differently, Jimenez et al. (2019) did not find morphological damages in preantral follicles *in vitro* cultured at different doses of gossypol but they found an increase in the production of estradiol-α receptors in the

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highest dose, relating it to intra-follicular steroidogenic activity.

Dabrowski et al. (2000) stated that the effect of gossypol upon fertility is related to the efficiency of gossypol to cross the gonadal-circulation barrier, depending on the permeability of the reproductive organs of each species, age and metabolic rate. Although these reproductive problems in the male bovines are broadly researched, considering problems in female, there is a lack of the reproductive damage caused by gossypol in female ovines and their descendants. The aim of this study was to evaluate the follicular development and postpartum hormonal concentrations of female ovines supplemented with cottonseed during the reproductive period; we also searched to assess the morphology, morphometry, follicular stereology and α-ER of ovarian follicles of female ovine descendants fed on cottonseed.

2. Materials and methods

The experiment was conducted at the Laboratory of Animal Nutrition (LANA) at the Center for Nuclear Energy in Agriculture (CENA), Campus Luiz de Queiroz, University of São Paulo (USP). This study was approved by the ethics committee for the use of animals in experimentation CENA/USP (008/2015).

Thirty-nine Santa Inês ewes were used, in good sanitary and general clinical condition (43.2 ± 2.0 kg of body weight) and (3.0 ± 0.1 of body score). The diets were elaborated according to the NRC (NRC, 2007), in order to meet the requirements of protein and metabolizable energy for the maintenance and reproduction with 14% CP and 7% EE. The difference between the two treatments was that one used soybeans (SB, $n = 21$) and another cottonseed (CS, $n = 18$). The composition was prepared according to Louvandini et al., 2019 and Moretti et al., 2019 (Table 1).

To inhibit the antinutritional factors the soybean grains seed was submitted in a drying oven (FANEM LTDA) to 90 °C for 1h30m based on studies by Monteiro et al. (2010) and Carvalho et al. (2013). The cottonseed used was with lint (with more fiber) and without any additional inactivation process. The concentrate consume was individual, carried during 10 months which contemplated pre-breeding season, breeding season, pregnancy, and lactation. The two experimental treatments were kept in pastures of *Panicum maximum cv aruana,* supplemented with hay of cost cross, mineral salt, and water *ad libitum.* The bromatological analysis of organic matter (OM), DM, CP, EE, NDF, ADF and mineral matter (MM) were carried monthly in the pasture, hay and concentrate, according to AOAC (2011).

2.1. Ultrasonography (follicular dynamics)

The *in vivo* ovarian ultrasonographic images were obtained with the CHISON® D-600VET ultrasound equipment and linear transducer (rectal probe) of 5 MHz frequency. Ultrasonography monitoring was performed every three days after 15 days postpartum. We evaluated: Total follicles, small follicles (<3.0 mm), medium follicles (3.1–5.9 mm) and large follicles (> 6.0 mm).

2.2. Hormonal dosage

For the progesterone and estradiol assays, radioimmunoassay techniques were used - RIA Kit[1](Beckman Coulter-ImmuChemTM Coated Tube, California, USA). Serum samples were collected every three days, along with ultrasound examination.

2.3. Evaluation of offspring ovarian development

After 60 days postpartum, lambs born to progenitors fed with CS ($n = 5$) and SB (n = 5) were selected. Lambs were not fed CS or SB. The lambs were breastfeeding and had creep-feeding of crushed corn and soybean meal in the proportion of 70:30 respectively. Lambs were submitted to laparotomy (unilateral ovariectomy). In order to perform the procedure xylazine, diazepam and ketamine were used as an anesthetic protocol. After the procedure, daily cleaning of the local was carried with chlorpyrifos, dichlorvos, and gentian violet. After a full recovery, the animals returned to the pickets with the rest of the herd.

The ovaries collected were washed in 70% ethanol and sodium chloride (0.9% NaCl). In the laboratory, the ovaries were divided and immediately fixed for morphological, morphometric, follicular stereology (histology) and presence of ER-α (immunoexpression analysis).

All pieces were fixed in Carnoy solution for 12 h, dehydrated in increasing concentrations of ethanol, diaphonized in xylene and embedded in paraffin (Synth, São Paulo, Brazil). Afterwards, we cut the ovine tissue pieces into 5-μm sections, each section was mounted on a glass slide and stained with periodic acid-Schiff and hematoxylin. The analysis of the ovarian sections employed a light microscope (Leica MLD7000, Wetzlar, Germany), with a camera (Leica DFC310 FX, Wetzlar, Germany) 10× and 40× objective. The classification of the ovarian follicles at different maturation stages according to Gartner & Hiatt (Gartner and Hiatt, 2003), Jimenez et al. (2016a), and Basso and Esper (2002). We described preantral follicles as:

- *Primordial follicles:* spherical or ovoid oocytes surrounded by a single layer of flattened granulosa cells (GCs). Some of them tended to cuboid-shaped;
- *Primary follicles*: spherical oocytes with a single layer of cuboid-shaped GCs;
- *Secondary follicles:* spherical oocytes with two or more layers of cuboid-shaped GCs;
- *Antral follicles:* with the antrum formation, being observed the formation of cumulus oophorus and corona radiata, and total development of external and internal theca cells.

For the qualitative analysis, we counted and classified as normal and degenerate follicles. Normal follicles were those with complete basement membrane, absence of pyknotic nuclei and without signs of oocyte and GCs degeneration. Degenerate follicles were those with the layers of granulosa and theca cell disorganized or hypertrophied and the oocyte in degeneration process (Jimenez et al., 2016b; Jimenez et al., 2016c).

2.4. Follicular dimensions

Follicle and oocyte diameters were analyzed in each vertical and horizontal region. The histological fields were captured on the light microscope (10× and 40× objective, Leica MLD7000, Wetzlar, Germany). The diameters were calculated with the Image J/Fiji 1.46 program (Ferreira and Rasband, 2012).

2.5. Quantitative See: Beckman Coulter direction insert: evaluation (follicular stereology)

In order to estimate the follicular stereology, we only counted the follicles whose oocyte nuclei were clearly visible in the sections studied. The number of total ovarian follicles was estimated according to Gougeon & Chainy (Gougeon and Chainy, 1987), from the correction factor below:

$$FP = \frac{FN \times NS \times CT}{NSO \times ø\ NOoc}$$

Where:PF = Follicular Population;FN = Follicle number (primordial, primary, secondary and antral counted in the ovary;NS = total number of sections;CT = cut thickness;NSO = number of sections observed;ø NOoc = mean diameter of oocyte nucleus (primordial, primary, secondary and antral).

[1] See: Beckman Coulter Direction insert: https://www.beckmancoulter.com/products/immunoassay (accessed 20 April 2020)

Table 1
Quantity offered and chemical composition of forage and concentrates.

Chemical Composition	DM	OM	CP	NDF	ADF	LIG	EE	MM	Gossipol Free
Hay	90.0	94.0	6.5	80.9	44.9	9.0	1.7	5.9	–
Forage	92.5	90.0	17.0	67.1	38.0	6.8	1.9	10.1	–
CO	93.2	96.1	24.5	47.7	12.9	9.4	19.3	3.9	–
CA|	94.0	96.4	24.9	58.6	42.6	15.8	21.0	3.6	300–360 mg/kg*

Hay and Forage *ad libitum*, CO and CA treatments Offered (500 to 600 g/kg). of organic matter (OM), dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin (LIG), ethereal extract (EE) and mineral matter (MM). CO - soybean concentrate; CA - cottonseed concentrate; *Free gossypol was determined according to the methodology adapted from (Botsoglou, 1991)

2.6. Immunoexpression analysis of α-ER

The immunoexpression analysis was evaluated according to Jimenez et al. (2019) We studied the presence of α-ER in ovine preantral follicles using the antibody Alpha / Estrogen Receptor (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 water bath (steamed) for 45 min at 100 °C. We blocked endogenous peroxidase activity using 3% hydrogen peroxide (H_2O_2) in methanol for 10 mins. In order to block non-specific binding, the samples were incubated to block (0.1% normal goat serum in PBS, 0.01% Tween) for 2 h. Subsequently, we incubated the cuts with primary anti-ER antibody (C88670, LSBioÂ®) in a humid chamber at 4 °C overnight. Slides were then washed in PBS and incubated with secondary antibody (Vectastain ABC kit Rabbit, PK-4001)[2] for 30 min. The reaction revealed by the addition of the diaminobenzidine (DAB-SK 4100) for 3 min. We stained the slides with Harris hematoxylin for 30 s, dehydrated in increasing concentrations of alcohols, immersed in xylene, mounted and analyzed under an optical microscope (Leica MLD7000, Wetzlar, Germany).

The immunoexpression of α-ER was analyzed digitally in the Image-Pro 10.0.5 program, evaluating the level of total pixels and specific pixels of the immunoexpression for primordial, primary, secondary follicles and corpus luteum. Five photos of each follicular classification were quantified per animal. The descriptive Immunoexpression was considered as positive when brown staining was detected in the preantral follicles and according to estrogen receptor tone light (+), medium (++) and strong (+++). We verified its cellular activity in the oocyte, granulosa cells, theca cells and corpus luteum of lambs ovary fragments born to progenitors fed cottonseed (CS) and soybean (SB).

2.7. Transmission electron microscopy analysis

Fragments of approximately 1 mm^3 were fixed in Karnovsky's solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer) at pH 7.2 for 4 h in room temperature. Then, we reserved in refrigerator in 0.1 M cacodylate buffer for analysis. Subsequently, we made three washes in the 0.05 M sodium cacodylate buffer. The fixation was in 1% osmium tetroxide in 0.1 cacodylate buffer, at pH 7.2 M, for 2 h at 4 °C. For contrast, the ovarian fragments were left overnight in 0.5% uranyl acetate at a temperature of 4 °C. On the next day, the dehydration was carried in acetone 30, 50, 70, 90% for 15 min and three times with 100% acetone for 10 min. The fragments passed through the process of infiltration and embedding in Resin Epon-812. We performed in the ultramicrotomy (Leica Ultracult UCT, Wetzlar, Germany) semi-thin sections (3 μm) and ultra-thin sections (70 nm) collected on copper grids (300 mesh mM), stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (Jeol Ge 1011, Akishima, Tokyo, Japan). We analyzed different follicular electromicrographs of the follicles ($n = 5$), showing characteristics of nucleus, oocyte, granulosa cells, theca cells, mitochondria, endoplasmic reticulum, Golgi apparatus, cytoplasmic and basal membrane, zona pellucida, transport or secretion vesicles, lysosomes, and peroxisomes (Jimenez et al., 2018).

2.8. Statistical analyses

Statistical analysis was performed using SAS v. 9.1® software (SAS Institute Inc., Cary, North Carolina, USA).

The data were analyzed in a completely randomized design with measurements repeated over time (from 15 to 60 days pos partum) in the MIXED procedure. The fixed effects were dietary treatments (SB or CS), days of (follicular size, progesterone and estradiol hormones) with the random effect of the experimental unit (animal).

The percentages of preantral follicles morphologically normal, primordial, primary, secondary and tertiary as well as those with follicular and oocyte diameter, were submitted to ANOVA.

The Comparisons between the means were performed using Tukey's test at 5% probability. The graphics were generated with the SIGMA-PLOT v.10 software.

3. Results

3.1. Ovarian analysis of progenitors

The results showed that after 15 days, the ovarian dynamics of the progenitors were not affected among the treatments and no differences were observed in the total number of large, medium and small follicles ($P > 0.05$; Figs. 1, 2).

In the hormonal analysis, estradiol levels showed that after 21 days postpartum, ewes with CS treatment had higher levels of estradiol than did SB treatment, and maintained this levels until 30 days postpartum ($P < 0.05$). On the other days evaluated, there were no differences between treatments ($P > 0.05$). The estradiol peaks in the ewe fed with CS treatment were on days 24, 36 and 51 postpartum and in intervals of 12 and 15 days, respectively. The ewes with SB treatment had lower estradiol levels, with peaks on days 18, 36, and 54 postpartum and with regular intervals of 18 days (Fig. 2). The progesterone peaks of the CS treatment were more prominent than were ewes under the SB treatment. However, there were no differences between treatments or postpartum time ($P > 0.05$; Fig. 3).

3.2. Ovarian analysis of offspring

In the 39 ewes evaluated, 46 lambs were born: 15 females and 9 males (CS treatment) and 8 females and 14 males (SB treatment).

Primordial, primary, secondary, antral, and total follicles were analyzed, as well as diameter, ultrastructural and immunoexpression evaluation of estradiol-α (ERα) receptors of follicles of lambs. The results showed that the normality, classification and follicular diameter of the lambs were not affected by consumption of treatments (CS or SB) of the progenitors, and the follicular stereology remained within the average of the ovarian population for ewes ($P > 0.05$, Table 2, Figs. 4 and 5).

The immunoexpression of α-ER was reactive for several ovarian structures such as corpus luteum, granulosa cells, theca cells and oocyte.

[2] See: Beckman Coulter Direction insert: https://vectorlabs.com/vectastain-elite-abc-kit-rabbit-igg.html (accessed 20 April 2020)

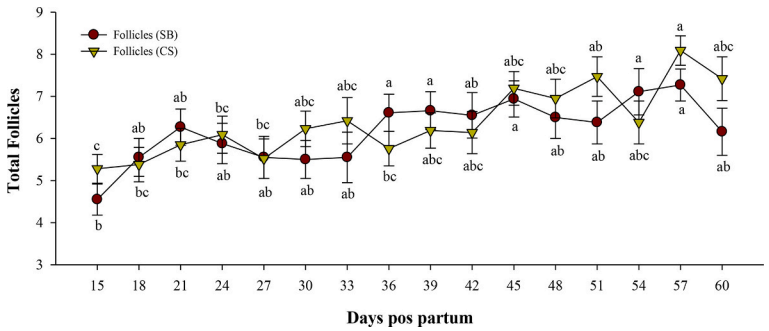

Fig. 1. Total number of ovarian follicles observed by ultrasonography in ewe fed with CS or SB after 15 days postpartum. a, b, c indicate the difference between the same treatment (P < 0.05); * There was no difference between treatments.

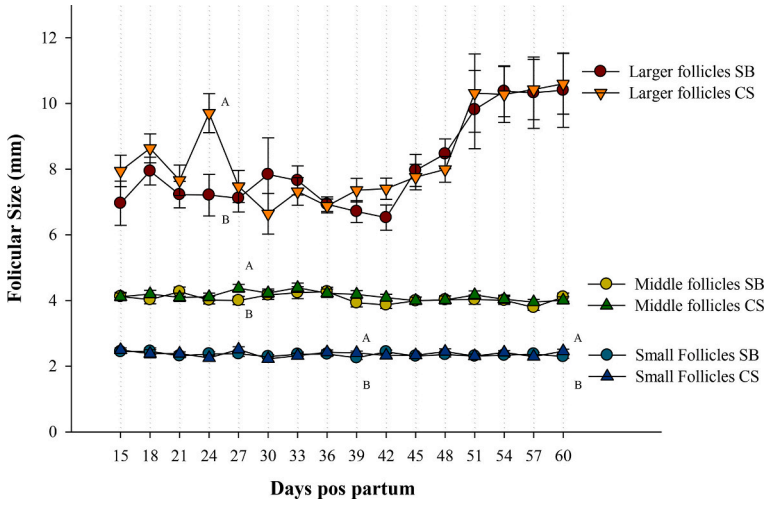

Fig. 2. Large, medium and small ovarian follicles observed by ultrasonography in ewe fed with CS and SB after 15 days postpartum. A, B indicate the difference between treatments ($P < 0.05$).

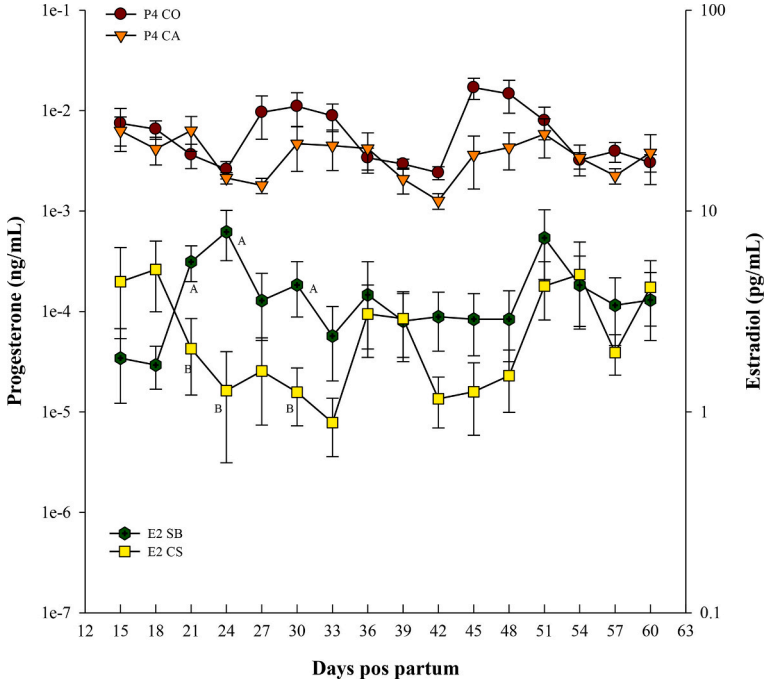

Fig. 3. Estradiol (a; pg / mL) and progesterone (b; ng / mL) concentrations in ewe fed with CS or SB after 15 days postpartum. A, B indicate the difference between treatments (P < 0.05).

Table 2
Normality, classification, diameter and follicular stereology (follicular population) of lambs born to progenitors fed cottonseed (CS) or soybean (SB).

Classification	Follicular classification (%)		Follicular Normality (%)		Follicle Diameter (μm)		Oocyte Diameter (μm)	
	CS	SB	CS	SB	CS	SB	CS	SB
Primordial	72.6 ± 3.3	78.1 ± 1.9	75.2 ± 5.8	76.4 ± 1.8	27.80.5^{B}	29.98 ± 0.8^{A}	18.7 ± 0.7	19.7 ± 0.6
Primary	75.2 ± 5.8	76.4 ± 1.8	65.4 ± 4.6	71.8 ± 3.6	41.4 ± 1.1	41.1 ± 2.0	23.1 ± 0.8	22.2 ± 0.7
Secondary	65.4 ± 4.6	71.8 ± 3.6	71.2 ± 9.5	81.6 ± 7.3	215.9 ± 22.1	201.4 ± 33.8	76.9 ± 6.9	68.41 ± 10.2
Tertiary	71.2 ± 9.5	81.6 ± 7.3	95.8 ± 3.0	95.2 ± 2.2	1139.2 ± 60.0	1250.3 ± 70.3	112.6 ± 4.1B	132.3 ± 3.5^{A}
	Total Normal Follicles (%)				Total Follicular Population (n = x)			
CS	72.6 ± 3.3				144.5			
SB	78.1 ± 1.9				139.5			

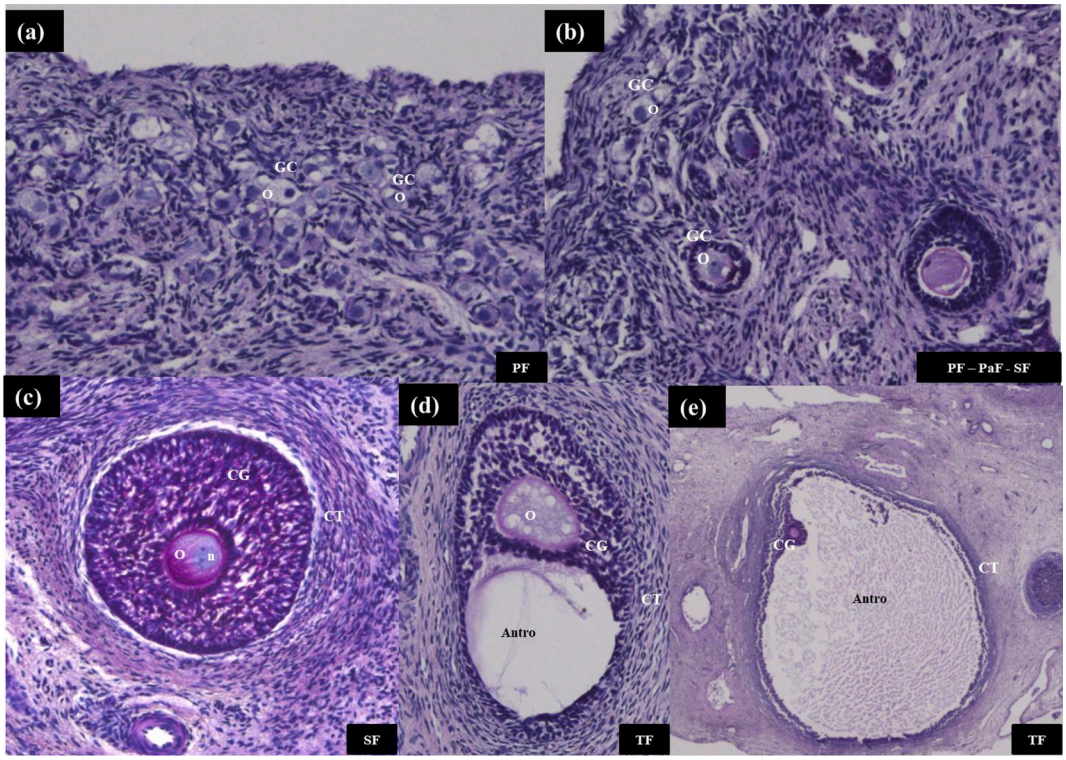

Fig. 4. Normal follicular morphology of lambs (offspring) born from progenitors fed with SB during the reproductive period. (a) primordial follicles; (b) primordial, primary, and secondary follicles; and (c, d) antral follicles. Granulosa Cell (GC), Oocyte (O), primordial follicles (PF), primary follicles (PaF), secundary follicles (SF), Theca Cell (TC).

Digital quantification of α-ER immunoexpression was performed with image analyzer (Image-Pro 10.0.5; Table 3) and descriptive immunoexpression qualification was classified as the reactive brown staining for α-ER by its intensity light (+), medium (++) and strong (+++; Figs. 6, 7). There was a higher percentage immunoexpression in all follicular structures of the ovary fragments of lambs born to parents who consumed CS (Table 3). In the descriptive immunoexpression the results showed that ovaries of lambs born to progenitors fed with SB treatment reacted with medium intensity for α-ER in structures as theca cells and corpus luteum. While the oocyte independent on follicular category showed light immunoexpression for α-ER. And ovaries of lambs born to progenitors fed with CS treatment had strong immunoexpression for α-ER in the corpus luteum and theca cells. In oocytes, immunoexpression were strong to medium, and in granulosa cells, it were light to absent (Table 3; Figs. 6, 7).

Electronmicrographs (n=5) per treatment were obtained from the ultrastructural analysis of ovarian lamb fragments of progenitors fed on SB and CS treatments can be observed in Fig. 8. No ultrastructural damage was evidenced by the consumption of progenitor diets. Several cytoplasmic organelles such as Golgi apparatus, secretory vesicles, ribosomes, smooth and rough endoplasmic reticulum, transport vesicles, lipid droplets, mitochondria, and a few vacuoles were observed. Granulosa cells in good conformation, cylindrical or ovoid oocytes, euchromatin and heterochromatin dispersed in the nucleus and nuclear and cytoplasmic membranes were well-demarcated (Fig. 8).

4. Discussion

The reproductive effects of gossypol on ruminants cause considerable controversy, especially in females animals where results are variable or scanty in some species. Thus, we observed the follicular effect of ovine females consuming CS and its maternal-descending effects. Studies performed in the same laboratory concluded that biochemical or hematological changes were not observed during the 10-month reproductive period (breeding season, pregnancy, and lactation) on the consumption of CS in Santa Inês ewes (Jimenez et al., unpublished data).

After 15 days postpartum, the total follicular dynamics was not altered by CS consumption. Likewise, the different follicular categories analyzed (small, medium and large) did not show differences with the ewes under SB treatment. In such way, after 300 days of diet intake, there was no feeding-affected reproductive performance in Santa Inês ewes after calving.

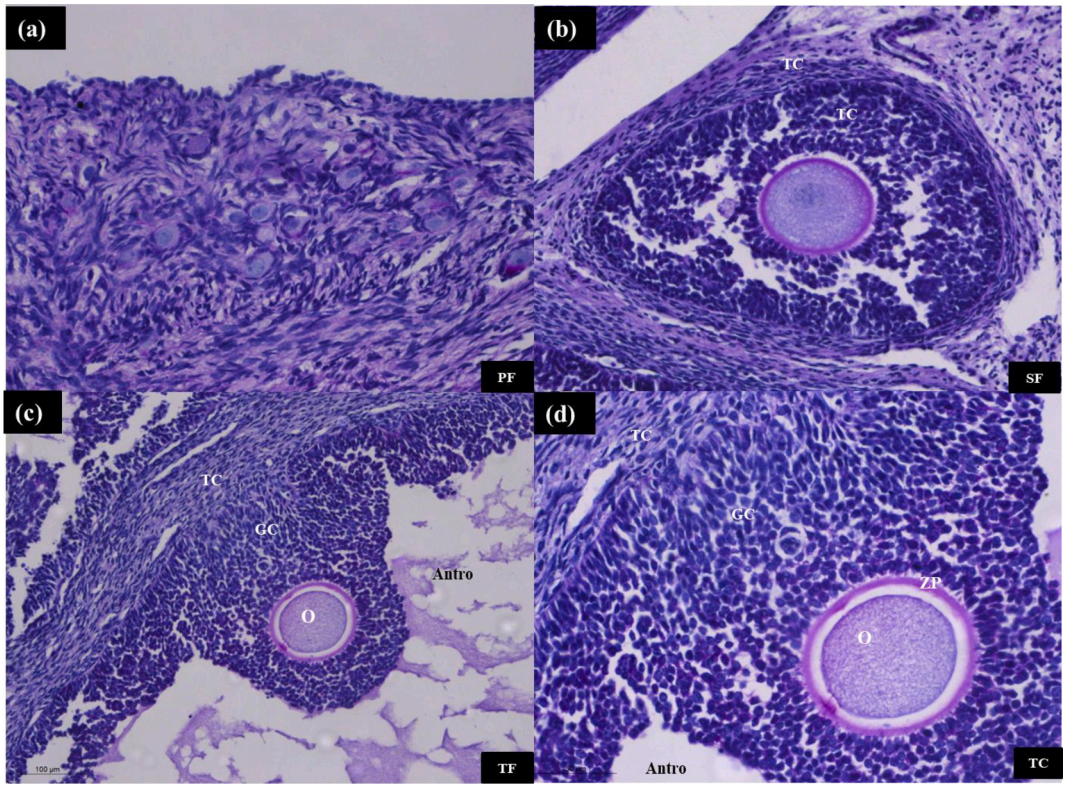

Fig. 5. Normal follicular morphology of lambs (offspring) born from progenitors fed with CS during the reproductive period. (a) primordial follicles; (b) secondary follicles; and (c, d) antral follicles. Granulosa Cell (GC), Oocyte (O), primordial follicles (PF), primary follicles (PaF), secundary follicles (SF), Theca Cell (TC), zona pellucida (ZP).

Research during the reproductive period in ewes showed that the CS consumption increased the concentration of immunoglobulin G (IgG) in the first milk secretions, suggesting a positive effect of feeding on the immunological quality of colostrum, antibacterial activity, and antioxidant potential. However, the same authors observed intensified oxidative stress, indicating a negative effect of CS (Moretti et al., 2019).

The estradiol levels in CS treatment were higher than the estradiol levels in SB treatment until 30 days postpartum. However, throughout the collection period estradiol remained within normal limits in the ovine ovarian cycle for both treatments. Some authors had similar data, evidenced levels of estradiol during natural estrus in an average of 10.32 pg / mL (Souza et al., 1997). Ciarlini et al. (2005) had different levels of estradiol during several lactations, from 3.45 to 8.57 pg / mL estradiol. In the present study, averages of 3.83 pg / mL and 2.61 pg / mL were observed for sheep from the CS and SB treatments respectively, ranging from 0.88 to 7.87 pg / mL.

The progesterone levels remained stable, but reduced, due to the period of active lactation for both treatments (Fig. 3). Lin et al. (1985) reported that rodents fed with gossypol had lower serum levels of estradiol and progesterone. Wu et al. (2004) expressed higher serum and ovarian concentrations of estradiol in hamsters treated with gossypol. Randel et al. (1996) revealed that progesterone concentrations in luteal tissue raised in heifers when free gossypol was increased in the diet. Gray et al. (1993) tested on heifers and cows (0, 0.5, 2.5, 5, 10 and 20 g / free gossypol animal) and they did not find differences in the concentration of progesterone in the luteal phase and progesterone and estradiol in the follicular fluid. In rats, Silva et al. (2002) reported that gossypol interfered in the maintenance process of gestation and in the number of pups born. In the same study, they reported that the interpretation of hormone results such as estradiol and progesterone was difficult because hamsters treated with gossypol showed normal cycles.

The recent works on nutrition *versus* reproduction interaction has concentrated most of the studies on matrix nutrition during the gestation period and its impact on several productive factors of the next generation. Such as fetal development, growth until weaning, carcass characteristics, reproductive performance, mammary gland growth, fleece weight, wool quality characteristics, offspring behavior and susceptibility of lambs to endoparasites (Bomfim et al., 2014; Kenyon et al., 2009).

Maternal nutrition during gestation may alter the development of reproductive organs of fetuses and reproductive performance in old animals (Bomfim et al., 2014). We observed in all follicular categories and follicular population within the parameters for the species, after 60 days postpartum, 144,000 and 139,000 follicles were found in ovary of lambs from SB and CS treatment progenitors, respectively. Similar results, had by Erickson (1986) and Lucci et al. (1999), stated that the follicular population in the ovary of lambs to birth is estimated at 150,000 to 160,000. In such a way, the study revealed that the CS ewe treatment did not cause any problems in ovarian morphology in the ovarian cells of their offspring.

The digital quantification and descriptive qualification of immunoexpression of α-ER showed an increase in α-ER in oocyte, granulosa cells, theca cells and corpus luteum of lambs born to progenitors fed cottonseed during 10 months. Jimenez et al. (2019) indicated that *in vitro* culture in the presence higher doses of gossypol there was a higher presence α-ER in ovary fragment of preantral follicles of ewe, indicating that the gossypol act in a way dose-dependent fashion. In swine indicated that the production of 17β-estradiol was inhibited in granulosa cell cultures when there was treatment with gossypol (Ohmura, 1996). The *in vitro* culture with gossypol affects the steroidogenic pathway in granulosa cells of cattle (Lin et al., 1985). And Akira et al. (1994) suggested that gossypol reduces estradiol production by inhibiting aromatase induction by FSH.

It is also worth highlighting the similar results *in vitro* with the MOIFOPA biotechnology of Jimenez et al. (2019) and the *in vivo* results of the present study, which are strong evidence that these two

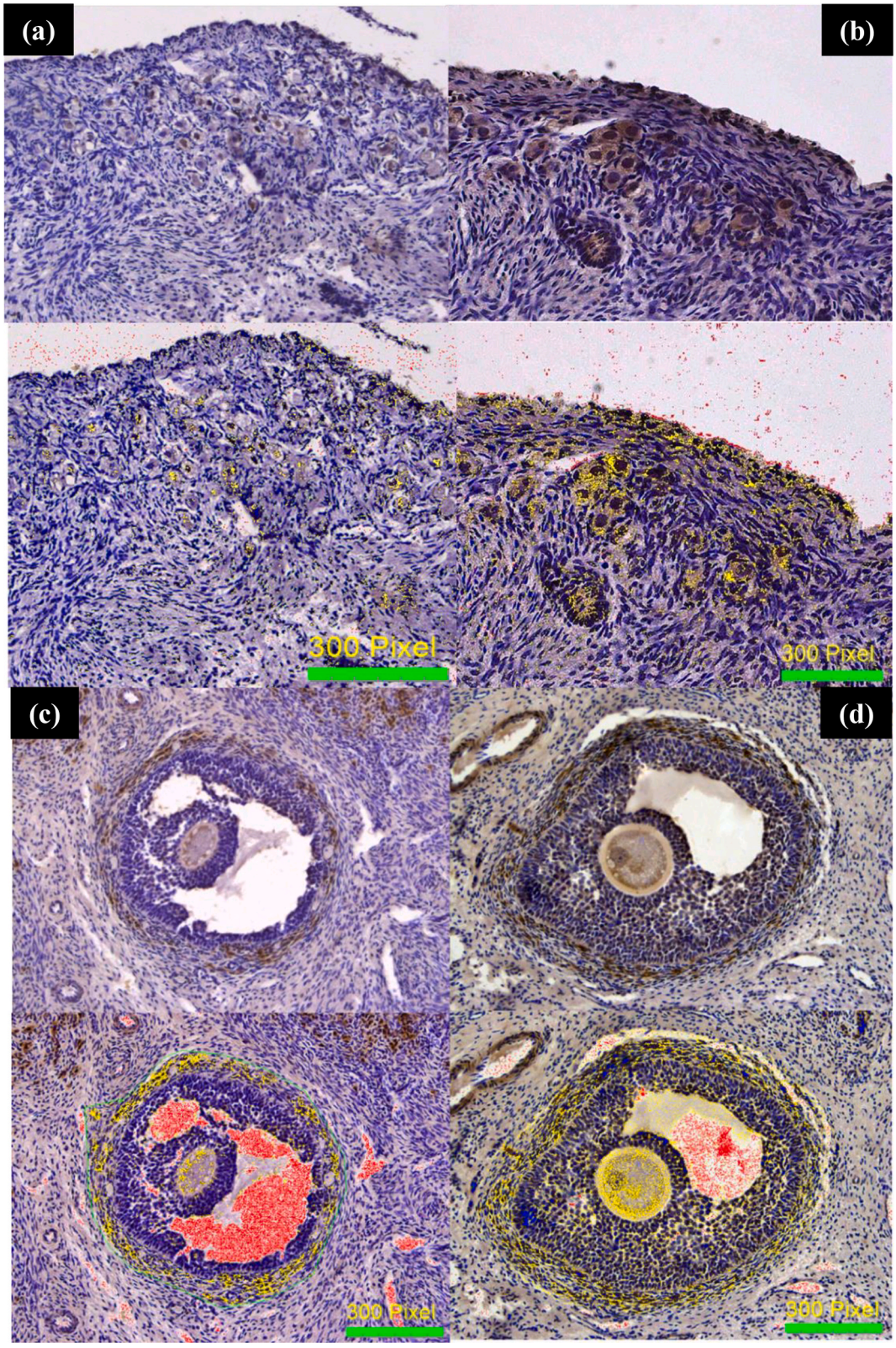

Fig. 6. Immunoexpression of estradiol-α receptors (α-ER) in lambs ovarian fragments born from progenitors fed with CS or SB during the reproductive period. Treatment SB (a,c) primordial and tertiary follicles. Treatment CS (b,d) primordial and tertiary follicles. The intensity of immunoexpression (brown) and digital immunoexpression (yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

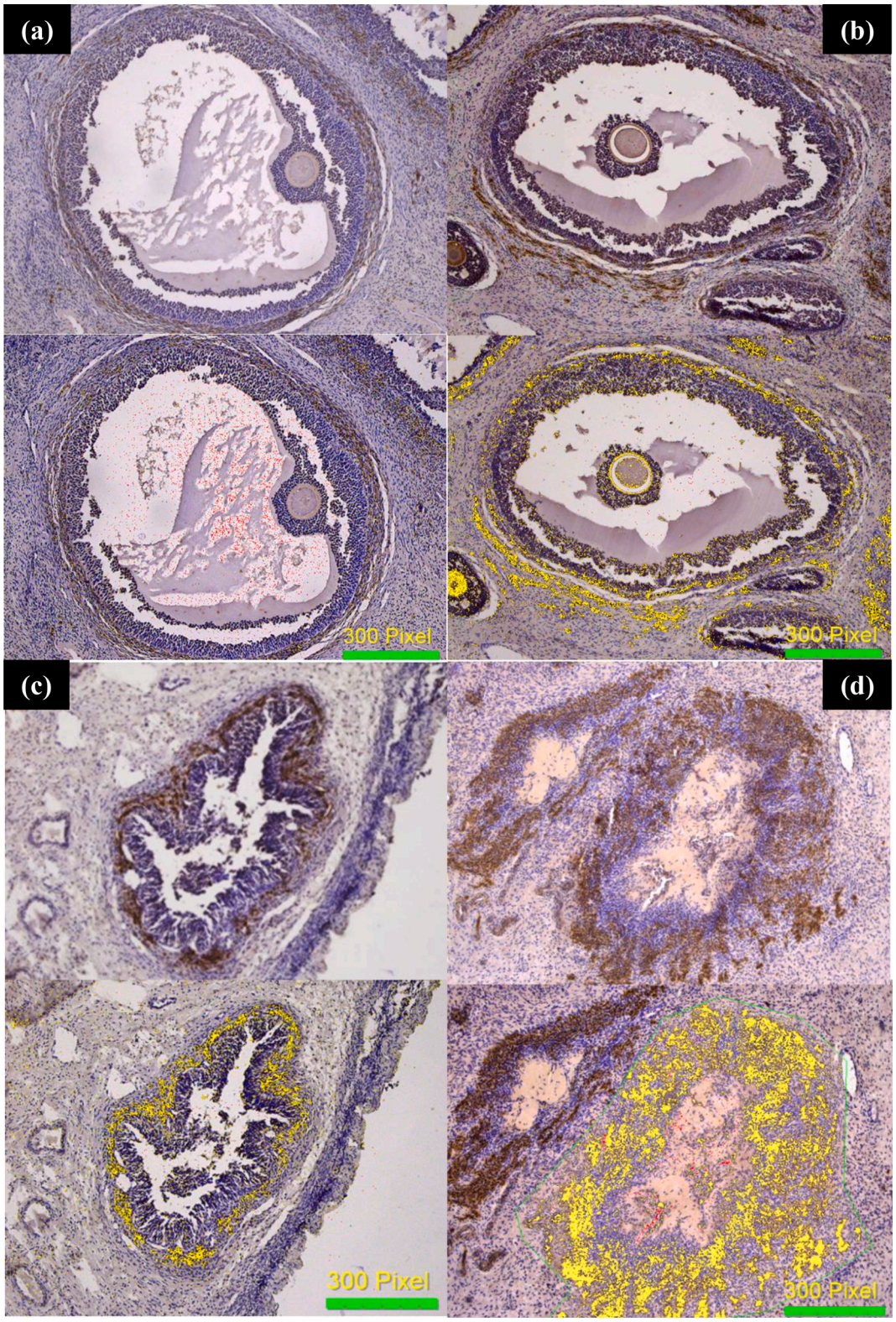

Fig. 7. Immunoexpression of estradiol-α receptors (α-ER) in lambs ovarian fragments born from progenitors fed with CS or SB during the reproductive period. Treatment SB (a,c) preovulatory follicles and luteum corpus. Treatment CS (b,d) preovulatory follicles and luteum corpus. The intensity of immunoexpression (brown) and digital immunoexpression (yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
The digital quantification and descriptive qualification of immunoexpression of α-ER in oocyte, granulosa cells, theca cells and corpus luteum of lambs born to progenitors fed cottonseed (CS) or soybean (SB). Digital quantification of α-ER immunoexpression.

Classification Follicular	Digital immunoexpression (%)		Descriptive immunoexpression (+) *			
	Total	Total Follicles	Oocyte	Granulosa Cells	Theca Cells	Corpus Luteum
SB Treatment						
Primordial	1.29	4.45	+			
Primary	0.33	2.05	+			
Secondary	1.20	2.49			+ +	
Tertiary	1.81	2.17			+ +	
Corpus luteum	0.84	4.11				+ +
CS Treatment						
Primordial	2.96	13.97	+++			
Primary	4.29	10.36	+++			
Secondary	4.67	7.46	+++	+	+	
Tertiary	4.80	6.58	+++	+	+ +	
Corpus luteum	3.13	4.72				+++

Intensity light (+), medium (++) and strong (+++).

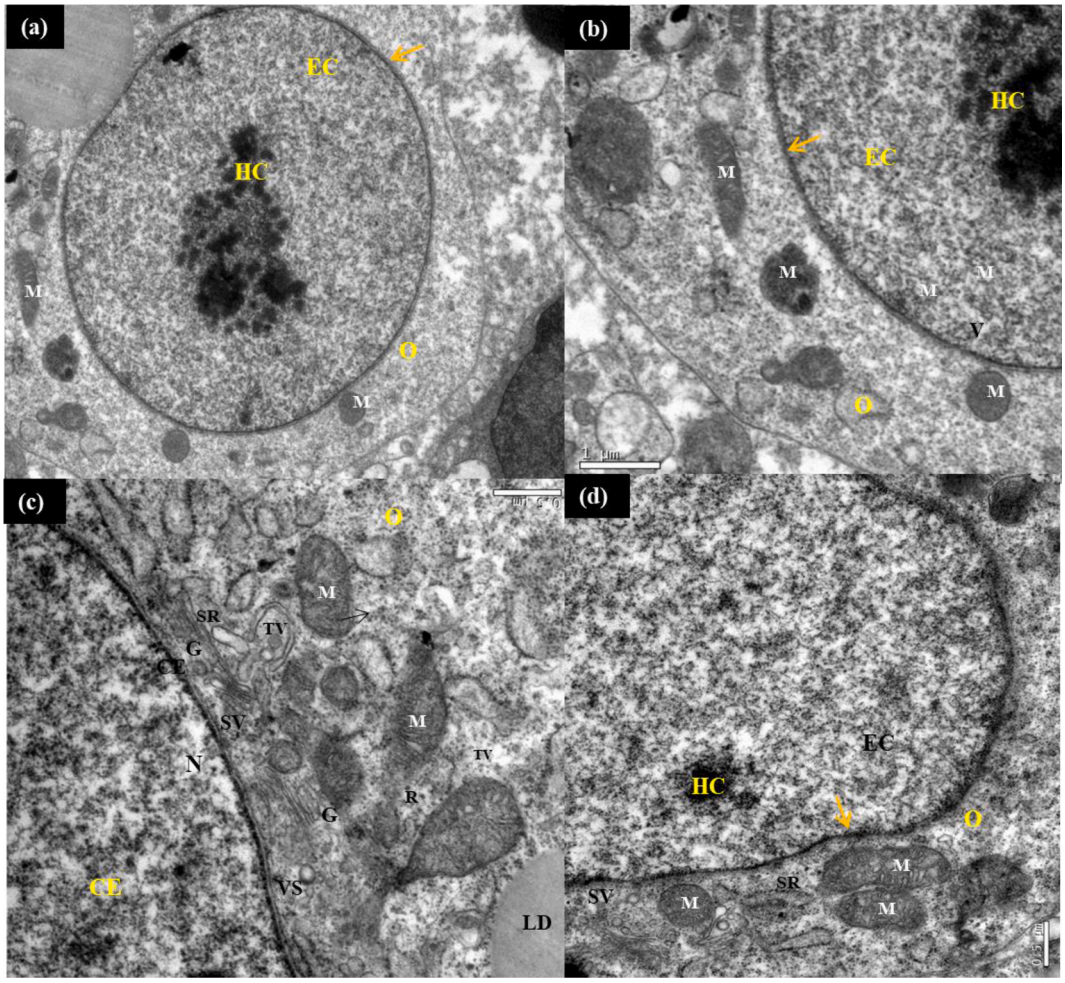

Fig. 8-. Electromicograph of lambs ovarian fragments born from progenitors fed with CS (a,b) or SB (b,c) during the reproductive period (b, c). HC = heterochromatin; EC = eurochromatin; N = nucleus; O = oocyte; nuclear membrane (yellow arrow); cytoplasmic membrane (black arrow), cytoplasmic degeneration (asterisk); TV = transfer vesicles; SV = secretory vesicle; M = mitochondria; SR = smooth endoplasmic reticulum; RR = rough endoplasmic reticulum; LD = lipid droplet; V = vacuoles; PN = picnotic nucleus; DC = degenerating cytoplasm, L: lysosomes, P: peroxisomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

techniques can be used together: *in vitro* for preliminary data and *in vivo* for definitive data, in agreement with Figueiredo (2008), who state that after the *in vitro* MOIFOPA technique, fewer animals could be used for *in vivo* research contributing to animal welfare.

5. Conclusions

It is concluded that cottonseed affected neither the maternal-descendant follicular dynamics nor the hormonal levels of the ewes. Lambs ovaries that consumed cottonseed had highest α-ER

immunoexpression in oocytes, granulosa cells theca cells, and corpus luteum, demonstrating the intra-follicular steroidogenic activity.

Declaration of Competing Interest

All authors have declared no conflict of interest for this study.

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