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Immunofluorescence Analysis of Estrogen and Progesterone Receptors and Ki-67 Nuclear Protein in Canine Uteri Treated with Medroxyprogesterone Acetate During Anestrus

Análisis de Inmunofluorescencia de Receptores de Estrógeno y Progesterona y Proteína Nuclear Ki-67 en Úteros Caninos Tratados con Acetato de Medroxiprogesterona Durante el Anestro

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SALINAS, P.; MIGLINO, M. A. & DEL SOL, M. A. Immunofluorescence analysis of estrogen and progesterone receptors and ki-67 nuclear protein in canine uteri treated with medroxyprogesterone acetate during anestrus. *Int. J. Morphol., 41(3)*:725-732, 2023.

SUMMARY: Estradiol and progesterone receptors play an essential role in the changes occurring in the uterus during the estrus cycle in dogs (*Canis lupus familiaris*). In order to investigate the potential effect of progestational agent medroxyprogesterone acetate (MPA) when is used during anestrus on the expression of estradiol receptors [ER], progesterone receptors [PR] and nuclear protein Ki67, we evaluated uterine tissue immunohistochemically. Uteri were grouped as nulliparous (control, n=11), multiparous (n=11) and treated with MPA (n=11; nulliparous with two treatments; 5mg/kg; i.m.). The amount and location of PR, ER and Ki67 were studied on the epithelial surface, apical and basal regions of the endometrium and myometrium using immunohistochemical techniques with a spectral confocal microscope and analyzed by ANOVA. Differences in ER were observed between the multiparous and MPA-treated groups in the apical region of the endometrium (p=0.0022). Differences in cell proliferation were detected between the nulliparous and multiparous groups (p=0.0037) and nulliparous and MPA-treated groups (p=0.0003) in the basal region of the endometrium. In conclusion, two doses of MPA (5mg/kg; i.m.) do not have a significant effect on the expression of ER and PR; however, they inhibit cell proliferation in the basal region of the endometrium, which includes the stroma, subepithelial cell layer, compact layer, and spongy layer. The clinical and long-term effect of this treatment should be evaluated in subsequent studies.

KEY WORDS: *Canis lupus familiaris*; Uterus; Medroxyprogesterone acetate; Estradiol; Progesterone; Ki67.

INTRODUCTION

The structure and function of the canine uterus is controlled mainly by steroid hormones such as estradiol [E2] and progesterone [P4], the effects of which are mediated by their respective receptors in the target organs (Brenner *et al*., 1990). These receptors are proteins located mainly in the cell nucleus; however, a cytoplasmic fraction has also been described (Ho & Liao, 2002; Tahir *et al*., 2013). The estradiol receptors [ER-alpha; ER-a] and progesterone receptors [PR] are regulated by ovarian steroid hormones. The endometrial epithelium and stroma (Vermeirsch *et al*., 2002; Galabova-Kovacs *et al*., 2004; Bartel *et al*., 2013) as well as the myometrium express ER-a and PR (Tan *et al*., 1999; Augsburger & Kürzi, 2004). In both *in vivo* and *in vitro* conditions it has been shown that steroid receptors in canine endometrial cells are regulated by estradiol and progesterone (Dhaliwal *et al*., 1999; Galabova-Kovacs *et al*., 2004). In dogs, ER-a expression in the luminal epithelium or stromal cells varies according to the estrus cycle, *i.e.*, it is more pronounced in the early proliferative stage and drops during the secretory phase (Dhaliwal *et al*.,1997). It has been reported that an estrogenic hormonal environment induces cell proliferation (Conti *et al*., 1981; Van Cruchten *et al*., 2004). With respect to PR expression, this increases during late proestrus and prior to ovulation; however, it decreases in the luteal progesterone present prior to ovulation and during the early luteal phase until the luteolysis (Fernandes *et al*., 1998).

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The synthetic progestin medroxyprogesterone acetate (MPA) is widely used to prevent estrus in dogs and to regulate the estrus cycle in ruminants (Yellon *et al*., 2009), and it is also considered an anti-neoplastic agent. It is known that MPA has a high affinity for PR in uteri of non-pregnant and pregnant females (Zhang *et al*., 1989). The canine uterus presents cyclical endometrial changes, characterized by hormone-mediated endometrial proliferative states (Van Cruchten *et al*., 2004), and studies have described the role of steroid receptors in regulating these changes (Vermeirsch *et al*., 2000). However, aspects related to the influence of MPA treatment on the regulation and distribution of ER and PR, and their effect on the cyclical endometrial changes present in the canine endometrium and myometrium are not known. Cell proliferation in the uterus has been investigated at different stages of the canine estrus cycle (Barrau *et al*., 1975; Spanel-Borowski *et al*., 1984; Van Cruchten *et al*., 2004). These studies have generally described patterns of endometrial proliferation during the onset of proestrus, but do not include data on anestrus. We have previously reported that two doses of MPA (5mg/kg; i.m.) induce quantitative changes in the architecture of the endometrium and myometrium in all the uterine segments, mainly morphological endometrial gland changes of the uterine corpus, increasing the surface area per unit of volume (Salinas *et al*., 2017a), without affecting the synthesis and distribution of collagen (Salinas *et al*., 2016, 2017b). However, these changes usually do not differ quantitatively from those observed in the uterus of multiparous bitches. We suggest that the intramuscular (i.m.) treatment of MPA when is during anestrus modifies the expression and distribution of ER-a and PR and, in addition, induces an increase in endometrial cell proliferation. The aim of this study was to evaluate the expressions of ER-a and PR in utero and the proliferation of endometrial cells by detecting the nuclear expression of the Ki67 protein in bitches exposed to the synthetic progestin MPA and to compare them with nulliparous and milliparous bitches exposed to luteal progesterone. Knowledge of the distribution of steroid receptors in the uterus associated with endometrial cell proliferation in dogs in anestrus can aid in understanding the tissue changes in dogs exposed to MPA in pathological conditions.

MATERIAL AND METHOD

This study was conducted at the Center for Excellence in Surgical and Morphological Studies (CEMyQ, in spanish) at the Universidad de La Frontera, Temuco, Chile. The animals were handled in accordance with guidelines for animal research as detailed in the NIH Guidelines for the Care and Use of Laboratory Animals (NIH, 1985) with the ethical approval from the Universidad Santo Tomás - Ethics Research Committee CE 081/2013.

Animals, samples and determination of estrus cycle. Complete uteri were obtained from 33 healthy adult female dogs (*Canis lupus familiaris*) from the University Animal Hospital, of no defined breed, subjected to ovariohysterectomy during anestrus (2 - 5 years). No gross abnormalities were present anywhere in these tracts. Uteri were classified as nulliparous (control; n = 11; older than 2 years, that had experienced at least one estrus cycle), multiparous (n = 11; older than 2 years, that had experienced at least two full-term gestations and involution of the uterus after the last gestation was complete) and MPA-treated bitches (n= 11; nulliparous exposed twice to contraceptive treatment during late anestrus). All the bitches included in this study demonstrated at least one estrus, with this information being determined from the owner's report. Treatment with the synthetic progestin depot preparation MPA (OVO-6® 50 mg, Drag Pharma Laboratory Invetec S.A., Chile) was begun during anestrus giving two treatments of 5 mg/kg i.m. body weight at 8-week intervals. A vaginal inspection and cytological examination of the vaginal floor were performed on bitches used in this study to determine the estrus cycle and confirm anestrus. Three days before the start of the treatment with MPA the stage of the estrus cycle was assessed. The anestrus was confirmed, therefore, using physical examination, vaginal cytology (Diff Quick®, Hartman Leddon Co, Philadelphia, PA), histological parameters of the uterus and ovaries (Galabova *et al*., 2003; Rehm *et al*., 2007) and confirmed by the absence of the corpus luteum in the ovary. Vaginal cytology showed sparse numbers of parabasal cells and variable numbers of neutrophils. The vaginal mucosa appeared thin and red with visible capillaries; the surface was easily traumatized and vaginal cytology was difficult to monitor without inducing bleeding with spurious erythrocytes in smears classified as being in anestrus (Concannon, 2009). In terms of uterus histological parameters, bitches in anestrus had simple low columnar or cuboidal epithelium, superficial stroma rich in cells, and the stroma of the deeper part of endometrium with less convoluted basal glands with simple cuboidal and in some cases columnar epithelium.

Preparation of the uterine tissue, immunofluorescence and confocal microscopy. Sections of uterine horn were obtained and fixed in 4 % formaldehyde v/v for 72h at room temperature. For confocal microscopy, uterine tissue was cut into 5-mm-thick sections using a microtome (Leica RM2265) and then washed 3 times for 10 minutes in Tris-phosphate buffer pH 7.8 (Tris 10 mM, NaCl 120 mM, Na2HPO4 8.4 mM, KH2PO4 3.5 mM). Next, the sections

were incubated at 4º C for 6 hours with the conjugated primary antibodies: anti-human Ki67 e-Fluor450 (eBioscience, Carpinteria, CA, USA), Progesterone Receptor - Alexa Fluor 488 (AA 525-575; antibodies-online Inc., Limerick, PA, USA), Estrogen Receptor alpha antibody - Alexa Fluor 647 (pTyr537; antibodies-online Inc., Limerick, PA, USA) and Hoechst nuclear marker (Biotium, ayward, CA, USA), which were prepared in Tris-phosphate BSA (1 %) -Triton X-100 (0.2 %). The dilutions and mixtures were as follows: a) Ki67-eFluor450 [1:100], ER-a-A647 [1:100] and Hoechst [1:500] and b) Progesterone-A488 [1:100] + ER-a-A647 [1:100] + Hoechst [1:500]. After the incubation time, the sections were again washed 3 times for 10 minutes in Tris-phosphate 10 mM buffer (pH 7.8) and finally mounted in the assembly medium for fluorescence (VectaShieldTM Mounting Media). Multi-labeled images were obtained by confocal spectral microscopy (LSM 780, Carl Zeiss).

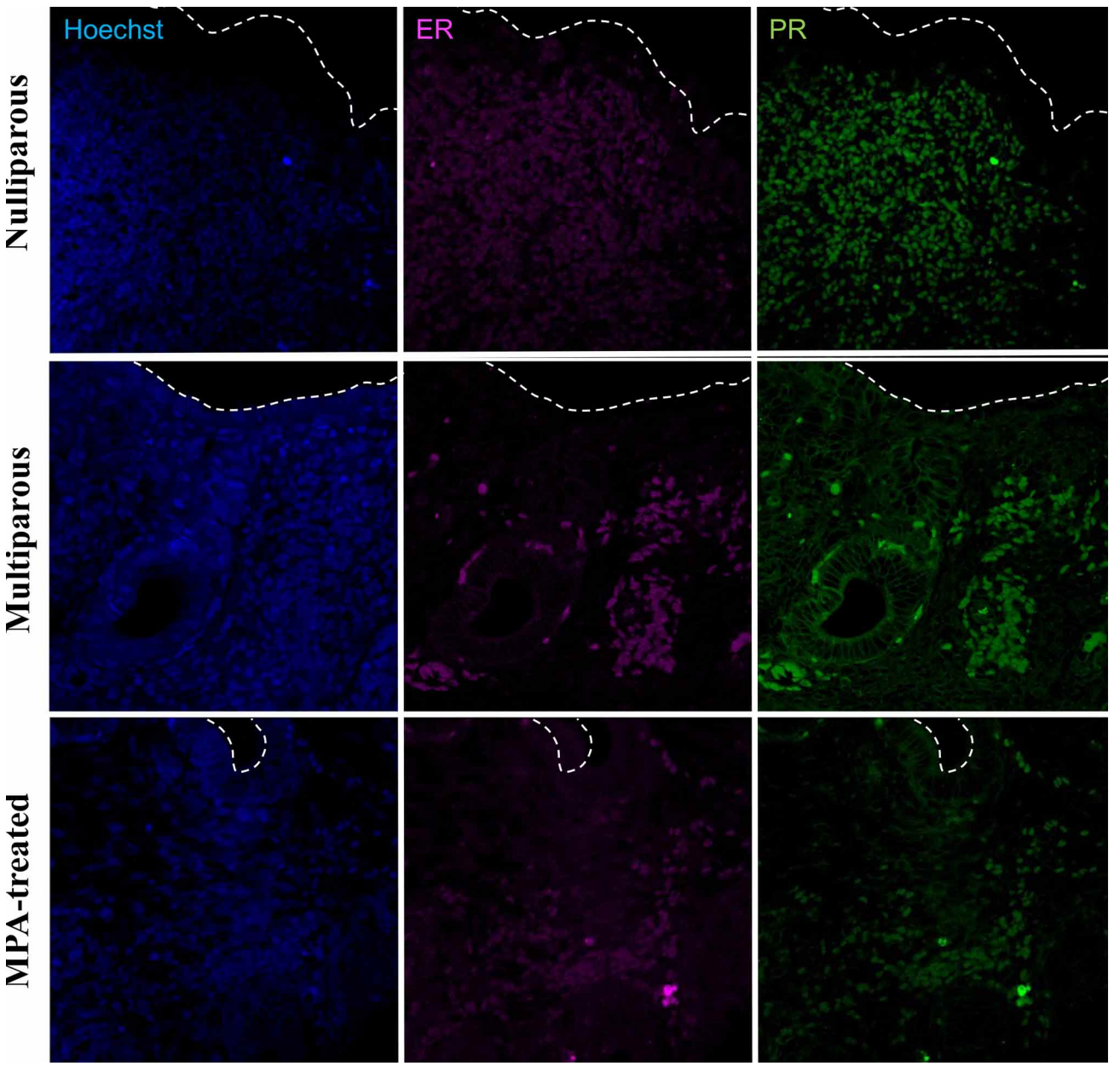

Fig. 1. Immunohistochemical detection of progesterone receptor (PR) and estradiol receptor (ER) in the apical region of the endometrium in uteri of nulliparous, multiparous and MPA-treated dogs. PR and ER-a is represented by green and magenta fluorescence, respectively (40X; Bar=50 µm).

Fluorescence quantification and statistical analysis. Endometrial analyzes were performed on the apical region (surface epithelium, includes secretory duct epithelium and glandular epithelium) and basal regions (stroma, includes subepithelial cell layer, compact layer, and spongy layer). After images around the endometrium and myometrium were obtained, the integrated density in the apical and basal regions of the endometrium, myometrium and background were quantified using ImageJ software. For the quantification of colocalized receptors, 10 fields were obtained per section using a 40X magnification, and at least three sections for each region per animal were counted. Control images were included to evaluate background staining. Data represent the mean of integrated density ± SD. The D'Agostino-Pearson test was used to evaluate data normality. Statistical comparisons between three groups of data were carried out using analysis of variance (ANOVA) among the three groups and Tukey's post-test of multiple comparisons was used. Statistical significance for all hypotheses tests was set at $p<0.05$. The data analysis was performed using GraphPad Prism 5.0 software for Mac OS X (GraphPad Software, San Diego CA).

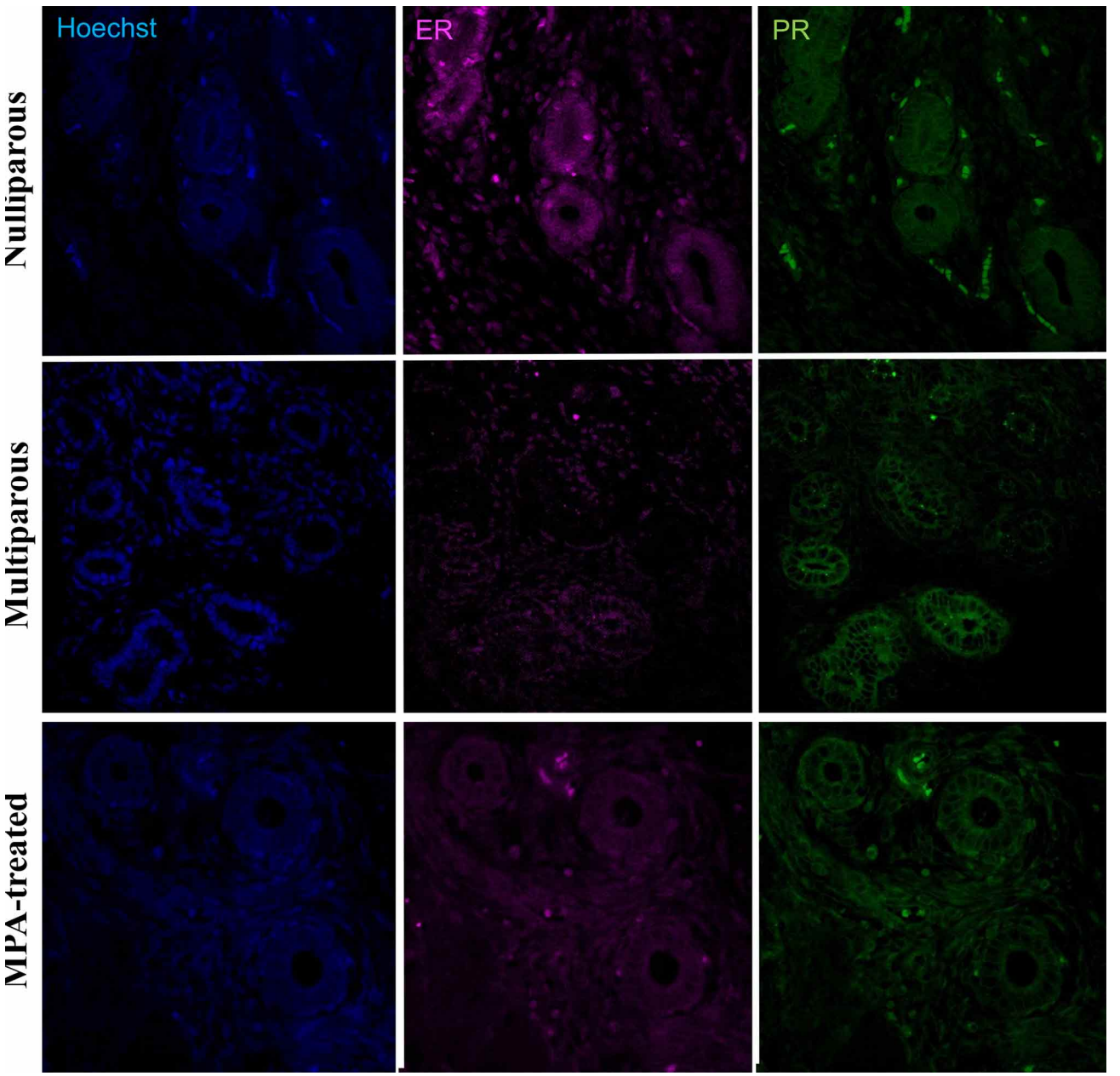

Fig. 2. Immunohistochemical detection of progesterone receptor (PR) and estradiol receptor (ER-a) in the basal region of the endometrium in uteri of nulliparous, multiparous and MPA-treated dogs. PR and ER-a is represented by green and magenta fluorescence, respectively (40X; Bar=50 µm).

RESULTS

PR receptor expression in the uterine horns. An intense green nuclear stain was observed in the three analyzed groups, demonstrating the presence of PR in different cell groups in the apical region of the endometrial fold, stromal cells of the lamina propria, glandular ducts and basal gland (Figs. 1 to 3). No differences were observed in the expression of PR in the lamina propria of the apical region (Fig. 4A; p=0.499), basal region (Fig. 4B; p=0.0513) or myometrium (Fig. 4C; p=0.1588; Table I) among groups. Low expression of PR was seen in epithelial cells on the surface of the endometrial fold in multiparous and MPA-treated groups. In addition, no differences were detected in the expression of PR between the apical and basal regions and the myometrium in each study group.

ER-a receptor expression in the uterine horns. Magenta fluorescent stain was observed in the three study groups, indicating the presence of ER-a in different cell groups, such as the apical region of the endometrial fold and stromal cells of the lamina propria. The superficial cells of the epithelium in the nulliparous group weakly expressed ER. Intense nuclear staining was distributed homogenously in the stroma of the lamina propria in the three study groups. In the multiparous and MPA-treated groups, generally, surrounding glands and ducts were observed in the base of the endometrial stroma (Figs. 1 to 3). Differences were observed in the apical region between the multiparous vs. MPA-treated group (p= 0.0022; Table I; Fig. 4). No differences were detected in the expression of ER-a between the apical and basal regions and the myometrium in each study group.

Ki67 nuclear protein expression. The expression of nuclear protein Ki67 it was not observed in cuboidal epithelial cells of the epithelium in the three study groups; in addition, moderate to strong expression was observed in the basal region of the endometrium. Proliferative activity was seen in the glands. The nulliparous group presented quantitative differences in the expression of Ki67 in the basal region of the endometrium compared to the multiparous group (p=0.037) and the MPA-treated group (p=0.0003; Table I; Fig.5).

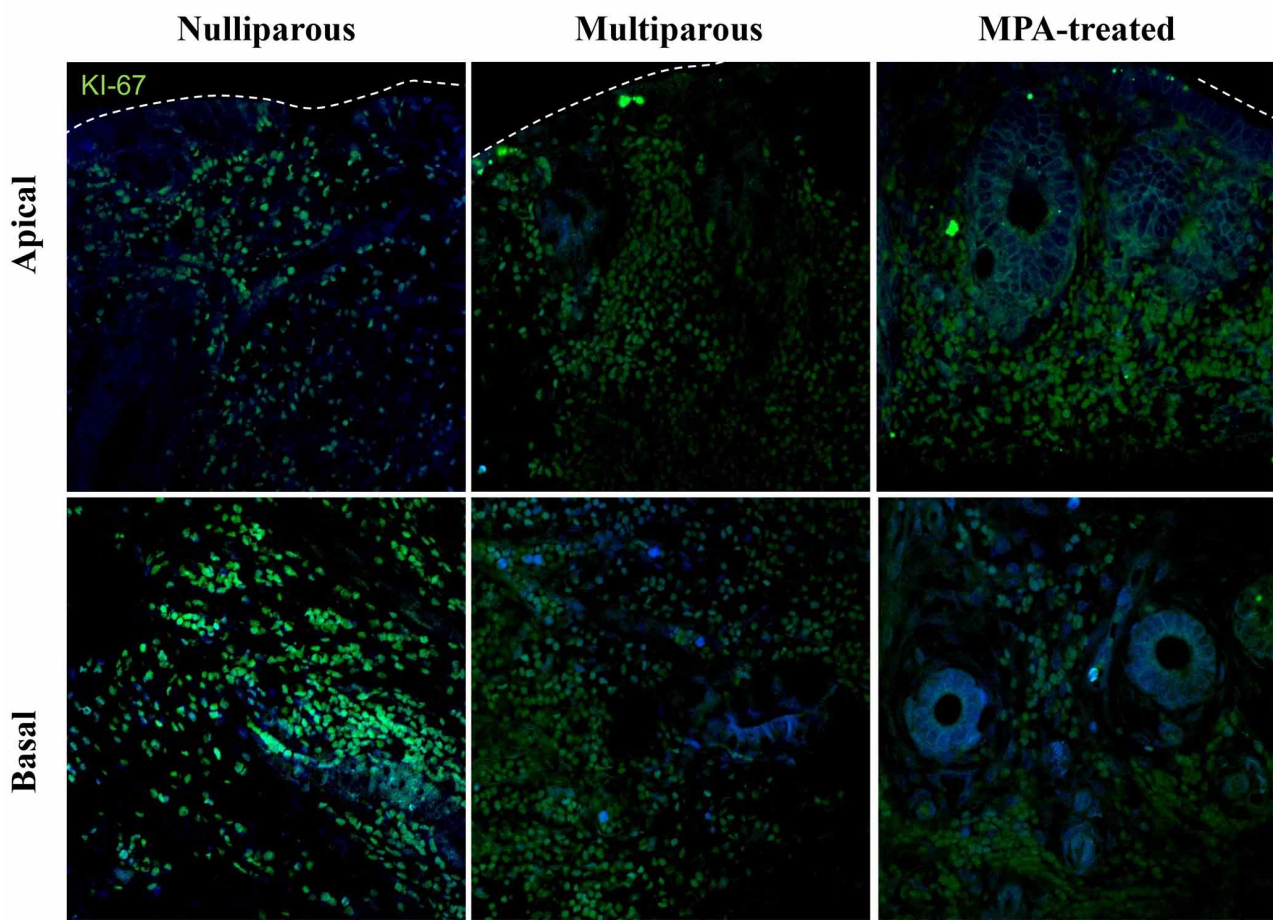

Fig. 3. Immunohistochemical detection of the nuclear protein Ki67 in the apical and basal regions of the endometrium in uteri of nulliparous, multiparous and MPA-treated dogs. Ki67 is represented by green fluorescence (40X; Bar=50 μm).

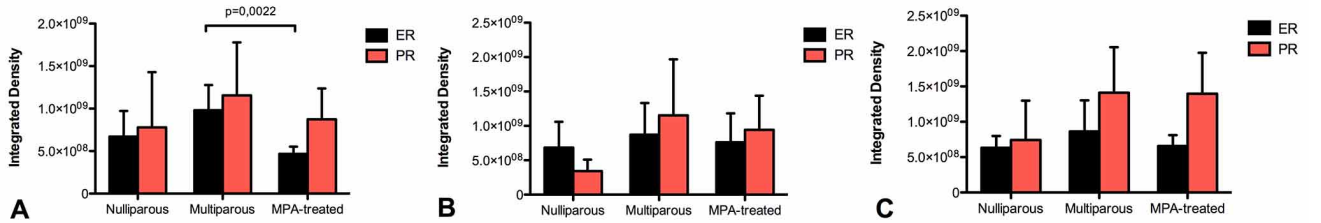

Fig. 4. Integrated density (mean ± SD) of progesterone receptor (PR) and estradiol receptor (ER-a) in (A) apical and (B) basal endometrium and (C) myometrium.

Table I. Integrated density (mean ± SD) of uterine expression of nuclear protein Ki67, progesterone (PR) and estradiol (ER-a) receptors (n=11).

		Nulliparous	Multiparous	MPA-treated	*p-value*
PR	*Apical endometrium*	779379370,6 ± 649352208,6	1154581504 ± 623556686,1	874587605,3 ± 363625496,9	0,4999
	Basal endometrium	408669866,6 ± 263324876,5	1152634880 ± 814443108,5	942847530,6 ± 496402551,2	0,0513
	Miometrium	742448832 ± 555788113,7	1411377715,2 ± 644950965,2	1395641770,6 ± 580305145,6	0,1588
ER-A	*Apical endometrium*	670259382,9 ± 302766066,4	981619541,3 ± 296304429,2[a]	467416192 ± 85186594,9 [a]	0,0096
	Basal endometrium	683685302,8 ± 375363510,3	872156458,6 ± 459229473,7	760996659,2 ± 420313228,9	0,7232
	Miometrium	631439786,6 ± 167054190,2	862666325,3 ± 440586766,3	656941525,3 ± 155210463,3	0,3355
Ki67	*Apical endometrium*	972367744 ± 297390735,8	646445013,3 ± 350094662,4	773532117,3 ± 374372167,1	0,2810
	Basal endometrium	1372619212,8 ± 393522423,9 [ab]	467888042,6 ± 378537363,0 [a]	373517312 ± 147593933,7[b]	0,0003

a, b Different letters in the same row indicate a significant difference by Tukey test ($p < 0.05$).

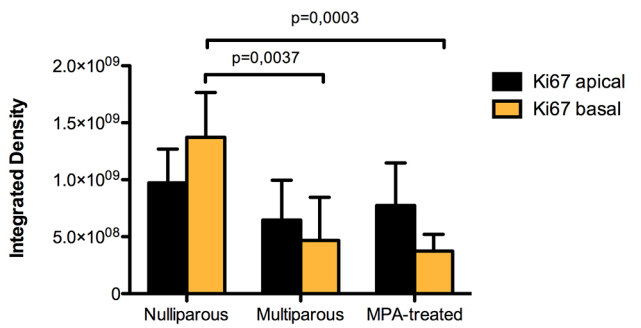

Fig. 5. Integrated density (mean ± SD) of endometrial expression of nuclear protein Ki67.

DISCUSSION

The present study evaluated the immunoreactivity and distribution of ER-a and PR in the canine uterus treated with MPA during anestrus and compared its effects with two physiological progestational conditions, nulliparity and multiparity. Furthermore, the effect on cell proliferation in the endometrium by measuring the expression of the nuclear protein Ki67 detected in proliferating cells was evaluated in all phases of the cell cycle. This is the first study to quantify the effect of contraceptive treatment with MPA on the expression of ER-a, PR and Ki67 in the endometrium and myometrium using immunohistochemical techniques. The uterine tissue responds differentially to estrogen and progesterone. The binding of estrogen to its receptor stimulates hyperplasia and hypertrophy (Geisert *et al*., 1993) and the binding of estrogen to its receptor induces differentiation and maturation (Graham & Clarke, 1997). Our results show similar effects between multiparous and MPA-treated uteri, suggesting a link between multiparity and treatment with MPA as a contraceptive at two doses of 5 mg/kg with respect to the expression of PR in dogs in anestrus. Generally, the expression of ER-a, PR and nuclear protein Ki67 did not exhibit any significant differences between uteri exposed to MPA and uteri exposed to physiological luteal progesterone under conditions of nulliparity and multiparity. This tissue response could be attributed to the incapacity of the two doses of 5 mg/kg to produce the progesterone-like regulatory effects of MPA on ER-a and PR.

With respect to the apical region of the endometrium, the multiparous and MPA-treated groups had positive staining for the expression of ER-a and PR in superficial cells of the epithelium, similar to what was reported for pregnant pigs. By contrast, in the nulliparous group weak staining of ER-a was observed in surface epithelial cells, consistent with that described in pregnant uteri in dogs (Vermeirsch *et al*., 2000); however, the three study groups showed low expression of PR and ER-a in the epithelial cells consistent with what was reported by Vermeirsh *et al*. (2002). The stromal cells presented positive staining with greater frequency for ER-a and PR than the surface epithelial cells, which may indicate that both hormones can act indirectly on the epithelial cells through the stromal cells. The results obtained in the multiparous and MPA-treated groups are

consistent with those described in the literature (Tani *et al*., 1999) regarding the association between ER-a expression and anestrus because the levels of estradiol are low and there are no manifestations of these in the reproductive system or in sexual behavior during this stage, which suggests treatment with MPA does not have a significant role in ER-a expression.

With respect to the basal region of the endometrium, no differences were observed between groups in the expression of ER-a and PR. Generally, in the multiparous and MPA-treated groups the expression of ER-a and PR was observed in stromal cells that surrounded some endometrial glands. In these cells, no differences were observed between groups in the expression of ER-a and PR. No differences were observed in the expression of steroid receptors in the myometrium either. In addition, although no statistical differences were detected, a predominance of PR over ER-a was detected in the multiparous and MPA-treated groups.

Our results generally do not demonstrate direct and differential effects of treatment with MPA compared to those observed in uteri exposed to luteal progesterone. This differs from what has been reported in other studies that evaluated the effects of MPA on the uterus (Hackenberg *et al*., 1993; Von Berky & Townsend, 1993; Abulafia *et al*., 1999). We hypothesize that there is a likely regulation of the expression of ER-a and PR regardless of the control of sexual hormones or their synthetic derivatives (Scarpin *et al*., 2009).

In terms of the evaluation of endometrial proliferation, our results reveal quantitative differences between nulliparous vs. multiparous and MPA-treated groups. A greater degree of cell proliferation was seen in the apical region of the endometrium in all groups; however, differences were detected in the basal region between the nulliparous and the other groups. During anestrus, cell proliferation of the superficial epithelium was detected in the three groups, which suggests continuous mitotic activity on the luminal surface and continuous regeneration of the epithelium after cell desquamation induced by metestrus (Galabova *et al*., 2003).

Cell proliferation was detectable on endometrial surface, but it was more intense in three groups on the basal region of the endometrium, particularly in the glands, which suggests the beginning of a proliferative phase, probably related to the onset of proestrus. Nevertheless, considering that high concentrations of estradiol are needed to support the mitotic activity of the endometrium, the mechanism responsible for cell proliferation observed in the absence of high levels of estradiol due to the application of MPA is unknown.

In short, an inverse effect was observed between cell proliferation and the expression of ER-a and PR in the three study groups, *i.e.*, those groups with the lowest expression of PR and ER-a had the greatest cell proliferation. In conclusion, this study shows for the first time in dogs that two treatments with MPA at a dose of 5mg/kg have no significant effect on the expression of ER-a and PR, but do have an inhibitory effect on cell proliferation in the endometrium. Therefore, the choice of dose and dosage can be important in terms of controlling a potential risk of neoplasia.

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SALINAS, P.; MIGLINO, M. A. & DEL SOL, M. A. Análisis de inmunofluorescencia de receptores de estrógeno y progesterona y proteína nuclear ki-67 en úteros caninos tratados con acetato de medroxiprogesterona durante el anestro. *Int. J. Morphol., 41(3)*:725-732, 2023.

RESUMEN: Los receptores de estradiol y progesterona juegan un rol fundamental en los cambios que se producen en el útero durante el ciclo estral de las perras (*Canis lupus familiaris*). El objetivo de este estudio fue evaluar las expresiones de ER-a y PR en el útero y la proliferación de células endometriales detectando la expresión nuclear de la proteína Ki67 en perras expuestas a la progestina sintética MPA y compararlas con perras nulíparas y multíparas expuestas a progesterona luteal. Úteros fueron agrupados como nulíparas (control, n=11), multíparas (n=11) y tratadas con MPA (n=11; nulíparas con dos tratamientos; 5 mg/kg; i.m.). La expresión de PR, ER-a y Ki67 fue evaluada en la regiones apicales y basales del endometrio y miometrio con un microscopio confocal espectral. Se observó diferencias en ER-a entre los grupos multíparas y tratados con MPA en la región apical del endometrio (p=0,0022). Se detectaron diferencias en la proliferación celular entre los grupos de nulíparas y multíparas (p=0,0037) y los grupos de nulíparas y tratados con MPA (p=0,0003) en la región basal del endometrio. En conclusión, dos dosis de MPA (5mg/kg; i.m.) no tienen un efecto significativo sobre la expresión de ER y PR; sin embargo, inhiben la proliferación celular en la región basal del endometrio, el cual incluye a estroma, capa de células subepiteliales, estratos compacto y esponjoso. El efecto clínico a largo plazo de este tratamiento debe ser evaluado en estudios posteriores.

PALABRAS CLAVE: *Canis lupus familiaris*; Útero; Acetato de medroxiprogesterona; Estradiol; Progesterona; Ki67.

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