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Journal of Steroid Biochemistry & Molecular Biology 84 (2003) 211–216

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Steroid hormone profile of canine inflammatory mammary carcinoma: a preliminary study☆

L. Peña[b], G. Silván[a], M.D. Pérez-Alenza[b], A. Nieto[b], J.C. Illera[a,*]

[a] *Department of Animal Physiology, School of Veterinary Medicine, University Complutense of Madrid, Madrid, Spain*

[b] *Department of Animal Pathology II, Veterinary Teaching Hospital, School of Veterinary Medicine, University Complutense of Madrid, Madrid, Spain*

Abstract

Inflammatory mammary carcinoma (IMC) is the most aggressive spontaneous type of mammary malignant tumor both in women and dogs. Latest studies in dogs indicate that different endocrine mechanisms seem to be involved in inflammatory carcinomas (IMCs). The aim of the present study was to characterize the steroid hormone profile of inflammatory carcinoma, and to compare it with mammary dysplasias, benign tumors and other malignant tumors. Eighty-six mammary samples (10 normal mammary tissue, 21 dysplasias, 26 benign, 22 malignant, and 7 IMC) from 30 female dogs were used. Hormone levels of progesterone (P4), 17β-estradiol (E2), androstenedione (A4), dehydroepiandrosterone (DHEA), and estrone sulphate (E1SO4) in tissue homogenates were measured by enzyme immunoassays (EIAs) techniques, previously validated for this species. IMC displayed the following steroid profile: P4: 13.80±0.56 μg/g; E2: 675.19±33.00 ng/g; A4: 631.73 ± 70.73 μg/g; DHEA: 702.22 ± 89.93 μg/g, and E1SO4: 2.84 ± 0.32 mg/g. All of these hormones were significantly higher ($P < 0.001$) compared with the hormone steroid profile determined for malignant, benign, dysplasias, and normal mammary tissue. The most relevant finding was the increased levels, two or three times, of both DHEA and E1SO4 in IMC respect to other groups ($P < 0.001$). These results, together with the highest immunohistochemical expression of P450scc found in IMC, suggest the hypothesis that an autocrine mechanism could be especially involved in the development of canine inflammatory carcinoma.

Keywords: Canine mammary inflammatory carcinoma; Steroid hormones; Progesterone; Estradiol; Androstenedione; Dehydroepiandrosterone; Estrone sulphate

1. Introduction

Normal and neoplastic mammary gland secrete steroid hormones and it is related with the development of tumors. A local synthesis of estrogens in normal mammary gland and tumors has been demonstrated [1–3]. The estrogens formed in the tumor can act as mitogen and promote tumor growth [4]. P450arom expression and activity have been demonstrated in breast carcinoma and breast cancer cells [4]. Normal and neoplastic breast tissues contain and produce several forms of androgens [5]. The effect of androgens and androgen precursors on mammary gland is not well known.

Inflammatory mammary carcinoma (IMC) is the most aggressive spontaneous type of mammary cancer both in women (usually named inflammatory breast carcinoma (IBC)) and in dogs (inflammatory mammary carcinoma). It is considered the most malignant type of mammary carcinoma with a fulminant clinical course and extremely poor survival rate [6,7]. The etiology of this neoplasm is unknown. Several epidemiological, clinical, pathological and genetic alterations have been specifically associated with the inflammatory mammary carcinoma in the woman and in the dog compared with other non-inflammatory mammary carcinomas [7–10]. Some endocrine-related characteristics have been indicated in canine IMC [7,11]. The occurrence of IMC is generally in the lutheal phase, previous treatments with progestagens are usually associated with a worse clinical course and increased histological malignancy and there is histological evidence of a relative high proportion of lipid-secreting tumors. These findings seem to indicate that probably different endocrine mechanisms seem to be involved in canine IMC and that IMC could be a source of steroids. The aim of the present study was to characterize the steroid hormone profile in tissue homogenates of IMC and to compare with mammary dysplasias, benign mammary tumors and other malignant mammary tumors non-IMC.

☆ Poster paper presented at the 15th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, "Recent Advances in Steroid Biochemistry and Molecular Biology", Munich, Germany 17–20 May 2002.

* Corresponding author. Present address: Department Fisiología Animal, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain. Fax: +34-91-3943864.

E-mail address: vefis07@sis.ucm.es (J.C. Illera).

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doi:10.1016/S0960-0760(03)00030-X

2. Materials and methods

2.1. Animals

Thirty female dogs (aged 7–14 years) with mammary dysplasias or tumors were clinically examined at the Veterinary Teaching Hospital of Madrid (VTHM). All dysplasias and non-inflammatory tumors were surgically treated. Inflammatory mammary carcinomas were clinically diagnosed and confirmed by tru-cut biopsy. In IMC cases, surgery was not the treatment of election, and only palliative therapy with anti-inflammatories and corticoids was applied. Cytology of vaginal smears was performed in each case to know the stage of the estrus cycle at sampling.

2.2. Sampling procedure

Eighty-six mammary samples (10 normal mammary tissue and 76 dysplasias and mammary tumors) were prospectively collected. Normal mammary gland tissues were obtained of 10 dogs (aged 7–10 years) without a history of mammary or endocrine disorder by tru-cut biopsies. Mammary dysplasias and non-inflammatory tumors ($n = 69$) were surgical or necropsy specimens submitted for diagnosis to the Pathology Service of the VTHM. Samples of IMC were obtained from tru-cut biopsies or at necropsies. In each case, two adjacent fragments of tissue were separated and processed for histopathology and immunohistochemistry, and steroid analysis.

2.3. Histopathology: immunohistochemistry of P450scc

The samples for histopathology and immunohistochemistry were fixed in 10% buffered formalin, paraffin embedded and cut in 4 μm sections, following routinary methods. Histopathological diagnosis was done on H&E sections following the WHO's classification for canine mammary tumors and dysplasias [12].

Immunohistochemistry was performed on deparaffined sections using the streptavidin–biotin-complex peroxidase method ($n = 43$). The primary antibody used was a polyclonal rabbit anti-P450scc (Chemicon Int., dilution 1/200; incubation overnight at 4 °C). The slides were subsequently incubated with anti-rabbit biotinylated secondary antibody (Vector Laboratories BA1000, 1:400, 30 min at room temperature) and with streptavidin conjugated with peroxidase (Zymed P50242, 1:400, 30 min, at room temperature). All washes and dilutions were made in Tris-buffered saline (TBS) (pH 7.4). The slides were developed with a chromogen solution containing 3,3′-diaminobenzidine tetrachloride (Sigma D5059) and H_2O_2 in TBS and counterstained in hematoxylin (Sigma GH5-2-16). Positive (normal canine testis) and negative control slides were used. Positive P450scc immunostaining intensity was also evaluated in each case as low (+), moderate (++), and intense (+++).

2.4. Assessment of steroid concentrations in tissues

2.4.1. Preparation of tissue homogenates

Samples for steroid analysis were maintained frozen until use. Mammary tissue of 0.5 g were homogenized in 4 ml of PBS (pH 7.2), centrifuged (3500 rpm, at 4 °C for 20 min) and the supernatants were collected and aliquoted individually (−30 °C), until hormone assays.

2.4.2. Dehydroepiandrosterone, androstenedione, testosterone, progesterone, estrone sulphate and 17β-estradiol enzyme immunoassay (EIA) of homogenates

Dehydroepiandrosterone (DHEA), androstenedione, testosterone, progesterone, estrone sulphate and 17β-estradiol levels of mammary tissue homogenates were assayed by competitive enzyme immunoassay (EIA) previously validated in our laboratory. Homogenate samples were prepared by diluting 10 μl of each homogenate in assay buffer (1:2500 for dehydroepiandrosterone, androstenedione, testosterone, progesterone and estrone sulphate assay or 1:500 for 17β-estradiol assay), and then extracted with 2 ml of diethyl ether (Sigma, St. Louis MO, USA), and 100 μl of the supernatant were evaporated under a nitrogen stream (Turbovap, ZIMARK, Hopkinton, MA, USA). Dehydroepiandrosterone, androstenedione, testosterone, estrone sulphate concentrations were expressed in μg/g, progesterone concentrations in mg/g, and 17β-estradiol concentrations were expressed in pg/g.

2.5. Statistical analysis

The Biomedical Data Program (BMDP, Statistical Software Inc., Los Angeles, CA, USA) was used for statistical analysis. Differences between individual means were analyzed by Pairwise *t*-test and Bonferroni post-test to determine whether values were statistically different. All values were expressed as mean ± S.E. In all statistical comparisons, $P < 0.05$ was accepted as denoting significant differences.

3. Results

3.1. Histopathology

Ninety-four mammary samples (10 normal mammary tissue, 21 dysplasias, 26 benign, 22 malignant, and 7 IMC) were analyzed. All mammary dysplasias were lobular hyperplasias. Benign tumors consisted in complex or simple adenomas, and mixed benign mammary tumors. The malignant tumors included several histologic subtypes: tubulopapillary, solid and lipid-rich carcinomas, and carcinosarcomas. All cytologic vaginal smears revealed characteristics of anestrus.

3.2. P450scc immunohistochemistry

Results of P450scc immunohistochemistry is detailed in Table 1.

Table 1
P450scc immunohistochemistry in canine normal mammary gland, dysplasias and tumors

	Number of samples analyzed	Negative	Positive		
			Low (+)	Moderate (++)	Intense (+++)
P450scc immunohistochemistry					
Normal mammary gland	5	0 (0)	1(20)	4 (80)	0 (0)
Mammary dysplasias	10	0 (0)	6 (60)	2 (20)	2 (20)
Benign tumors	15	0 (0)	5 (33.3)	8 (53.3)	2 (13.3)
Malignant non-IMC tumors	10	1 (10)	4 (40)	3 (30)	2 (20)
Inflammatory mammary carcinomas (IMC)	3	0 (0)	0 (0)	1 (33.3)	2 (66.7)
Total	43				

Values in parenthesis are the percentage values.

3.2.1. Normal mammary gland

In general, the normal mammary gland showed a moderate (++) P450scc immunoexpression. P450scc was detected in the cytoplasms of epithelial and myoepithelial cells in ducts and acini with uniform distribution among the different mammary lobules. Stromal cells were slightly positive or negative. Muscular cells of the wall of arterioles were intensely positive (Fig. 1).

3.2.2. Mammary gland dysplasias

Most of the dysplasias had a low (+) level of P450scc expression compared with the normal mammary gland. Only in two cases the immunostaining was considered intense (+++). The distribution of immunostaining was homogeneous among the cells and the mammary lobules.

3.2.3. Benign mammary tumors

The expression of P450scc was moderate (++) or intense (+++) in most of the cases (10/15), showing a marked heterogeneous distribution among cells and lobules. Generally, proliferated myoepithelial cells forming mixoid areas (in complex type of adenomas) expressed less P450scc than the epithelial counterpart. Cartilaginous cells in mixed benign tumors showed or not P450scc immunoexpression in different tumors (Fig. 2).

3.2.4. Malignant tumors (non-IMC)

A marked heterogeneous distribution of P450 immunostaining was found in malignant tumors. A reduction of P450scc immunostaining was observed in this group of tumors compared with that found in normal mammary gland and benign tumors. The only negative case of all the samples analyzed was a malignant tumor (papillary carcinoma); four malignant tumors (4/10) showed a low immunoexpression of P450scc (Fig. 3).

3.2.5. Inflammatory mammary carcinomas (IMC)

In the three IMC cases studied, p450scc was heterogeneously expressed and considered moderate (++) in one case and intense (+++) in the two remaining cases. Highly malignant independent epithelial cells invading the dermis showed an intense immunostaining (Fig. 4).

3.3. Steroids concentrations in tissues

The steroid hormone concentrations in tissue homogenates is depicted in Table 2. Hormone levels of progesterone (P4), 17β-estradiol (E2), androstenedione (A4), dehydroepiandrosterone and estrone sulphate (E1SO4) were

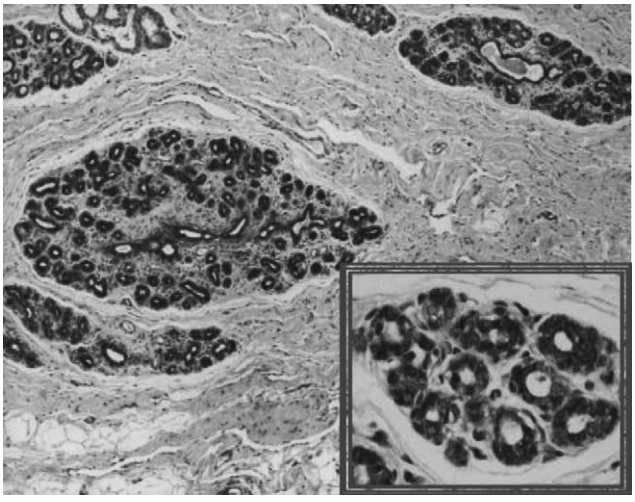

Fig. 1. Normal mammary gland. P450scc immunohistochemistry.

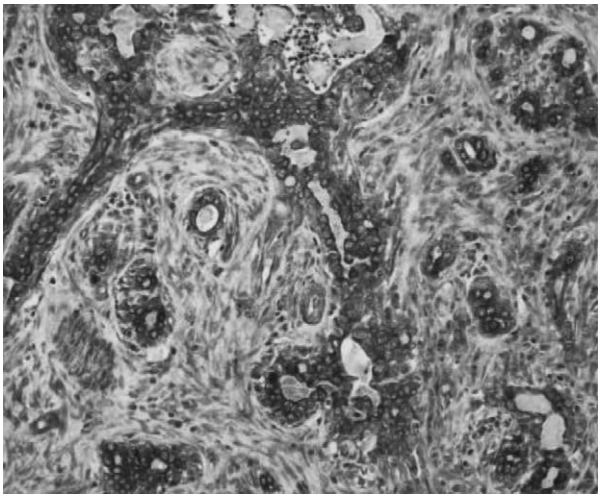

Fig. 2. Benign mammary tumor (complex adenoma). P450scc immunohistochemistry.

Table 2
Steroid hormone concentrations in tissue homogenates

	Normal mammary tissue	Dysplasia	Benign tumor	Malignant tumor non-IMC	Inflammatory mammary carcinoma (IMC)
Progesterone (μg/g)	1.48 ± 0.13 a	2.49 ± 0.08 b	1.44 ± 0.05 ac	5.40 ± 0.36 d	13.80 ± 0.56 e
Dehydroepiandrosterone (μg/g)	67.70 ± 6.22 a	83.70 ± 3.54 b	70.19 ± 2.74 ac	244.65 ± 7.94 d	702.22 ± 89.93 e
Androstenedione (μg/g)	32.62 ± 5.65 a	50.89 ± 6.56 ab	35.93 ± 3.70 abc	158.52 ± 6.02 d	631.73 ± 70.73 e
Estrone sulphate (μg/g)	398.74 ± 9.64 a	545.78 ± 28.99 b	430.59 ± 21.13 abc	1118.53 ± 45.07 d	2844.96 ± 326.95 e
17β-estradiol (μg/g)	133.29 ± 14.71 a	141.05 ± 4.72 ab	128.58 ± 2.88 abc	260.96 ± 10.69 d	675.19 ± 33.00 e

Hormone values with different letters denoting statistical differences (a vs. b, c, $P < 0.05$; a–c vs. d, e, $P < 0.01$).

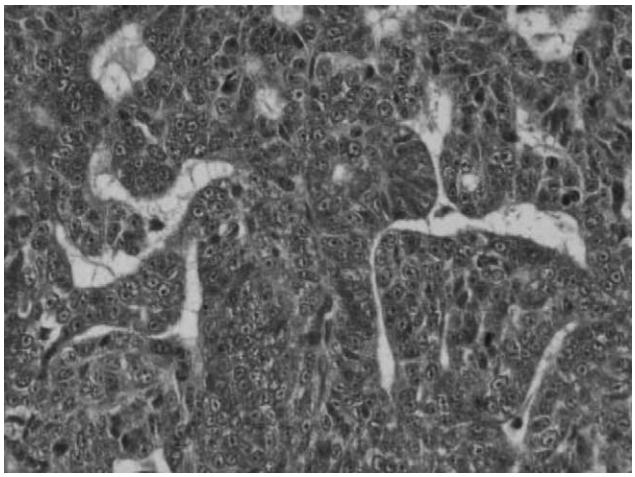

Fig. 3. Malignant mammary tumor non-inflammatory mammary carcinoma (papillary carcinoma). P450scc immunohistochemistry.

significantly higher ($P < 0.001$) in IMC compared with the hormone steroid profile determined for malignant non-IMC tumors, benign tumors, mammary dysplasias and normal mammary gland. Levels of all the steroids analyzed were elevated two or three times in IMC respect to other malignant tumors non-IMC. Concentrations of both DHEA and E1SO4 were especially elevated in IMC respect to other groups.

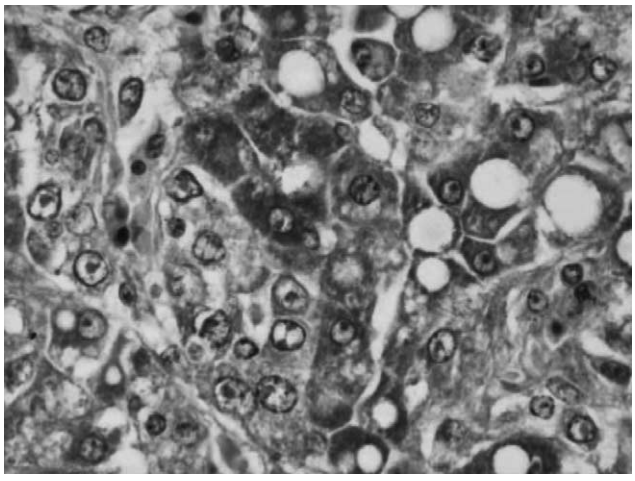

Fig. 4. Mammary carcinoma-inflammatory carcinoma. P450scc immunohistochemistry.

4. Discussion

Inflammatory mammary carcinoma is a rare type of mammary cancer that clinically resembles a mastitis or dermatitis, with a very poor prognosis both in humans (inflammatory breast carcinoma) and in dogs (inflammatory mammary carcinoma) [6,7]. Little is known about the etiology and pathogenic mechanisms involved in the apparition and development of this type of neoplasm. Lately, some mechanisms have been suggested to be especially involved in IBC and IMC respect to other non-inflammatory malignant mammary tumors [7,9]. In dogs, some histopathologic evidences indicate a lipid secretion by IMC cells, possibly steroids [11]. In this study, different steroid hormones have been detected in homogenates of 86 samples of normal, dysplastic and neoplastic mammary gland. IMC presented significantly the highest amounts of all the hormones studied (P4, E_2, A_4, DHEA and E_1SO4) compared with normal and dysplastic mammary gland, benign tumors and malignant tumors non-IMC.

Sex steroid formation in peripheral tissues is well documented in humans [13]. Normal and neoplastic mammary gland is considered by some authors as an endocrine tissue, particularly by the estrogen synthesis [3]. Intratissue estrogen biosynthesis and aromatasa expression and activity in human breast cancer is well documented [1–4,14–16]. The activity of other steroidogenic enzymes involved in the formation of estradiol has been also demonstrated in breast cancer tissues [3]. Steroid hormone content in peripheral tissues is not referred in the dog. In this section, the comparison of our results with those obtained in the woman should be taken carefully and they only include studies on premenopausal women (menopasusal state is very rare even in old bitches). Estradiol content in canine malignant mammary tumors was significantly higher than in normal mammary gland. Concentrations of estradiol in IMC were also significantly higher than in other malignant mammary tumors non-IMC. Increased levels of estradiol in malignant

respect to normal and benign human breast tissues have been described [14,17].

Estrone and estrone sulfate contents can represent a reservoir for biologically active estrogens in mammary tumors, since breast tissue also contains a sulfatase enzyme that can convert estrone sulfate to estrone and finally produce estradiol [18–20,24]. There is direct evidence that estradiol in breast cancer tissues is produced mainly, at least 10-fold higher than the aromatase pathway, via sulfatase enzyme (steroid sulfatase, STS), using estrone sulfate as precursor [18–21]. Our study revealed high concentrations of estrone sulfate in canine mammary tissues, increased with the histological malignancy. The maximum amounts of E1SO4 were found in IMC and malignant mammary tumors non-IMC, being the mean between these groups significant. Large amounts of estrone have been detected in human mammary tissues, although the results indicating higher levels of estrona in carcinomatous tissues are contradictory [14,17,19]. Other authors did not find differences in the levels of E1SO4 between benign and malignant breast tumors [21].

Normal and neoplastic human breast tissues contain and produce also several forms of androgens [5,16,17,22,23]. In the present study, levels of dehydroepiandrosterone (DHEA) and androstenedione were significantly elevated in IMC respect to other malignant non-IC mammary tumors and respect to normal mammary gland. DHEA, DHEA sulphate (DHEAS) and Adiol (5-androstene-3β, 17β-diol) of adrenal origin have been detected at high amounts in normal and cancerous breast tissues [5]. Other authors indicated lower concentrations of androgens in cancerous breast tissue than in normal breast [1]. DHEA can have effect on breast cancer cells by the final production of estrogens in situ or by the activation of the estrogen receptor alpha [4]. DHEAS is also substrate for the formation of ADIOL via STS, although DHEAS can stimulate the growth of mammary carcinoma cells by an aromatase-independent pathway [24]. Studies using [3H] androstenedione (A) demonstrated that this substrate can be aromatized to estrone (E1) in homogenates of breast carcinoma [2]. If the adrenal cortex secretes DHEA and A4 in dogs remains controversial [13,25]. In our opinion, a high proportion of the androgens found in the canine mammary tissues could be attributed to a local synthesis.

At our knowledge there are not previous studies concerning progesterone content in normal and neoplastic mammary gland. Progesterone was also increased significantly in malignant non-IMC mammary tumors and in IMC.

Results of our study indicate a higher concentration of all the studied hormones in malignant tumors and especially in inflammatory mammary carcinoma. The most relevant finding was the increased levels, two or three times, of DHEA and E1SO4 in IMC respect to other groups ($P < 0.001$). If the steroid production of the canine mammary gland and tumors causes an elevation of these hormones in the blood stream is still not studied. The preliminary results on steroidogenic enzymes immunoexpression concerned to P450scc, which has shown to be present in the majority of samples analyzed. The cholesterol side chain cleavage cytochrome P450 (P450scc) is a mitochondrial enzyme that catalyzes the conversion of cholesterol to pregnenolone and is the first step in the steroidogenic cascade. We can assume that at least a portion of the steroid hormones found in the mammary tissues, were produced “in situ”. In 42 out of the 43 samples studied there was P450scc expression although the increased levels of different hormones was not correlated with the intensity of expression of P450scc. Inversely, in malignant tumors non-IBC (with high levels of all the steroid hormones analyzed) there was a lower expression of P450scc than in benign tumors. Nevertheless, inflammatory mammary carcinomas, which had the highest levels of the steroid hormones analyzed, showed a high expression of this steroidogenic enzyme. The immunostaining of P450scc and aromatase in human breast carcinomas [4,26] is less than the reported in canine IMC and concerned mainly to stromal and adipose cells.

There are no direct studies about the possible content of steroid hormones in human IBC. Recent studies have described a worse survival rate in post-menopausal obese IBC patients compared with post-menopausal non-obese IBC patients [27]. In our opinion this fact could be due to that adipose tissue is an important source of sex steroids in post-menopausal women [28], as we are discussing here, steroid hormones seem to play a relevant role in inflammatory mammary carcinoma. Direct endocrine responsiveness of IBC cells is only known by the presence of estrogen and progesterone receptors (ER and PR), in some cases. Positive ER and PR IBC-tumors have a better prognosis than negative tumors [29,30]. In canine IMC, a relative high proportion of IMC studied were PR-positive (71.4%) while none was considered ER-positive [11]. The presence of intratissue estrogen levels in ER negative breast cancer has been described [3], indicating that in these tumors other mechanism than direct action of estrogens on tumor cell can occur.

The dog has been proposed as a spontaneous model of inflammatory mammary carcinoma, since it is the unique animal specie in which this type of tumor occurs naturally, and several similar clinical and pathologic characteristics between IMC and IBC have been indicated [7,11]. In our opinion, the results of the present study concerning endocrine mechanisms involved in the IMC development, should be taken carefully in the extrapolation to the women. A different endocrine status exists in the two species: less number of estrus in the dog (1–2 per year), causing probably less influence of estrogens, and absence of a menopausal status in the bitch.

Our results suggest the hypothesis that an autocrine mechanism could be especially involved in the pathogenesis of canine inflammatory carcinoma. Future studies about the production and metabolism of steroid hormones of inflammatory mammary carcinoma could be useful to the development of new therapies directed to the block of determined steroidogenic enzymes.

Acknowledgements

This study has been partially financed and supported in part by the research project of the Complutense University no. PR-269/98-8178. We thank Pedro Aranda and Mario Hernando for their technical help.

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