

Assessment of Prolactin, Steroid Hormone Levels in Tissue Homogenate, Serum and Receptor Distributions in Canine Mammary Tumours ^[1]

Sinem Özlem ENGİNLER ¹ Tuğba Seval Fatma TOYDEMİR ¹ Atilla ATEŞ ²
Iraz AKIŞ ² Haydar ARMAĞAN ³ Kemal ÖZTABAK ² İsmail KIRŞAN ¹
Mehmet Can GÜNDÜZ ¹ İbrahim FIRAT ⁴

^[1] This study was supported by "Research Fund of Istanbul University with the grant number 8802

¹ Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Avcılar, Istanbul - TURKEY

² Department of Biochemistry, Faculty of Veterinary Medicine, Istanbul University, TR-34320Avcılar, Istanbul - TURKEY

³ Department of Neurology, Istanbul Faculty of Medicine, Istanbul University, TR-34080 Çapa, Istanbul - TURKEY

⁴ Department of Pathology, Faculty of Veterinary Medicine, Istanbul University, TR-34320Avcılar, Istanbul - TURKEY

Makale Kodu (Article Code): KVFD-2013-10579

Summary

The aim of the present study is to determine variations of estrone sulphate (SO₄E1), progesterone (P4), 17-beta estradiol (E2), testosterone (T) and prolactin (PRL) levels in serum, mammary tissue homogenates as well as variations in the distribution of androgen (AR), estrogen alpha (ER α), progesterone (PR) and prolactin (PRL-R) receptors in mammary tumours of dogs. Thirty bitches from different breeds and ages with mammary lesions (experiment group) and 10 healthy bitches (free of any mammary lesions=control group) were used in this study. All the dogs included to the study were in anoestrus. Mammary lesions of experiment group were surgically removed and 10 normal mammary tissue samples were collected by surgical biopsy from control group dogs. Fifty-seven mammary tissue samples were obtained and were divided into two fragments for histopathology/immunohistochemistry and for hormone determinations in tissue homogenate. Besides, blood samples were collected from all dogs before the surgeries to detect hormonal variations. Student t-test was used for serum samples, one way ANOVA and tukey test were used in tissue homogenate for statistical analysis. According to current study results; PRL levels in serum were found significantly higher ($P<0.01$) in the experiment group compared to the control group. In tissue homogenate samples, T levels were found to be significantly ($P<0.01$) higher in malignant mammary tissue samples. PR was intensely expressed in 23, PRL-R was expressed in 13, ER α was expressed in 9 and AR was expressed in 6 cases of canine malignant mammary tumours (CMTs). In conclusion, PRL levels in serum, T in tissue can act on the formation of canine mammary tumours which may have different features of developments in different mammary glands of the same dog and with the predominance of PR expression in CMT.

Keywords: Prolactin, Receptor distribution, Serum, Steroid hormone, Tissue homogenate

Meme Tümörlü Köpeklerde Serumda, Doku Homojenatında Prolaktin ve Steroid Hormon Seviyeleri ile Reseptör Dağılımlarının Belirlenmesi

Özet

Bu çalışmanın amacı östron sülfat (SO₄E1), progesteron (P4), östradiol 17-beta (E2), testosteron (T) ve prolaktin (PRL) seviyeleri değişikliklerinin serumda ve doku homojenatında, androjen (AR), östrojen alfa (ER α), progesteron (PR) ve prolaktin (PRL-R) reseptör dağılım değişikliklerinin köpeklerde meme tümörlerinde saptanmasıdır. Değişik ırk ve yaşta meme lezyonlu otuz köpek (çalışma grubu) ve 10 sağlıklı köpek (herhangi bir meme lezyonu olmayan=kontrol grubu) çalışmada kullanıldı. Çalışmaya katılan tüm köpekler anöstrustaydı. Çalışma grubu köpeklerin meme lezyonları cerrahi olarak uzaklaştırıldı ve 10 normal meme dokusu örneği kontrol grubu köpeklerden cerrahi biyopsi ile toplandı. Elli-yedi meme dokusu örneği toplandı, histopatoloji/immunohistokimya ve doku homojenatında hormon değişikliklerinin saptanması için iki parçaya ayrıldı. Bunun dışında, tüm köpeklerden ameliyat öncesi kan örnekleri toplandı. Serum örnekleri için student t-test, one way ANOVA ve tukey testi de doku homojenatında istatistik analiz için kullanıldı. Bu çalışmanın sonuçlarına göre, serumda PRL seviyeleri çalışma grubunda önemli olarak ($P<0.01$) yüksek bulundu. Doku homojenatı örneklerinde, T seviyeleri kötü huylu meme dokusu örneklerinde önemli olarak ($P<0.01$) yüksek bulundu. Kötü huylu köpek meme tümörlerinde (KMT) PR 23, PRL-R 13, ER α 9 ve AR 6 olguda yoğun olarak ifade edildi. Sonuç olarak serumda PRL, dokuda T seviyeleri köpek meme tümörü oluşumunda rol oynayabilir bu da kötü huylu meme tümörlerinde PR baskınlığı ile aynı köpekte farklı meme bezlerinde farklı yapıda gelişimlerin oluşumunda etkili olabilir.

Anahtar sözcükler: Prolaktin, Reseptör Dağılımı, Serum, Steroid Hormon, Doku Homojenatı



İletişim (Correspondence)



+90 212 4737070/17137



soapaydin@hotmail.com

INTRODUCTION

Mammary tumours are the most common type of tumour in female dogs, women and cats [1-7]. Mammary tumours mostly affect middle-aged and elderly bitches [4], between the age 6 to 10 [3], median age; 10 to 11 years [6]. Approximately 53.3% of the mammary tumours in bitches are malignant [3,6,7]. Although the aetiology of canine mammary tumours is not clear, steroid hormones, some growth factors and their receptors were reported to be responsible for the occurrence of this entity [6]. High amounts of SO4E1 detected in canine inflammatory mammary carcinoma (IMC) can be due to the conversion of dehydroepiandrosterone (DHEA), androstenedione and testosterone to oestrone and oestradiol and oestradiol into SO4E1 by enzymes aromatase, steroid sulphatase and estrogen sulfotransferase respectively [8]. Estrogens produced by the tumour can act as mitogen and promote tumour growth [9]. Normal and tumoural mammary tissues contain and produce several forms of androgens [9]. Androgens may affect mammary tumour formation by binding to AR or indirectly through their transformation to estradiol [8]. The mammary tumours in dogs may be multiple and may have different histological features within or among the different tumour sites [3]. Immunohistochemistry (IHC) is an assistant diagnostic method evaluating the degree of malignancy in the tumours including canine mammary tumours [10-12]. The aim of the present study is to determine variations of estrone sulphate (SO4E1), progesterone (P4), 17-beta estradiol (E2), testosterone (T) and prolactin (PRL) levels in serum, mammary tissue homogenates as well as variations in the distribution of androgen (AR), estrogen alpha (ER α), progesterone (PR) and prolactin (PRL-R) receptors in mammary tumours of dogs.

MATERIAL and METHODS

Animals

Physical examination and vaginal cytology was performed to all dogs. Only the dogs in anoestrus were included in the study. For this study permission from "Istanbul University Animal Researches and Ethic Committee" was obtained with verdict number; 126, on date: 29.07.2010. All animals in the study (experimental group, n=30) and (the control, n=10) from different breeds were presented to our clinic. The dogs in the experimental group were 5 to 18 years of age, between 3-36.4 kg. Dogs aged 9 to 14 years and between 17-38 kg were used as controls. The age of the dogs used in the current study was decided according to the previous reports about canine mammary tumours. Mammary lesions of the experimental group were surgically removed and 10 normal mammary tissue samples were obtained by surgical biopsy from left inguinal mammary lobes of the control dogs under general anesthesia.

Fifty-seven mammary tissue samples were obtained and were separated in 2 fragments for histopathology (HP)/IHC, and hormonal determinations in tissue homogenates. A sample was frozen in liquid nitrogen, stored in -86°C until homogenization. The other sample was sent to Pathology Department for HP and IHC. Fasted 10 ml blood samples were collected from 40 dogs to evaluate steroid hormones and prolactin levels before operations and serum were stored -20°C until hormone assays. Liquid nitrogen frozen tissue samples were freed from all skin and fat. For 1 mg tissue 10 ml Phosphate- buffered saline (PBS; Ph 7.4) was added and was homogenized with a homogenization device (MICCRA-D1, ART Prozess&Labortechnik GmbH&Co. KG., Germany) until there is no significant tissue mass. After 2000-3000 rpm centrifugation for 20 min supernatants were collected, hormonal analysis was performed. All of the obtained tissues were homogenized except if the tissues had the same histopathological features even collected from different mammary lobes of the same dog only one tissue was chosen and used for homogenization. In serum and tissue homogenization samples SO4E1 (Canine SO4E1 elisa kit, catalog no: CSB-E13430c), P4 (Canine P4 elisa kit, catalog no: CSB-E07285c), E2 (Canine E2 elisa kit, catalog no: CSB-E06846c), T (Canine T elisa kit, catalog no: CSB-E06893c), PRL (Canine PRL elisa kit; catalog no: CSB-E14930c) levels were detected by EIA. While P4, T, SO4E1 were determined in ng/ml, E2 was determined in pg/ml and PRL was determined in μ U/ml both in mammary tissue homogenates and serum samples.

*Dogs were classified in four groups to evaluate serum hormone determinations according to their histopathological features of all mammary lesions: **Group 1:** Dogs with mix mammary lesions (for example; first dog had mastitis, complex adenoma, chronic active mastitis, carcinoma in situ in her different mammary lobes) (n=5); **Group 2:** Dogs with dysplasias; non-tumoral lesions (n=1); **Group 3:** Dogs with malignant mammary tumours in different mammary lobes (n= 24); **Group 4:** Control dogs (n=10); Total (n= 40).*

*Mammary tissues were classified in five groups to evaluate hormonal determinations in tissue homogenates from different mammary lobes of dogs: **Group 1:** Mastitis and chronic active mastitis (n= 2); **Group 2:** Benign mammary tumours (n=3); **Group 3:** Malignant mammary tumours (n=39); **Group 4:** Dysplasias; non-tumoral lesions (n=3); **Group 5:** Control dogs (n=10); Totally (n=57). Statistical measurements were not possible due to small numbers with the same characteristic of tissue samples; Group 1, 2, 4 were therefore combined and Group C (n=8) was generated. Consequently, for tissue homogenization there would be group A (malignant mammary tumours), group B (control dogs) and group C (mastitis, chronic active mastitis, benign mammary tumours, dysplasias and non-tumoural lesions).*

Histopathology and Immunohistochemistry

All mammary gland samples were fixed in 10% buffered formalin and routinely processed. Histopathological diagnosis was established on H&E stained sections according to the WHO's classification for canine mammary tumours and dysplasias [13]. IHC was performed with 38 canine malignant mammary tumours using streptavidin biotin immunoperoxidase method with a commercially available HRP detection kit (ABCAM, ab94705) according to the manufacturer's instructions. Only in one case (25-6), IHC was performed on the section related to lobular hyperplasia and adenosis in order not to extract the case from the study. Antibodies against AR, PR, PRL-R and ER were used to detect the distribution and immunostaining intensity of these antigenic products (Table 1). Immunoreactivity was visualised with diaminobenzidine (DAB, ABCAM, ab94665) and the sections were counterstained with Mayer's Hematoxyline. The substitution of the specific primary antibodies by PBS served as the negative control. The intensity and pattern of immunoreactivity

were determined in 10 areas under 40x magnification. The intensity of staining was scored as weak (+), moderate (++) and abundant (+++).

Statistical Analysis

Student t-test was used for serum samples utilizing SPSS 13.0 programme. One-way ANOVA and tukey test were used in tissue homogenates for statistical analysis.

RESULTS

In this study, serum prolactin levels were found to be higher ($P<0.01$) in the experiment group when compared to the control group. Also in tissue homogenate samples T levels were found to be higher ($P<0.01$) in malignant mammary tumour samples when compared to the control, mastitis, dysplasias, benign mammary tumours and non-tumoural lesions. Prolactin and steroid hormone levels in serum and tissue homogenate samples are given in Table 2, Table 3. In current study, from 40 dogs 57

Table 1. Details of the immunohistochemical reagents used in the study

Tablo 1. Çalışmada kullanılan immunohistokimyasal ayırıcıların detayları

Antigen Marker	Clone	Manufacturer	Dilution	Antigen Retrieval	Incubation Period
AR	441	Abcam	1:25	HIER	1 h at RT
ER α	33	Abcam	1:100	HIER	1 h at RT
PR	0111R	BIOS	1:200	HIER	1 h at RT
PRL-R	39279	Gene Tex	1:500	HIER	1 h at RT

AR: Anti-androgen receptor antibody, ER α : Anti-Estrogen receptor alpha antibody, PR: Anti progesterone antibody, PRL-R: Prolactin antibody, HIER: Heat induced antigen retrieval, RT: Room temperature

Table 2. Prolactin and steroid hormone levels in serum samples

Tablo 2. Serum örneklerinde prolaktin ve steroid hormon seviyeleri

Parameters	Experiment Group (n=30) (mean \pm SE)	Control Group (n=10) (mean \pm SE)	Significance
E2 (pg/ml)	70.93 \pm 12.28	51.51 \pm 14.89	NS
SO4E1 (ng/ml)	119.40 \pm 32.10	93.00 \pm 49.92	NS
P4 (ng/ml)	31.1 \pm 88.17	28.12 \pm 11.22	NS
T (ng/ml)	0.46 \pm 0.15	0.47 \pm 0.26	NS
PRL (μ IU/ml)	402.63 \pm 34.69	202.79 \pm 22.92	**

NS: $P>0.05$; ** $P<0.01$

Table 3. Prolactin and steroid hormone levels in tissue homogenates

Tablo 3. Doku homojenatında prolaktin ve steroid hormon seviyeleri

Parameters	Group A: Malignant Mammary Tumours (n=39) (mean \pm SE)	Group B: Control Group (n=10) (mean \pm SE)	Group C: Mastitis, Chronic Active Mastitis, Benign Mammary Tumours, Dysplasias; Non-tumoural Lesions (n=8) (mean \pm SE)	Significance
E2 (pg/ml)	99.34 \pm 3.80	85.26 \pm 3.03	110.39 \pm 13.09	NS
SO4E1 (ng/ml)	7.43 \pm 1.52	1.72 \pm 0.40	8.61 \pm 1.85	NS
P4 (ng/ml)	0.65 \pm 0.10	0.30 \pm 0.45	0.97 \pm 0.22	NS
T (ng/ml)	0.14 ^b \pm 0.02	0.08 ^b \pm 0.02	0.36 ^a \pm 0.14	**
PRL (μ IU/ml)	65.41 \pm 10.23	31.77 \pm 9.10	32.02 \pm 13.37	NS

Mean values within the same row with different superscript small letters are different, NS: $P>0.05$; ** $P<0.01$

Table 4. The detailed data of the mammary lesions for tissue homogenization and immunohistochemistry								
Tablo 4. Doku homojenizasyonu ve immunohistokimya için meme lezyonlarının detaylı bilgileri								
Bitch n= 40	Lesion Localizations*, Selected Lobes for Homogenization	Lesion Type	Total Tissue Sample (n=57)	Case Number- Mammary Lobes Involved	AR 6/38	ERα 9/38	PR 23/38	PRL-R 13/38
No1	3*, 6*, 7*, 8*	3: Mastitis, 6: Complex adenoma, 7: Chronic active mastitis, 8: Carcinoma in situ	4	1-8	-	+++	+++	-
No 2	8*	8: Lobular hyperplasia and ductal hyperplasia and mastitis and ductal carcinoma (was all detected in the same lobe and evaluated as malignant mammary tumour by pathologists)	1	2-8	+	+	+++	+
No 3	4*, 5*, 8*	4: Ductal hyperplasia, 5: Dermatofibrosarcoma, 8: Carcinoma in situ	3	3-5	-	-	+++	+++
			3	3-8	-	-	+++	-
No 4	3*, 7*, 9*	3: Ductal carcinoma, 7: Simple tubular carcinoma, 9: Benign mix tumour	3	4-7	-	-	+++	---
No 5	4*, 5*	4: Carcinosarcoma, 5: Tubulopapillary carcinoma	2	5-4	-	-	++	-
				5-5	-	-	+++	-
No 6	6*, 7*	6: Ductal Adenoma, 7: Chondrosarcoma and fibroadenomatosis	2	6-7	-	-	-	-
No 7	2*, 5*, 7*	2: Complex carcinoma, 5: Complex spindle carcinoma, 7: Spindle cell carcinoma	3	7-2	-	-	-	+++
				7-5	-	-	-	++
				7-7	++	-	-	+++
No 8 ^a	7*	7: Complex adenocarcinoma	1	8-7	-	+	+	+++
No 9 ^a	5*	5: Carcinosarcoma	1	9-5	-	-	-	+++
No 10 ^a	3*	3: Complex adenocarcinoma	1	10-3	-	+++	+	+++
No 11	6*	6: Anaplastic simple carcinoma	1	11-6	-	-	-	++
No 12	8*	8: Simple tubulopapillary adenocarcinoma	1	12-8	-	-	-	+
No 13 ^a	5*	5: Tubulopapillary adenocarcinoma	1	13-5	-	-	-	+
No 14 ^a	5*	5: Malignant mix tumour	1	14-5	-	+	-	+++
No 15 ^a	6*	6: Complex adenocarcinoma	1	15-6	-	-	-	-
No 16	5*, 7*	5: Simple adenocarcinoma 7: Adenosquamous carcinoma	2	16-5	-	-	+++	-
				16-7	-	-	+	-
No 17	5*	5: Anaplastic simple carcinoma	1	17-5	-	-	-	-
No 18 ^a	5*	5: Complex carcinoma	1	18-5	+	+	++	-
No 19 ^a	5*	5: Tubulopapillary carcinoma	1	19-5	-	-	+++	-
No 20	5*, 7*	5: Complex adenocarcinoma, 7: Tubulopapillary solid adenocarcinoma	2	20-5	-	-	-	-
				20-7	-	-	+++	-
No 21	4*, 8*	4: Simple Solid Carcinoma, 8: Complex carcinoma	2	21-4	-	-	-	-
				21-8	+	+	+++	-
No 22 ^a	4*	4: Anaplastic simple carcinoma	1	22-4	-	-	+++	-
No 23	6*	6: Simple Tubular Carcinoma (MMT)**	1	23-6	-	-	+	-
No 24	1*, 4*	1: Complex carcinoma, 4: Solid carcinoma	2	24-1	-	+	-	+++
				24-4	+	-	+++	-
No 25	6*	6: Lobular hyperplasia and adenosis	1	25-6	-	+	++	-
No 26	2*	2: Simple tubulopapillary adenocarcinoma	1	26-2	-	-	+	-
No 27	4-5**	4-5: Solid adenocarcinoma	1	27/4-5	-	-	-	-
No 28	3*, 6*, 7*	3: Cystic tubular carcinoma, tubulopapillary type complex carcinoma, 6: Adenomatous hyperplasia, 7: Adenomatous hyperplasia and tubular adenocarcinoma	3	28-7	-	-	+++	-
No 29	4-5**	4-5: Tubulopapillary adenocarcinoma	1	29/4-5	+	-	+++	-
No 30 ^a	3*	3: Complex adenocarcinoma	1	30-3	-	-	++	-
Control 1-10	6*	6: Normal Mammary Tissue	10	C-1	-	-	-	-
				C-2	+	+	+++	+
				C-3	-	-	-	-
				C-4	-	+	-	-
				C-5	-	-	-	-
				C-6	-	-	+++	+
				C-7	-	-	-	-
				C-8	-	-	+	-
				C-9	-	+++	++	-
				C-10	-	-	-	-

* 1: right axillar, 2: right thoracal, 3: right cranoabdominal, 4: right caudoabdominal, 5: right inguinal, 6: left inguinal, 7: left caudoabdominal, 8: left cranoabdominal, 9: left thoracal, 10: left axillar mammary glands, ** Tumoral lesions are between these two lobes (4-5), -: Most of the bitches had multiple neoplastic lesions on mammary glands and their neoplasias were all in same features histopathologically, so one neoplastic gland was selected and homogenized, +: weak, ++: moderate, +++: abundant

mammary lesions (47 had different features histopathologically and 10 normal mammary tissue) were obtained. For homogenization all of the obtained tissue samples were used. Mammary lesions, affected mammary lobes, the lobes selected for immunohistochemistry and tissue homogenization were given in [Table 4](#) in detail. Different than homogenization samples the sections on two of the slides for IHC were dissolved during immunohistochemistry (4-3 and 28-3) and we excluded these samples for IHC in [Table 4](#). A statistically significant difference ($P<0.01$) was determined between the control and experimental groups for PRL levels in serum samples. With regard to T level in tissue

homogenate, group C had higher mean concentration when compared to group A and B ($P<0.01$). In this study, PR was intensely expressed in 23, PRL-R was expressed in 13, ER α was expressed in 9 and AR was expressed in 6 cases of canine malignant mammary tumours (CMTs). Coexpression of AR/PRL-R, AR/ER, AR/PR, PR/ER, PR/PRL-R, ER/PRL-R was immunohistochemically found in 2, 3, 5, 7, 4 and 5 of the specimens evaluated within the experimental group, respectively. There was a single case which revealed (+) immunoreactivity with all receptor markers in both groups. The labeling for PR was mostly cytoplasmic ([Fig. 1](#)), whereas the nuclear membrane was also stained with PRL ([Fig. 2](#)), AR, and ER α antibodies.

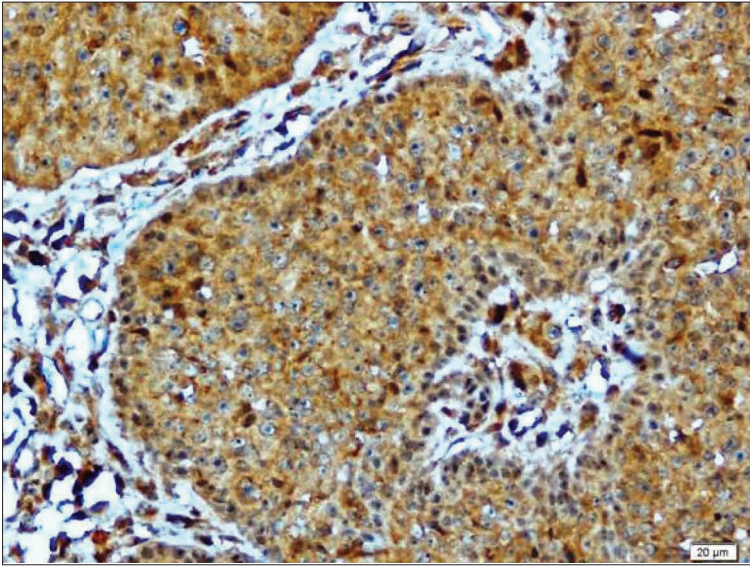


Fig 1. Complex carcinoma, Positive immunoreaction for PR cytoplasmic staining. Streptavidin-biotin immunoperoxidase. Bar: 20 µm

Şekil 1. Kompleks karsinoma, PR'nün sitoplazmik boyanması için pozitif immunreaksiyon, Streptavidin-biyotin immunoperoxidaz. Bar: 20 µm

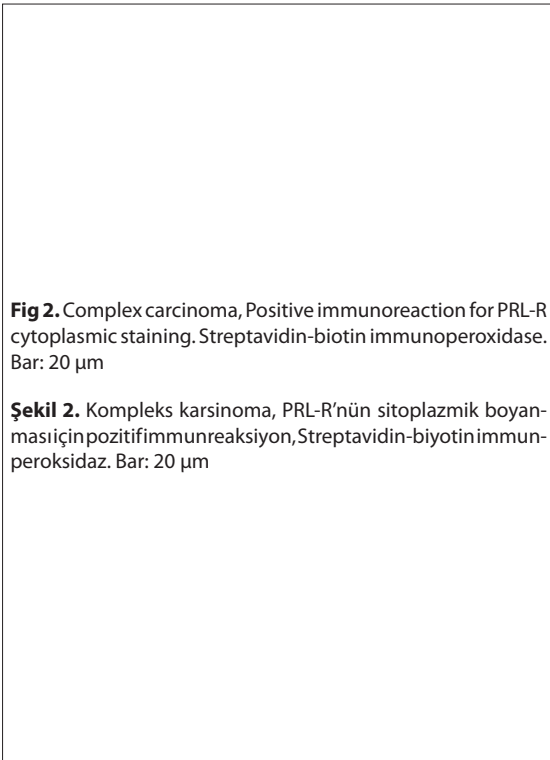
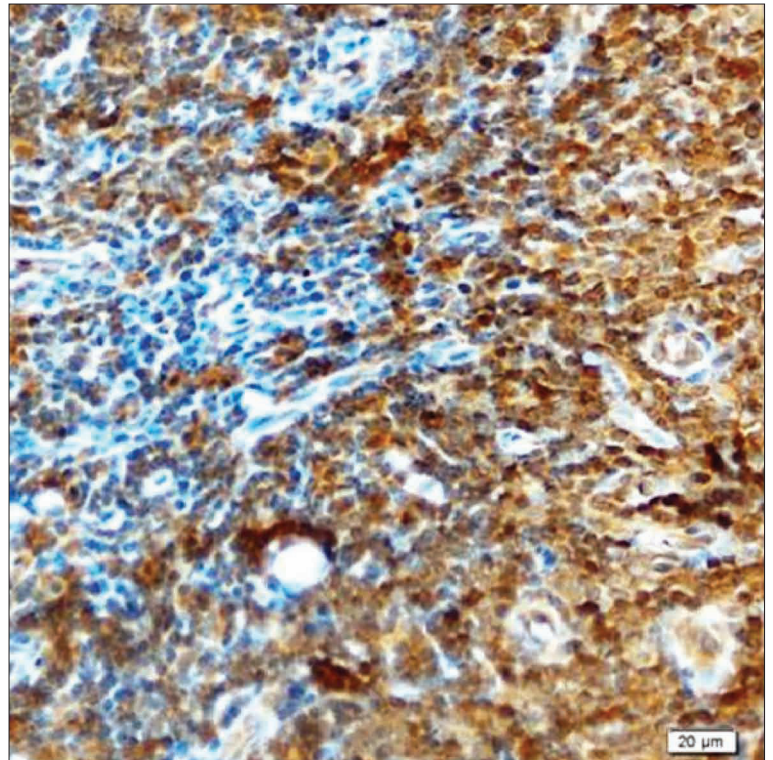


Fig 2. Complex carcinoma, Positive immunoreaction for PRL-R cytoplasmic staining. Streptavidin-biotin immunoperoxidase. Bar: 20 µm

Şekil 2. Kompleks karsinoma, PRL-R'nün sitoplazmik boyanması için pozitif immunreaksiyon, Streptavidin-biyotin immunoperoxidaz. Bar: 20 µm



DISCUSSION

Steroid hormones are important in mammary gland development, possibly in the formation of neoplastic tissue in mammary glands [5]. In the studies conducted in dogs serum E2, SO4E1, T [5,8,14] and P4 [5,8] levels were found higher ($P < 0.01$) in CMTs when compared to the control group. In this study E2, SO4E1 and P4 levels were not found to be different in comparison to the control group. This difference may be arisen due to the malignancy of the tumours obtained from their study of canine inflammatory mammary carcinoma [8,14]; they had compared canine inflammatory carcinoma unlike this study. Serum T level was found to be insignificant in this study in tumoural group when compared to the control group, this result contradicts with Sanchez-Archidona et al. [8] and Illera et al. [14]'s results. They found statistically significant difference between control and tumoural group and they indicated higher serum T concentrations in malignant tumours, this result may be arisen because of the histopathological features of the obtained mammary tumours in their study. Illera et al. [14] reported higher T levels (287.43 ± 6.89 ng/ml) in tissue homogenate samples than this study's results, it might have been arisen because of the tumoural feature (inflammatory mammary carcinoma) of canine mammary tumours. According to Liao and Dickson [15] normal and cancerous mammary tissues contain and produce many kinds of androgens, and Maggiolini et al. [16] reported that androgens have effects on mammary tumour formation through binding directly to AR or indirectly by conversion of androgens to oestradiol. In this study it can be attributed to either androgens not used in the tissue or would be redirected to the blood. We found serum PRL levels of dogs with mammary tumours as 402.63 ± 34.69 μ U/ml, which was significantly higher ($P < 0.01$) when compared to that of the control group (202.79 ± 22.92 μ U/ml). Likewise, Queiroga et al. [5] reported lower serum PRL levels 3.086 ± 0.8 ng/ml in control group when compared to dogs with malignant tumours (5.61 ± 0.85 ng/ml) in which these results were statistically significant ($P = 0.01$). Queiroga et al. [5] reported high PRL levels in malignant mammary tissue homogenates 49.61 ± 5.21 ng/g when compared to dysplasias 16.32 ± 3.0 ng/g, benign tumours 12.72 ± 1.92 ng/g and control tissues 2.43 ± 0.64 ng/g, their findings were significant ($P < 0.01$) statistically, in this study our findings are in line with them 65.41 ± 10.23 μ U/ml in malignant tissue homogenates; 32.02 ± 13.37 μ U/ml in mastitis, benign tumours and dysplasias and 31.77 ± 9.10 μ U/ml was detected in control tissues, respectively but not found statistically significant. Steroid and peptide hormones demonstrate their action by binding to their cognate receptors. In normal mammary tissues of dogs ER, PR and PRL-Rs found previously [1]. Normal and neo-plastic mammary tissues have been reported to express estrogen and progesterone receptors.

The respectively low number of the cases stained positively for ER can be associated with the type of the antigen marker selected for this study. The expression of ER α was found to be lower than that of ER β in a recent study, as well [17]. We consider that the predominance of immunoreactivity for PR confirmed the proliferative effect of progesterone hormone in the development of mammary tumours [18]. Normal canine mammary tissues and benign lesions of the mammary gland were found to express high levels of PRL-Rs. Malignancy is controversially correlated with the expression of this receptor, which was compatible with our findings [19]. The lack of immunoreactivity for AR in most of the cases was associated with the shortfall of the selected clone of the receptor marker applied in this study. Therefore we recommend a different antigenic marker for AR for further studies. Furthermore, our findings revealed a lack of correlation amongst ER, PR, PRL-R and/or AR expression, which could be expected in tumoral tissues [20]. However the relatively low number of the control samples which positively reacted with the receptor markers used in the study can be associated with the insufficient amount of mammary tissues available in the biopsy specimens. In this study different kinds of tumoural or/and non-tumoural lesions were obtained in different mammary lobes of the same dog, as explained in Table 4. However, the researchers did not state the exact lobular localization of the tumoral mass in previous studies [5,9,10,14,21]. Golshahi et al. [22] have used streptavidin-biotin-peroxidase technique for IHC of ER α for chondrosarcoma in mammary gland of a dog and they found this tumour was not expressed ER α like most of the cases in this study. Toniti et al. [20] used ER and PR like in this current study but they used it in different dilutions and they concluded that immunohistochemical studies did not have any correlation to estrogen and/or progesterone receptors expressions in canine mammary tumours. On the basis of our findings it can be concluded that IHC did not aid in the elucidation of the hormonal mechanism of the canine mammary tumors even to some extent although predominance of PR expression was revealed in CMT in the present study and further studies are needed. Besides there was no direct correlation between serum samples, tissue homogenates and immunohistochemical features of the evaluated samples in terms of AR, ER, PR and PRL-R expression. Therefore, it can be stated that steroid hormone levels in the sera and T levels in the tissue might have contributed to the formation of canine mammary tumours. However, only PRL and T effects were found to be significantly higher in serum and tissue homogenate samples. Further studies should be planned to detect the effects of these two hormones on the development canine mammary neoplasia. On the other hand, this study confirmed that there might be different features of developments in different mammary lobes of the same dog and IHC revealed the predominance of PR expression in CMT.

REFERENCES

1. Rutteman GR, Misdorp W: Hormonal background of canine and feline mammary tumours. *J Reprod Fertil Suppl*, 47, 483-487, 1993.
2. Sorenmo KU, Shofer FS, Goldschmidt MH: Effect of spaying and timing of spaying on survival of dogs with mammary carcinoma. *J Vet Intern Med*, 14, 266-270, 2000.
3. Hellmen E: Complex mammary tumours in the female dog: A review. *J Dairy Res*, 72, 90-97, 2005.
4. Millanta F, Calandrella M, Bari G, Niccolini M, Vannozi I, Poli A: Comparison of steroid receptor expression in normal, dysplastic, and neoplastic canine and feline mammary tissues. *Res Vet Sci*, 79, 225-232, 2005.
5. Queiroga FL, Perez-Alenza MD, Silvan G, Pena L, Lopes C, Illera JC: Role of steroid hormones and prolactin in canine mammary cancer. *J Steroid Biochem Mol Biol*, 94, 181-187, 2005.
6. Rivera P, Melin M, Biagi T, Fall T, Haggström J, Lindblad-Toh K, von Euler H: Mammary tumor development in dogs is associated with BRCA1 and BRCA2. *Cancer Res*, 69 (22): 8770-8774, 2009.
7. Hsu WL, Huang YH, Chang TJ, Wong ML, Chang S-C: Single nucleotide variation in exon 11 of canine BRCA2 in healthy and cancerous mammary tissue. *Vet J*, 184 (3): 351-356, 2010.
8. Sanchez-Archidona AR, Jimenez MA, Perez-Alenza D, Silvan G, Illera JC, Pena L, Dunner S: Steroid pathway and oestrone sulphate production in canine inflammatory mammary carcinoma. *J Steroid Biochem Mol Biol*, 104, 93-99, 2007.
9. Pena L, Silvan G, Perez-Alenza MD, Nieto A, Illera JC: Steroid hormone profile of canine inflammatory mammary carcinoma: A preliminary study. *J Steroid Biochem Mol Biol*, 84, 211-216, 2003.
10. Gatter KC: Diagnostic immunohistochemistry: Achievements and challenges. *J Pathol*, 159, 183-190, 1989.
11. Folpe AL: Practical applications of immunohistochemistry in the diagnosis of soft tissue neoplasms. *Surg Oncol*, 8, 197-203, 1999.
12. Folpe AL, Gown AM: Immunohistochemistry for analysis of soft tissue tumors. In, Weiss SW, Goldblum JR, Sharon WW (Eds): *Enzinger and Weiss's Soft Tissue Tumors*. 4th ed., 199-245, Mosby, St Louis, USA, 2001.
13. Misdorp W, Else R, Hellmen W, Lipscomb TP: *Histological Classification of Mammary Tumors of the Dog and Cat*. Second series, Vol. VII. 11-29, Published by the Armed Forces Institute of Pathology, Washington, DC, 1999.
14. Illera JC, Perez-Alenza MD, Nieto A, Jimenez MA, Silvan G, Dunner S, Pena L: Steroids and receptors in canine mammary cancer. *Steroids*, 71, 541-548, 2006.
15. Liao DJ, Dickson RB: Roles of androgens in the development, growth and carcinogenesis of the mammary gland. *J Steroid Biochem Mol Biol*, 80, 175-189, 2002.
16. Maggiolini M, Donze O, Jeannin E, Ando S, Picard D: Adrenal androgens stimulate the proliferation of breast cancer cells as direct activators of estrogen receptor α . *Cancer Res*, 59, 4864-4869, 1999.
17. Ginsburg E, Vonderhaar BK: Prolactin synthesis and secretion by human breast cancer cells. *Cancer Res*, 55 (12): 251-255, 1995.
18. Shaw-Bruha CM, Pirruccello SJ, Shull JD: Expression of the prolactin gene in normal and neoplastic human breast tissues and human mammary cell lines: Promoter usage and alternative mRNA splicing. *Breast Cancer Res Treat*, 44, 243-253, 1997.
19. Michel E, Feldmann SK, Kowalewski MP, Bley CR, Boos A, Guscelli F, Reichler IM: Expression of prolactin receptors in normal canine mammary tissue, canine mammary adenomas and mammary adenocarcinomas. *BMC Vet Res*, 8 (72): 2-8, 2012.
20. Toniti W, Buranasinsup S, Kongcharoen A, Charoonrut P, Puchadapirom P, Kasordorkbua C: Immunohistochemical determination of estrogen and progesterone receptors in canine mammary tumors. *Asian Pac J Cancer Prev*, 10 (5): 907-911, 2009.
21. Pena L, Silvan G, Perez-Alenza MD, Rodriguez-Bertos A, Nieto A: Canine inflammatory mammary carcinoma: Histopathology, immunohistochemistry and clinical implications of 21 cases. *Breast Cancer Res Treat*, 78, 141-148, 2003.
22. Golshahi H, Tavasoly A, Rezaie A, Farhadi M: Immunohistochemical and histopathological findings of chondrosarcoma in mammary gland of a dog: A case report. *Kafkas Univ Vet Fak Derg*, 19 (6): 1053-1056, 2013. DOI: 10.9775/kvfd.2013.9215