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Bitches with Mammary Tumors: the Role of Melatonin, Melatonin Receptor 1A and Tumor Apoptosis

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ABSTRACT

***Background*:** Melatonin (N-acetyl-5-methoxytryptamine) is the main biologically active substance secreted by the pineal gland. The effects of melatonin depend on its binding to specific receptors in target tissues. Melatonin can directly affect tumor cells and affect their proliferation rates. Studies on canine mammary tumor cell cultures have reported that melatonin reduces cancer cells. Most of the physiological effects of melatonin are mediated by its interaction with 2 high-affinity G protein coupled rectors, termed melatonin receptors type 1 and type 2. Mammary tumors, most of which are malignant, are the most common neoplasms in intact bitches. Estimation of the proliferation potential of a tumor is useful for determining tumor malignancy. Also, Ki-67 is the preferred biomarker to determine proliferation and apoptosis in many types of canine mammary tumors.

***Materials, Methods & Results*:** It was aimed at determining the difference in the amounts of melatonin (MEL) and melatonin receptor 1A (MTR1A) in the mammary tissues with different tumor types and to reveal its relationship with the tumor development mechanism, evaluating by apoptotic index (AI) and Ki-67 index. The study groups consisted of malignant epithelial tumors (Group MET; n=36) and hyperplasia & dysplasia (Group H&D; n=23). The samples were evaluated in 2 periods: Spring-Summer (SSP) (Group MET; n=23, Group H&D; n=7) and Autumn-Winter (AWP) (Group MET; n=13, Group H&D; n=16). MEL level in group MET was higher than in group H&D in both seasonal periods ($P < 0.01$). In Group MET, a significant rise in MEL level was observed in SPP than in AWP ($P < 0.05$). MTR1A level in group H&D was higher than in group MET in both seasonal periods ($P < 0.05$). In Group H&D, MTR1A level in SSP was higher than in AWP ($P = 0.05$). In Group MET, a significant rise in MTR1A level was observed in AWP than in SSP ($P < 0.05$). The Ki-67 index in Group MET was higher compared to group H&D in both seasonal periods ($P < 0.001$). In Group H&D, Ki-67 index in AWP was higher than in SSP ($P < 0.001$). In Group MET, a significant rise in Ki-67 index was observed in SSP than in AWP ($P < 0.001$). The AI value of the MET group tended to be higher than that of the H&D group ($P = 0.07$). In Group H&D, AI in AWP was higher than in SSP ($P < 0.05$). In Group MET, a significant rise in AI index was observed in SSP than in AWP ($P < 0.05$).

***Discussion*:** Clinical similarities between human and canine mammary tumors (CMT) include age of onset, hormonal etiology, and disease course. In this respect, this study, which evaluated the amounts of Ki-67, AI, melatonin, and MTR1A in canine mammary tissue, serves as a role model for human breast cancer studies and provides important data, particularly regarding the amounts of endogenous melatonin and MTR1A in canine mammary tumor tissue. Ki-67 index values and endogenous melatonin concentrations were found to be higher in malignant epithelial tumors than in hyperplasia-dysplasia tumors, while AI values tended to be higher in malignant epithelial tumors. Furthermore, MTR1A concentrations were found to be higher in hyperplasia-dysplasia tumors than in malignant epithelial tumors. Overall, the findings indicate that endogenous melatonin levels and melatonin receptor expression are influenced by both seasonal rhythms and tumor biology; however, the role of melatonin within malignant mammary tissue appears to be a potential yet not fully elucidated modulatory effect.

Keywords: canine, apoptosis, dog, Ki-67, mammary tumor, melatonin.

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INTRODUCTION

The pineal gland is a photoneuroendocrine transducer that transmits light information into humoral signals [6]. In humans, melatonin has been reported to control mammary gland development and down-regulate some pituitary and gonadal hormones responsible for the growth of hormone-dependent breast tumors [6,42]. It has been reported that the pineal hormone melatonin may protect against breast cancer and that melatonin works through receptors and different second messenger pathways to reduce cellular proliferation and induce cellular differentiation [21]. Kaplan *et al*. [28] reported in a study that melatonin has oncostatic and antiproliferative effects. There are many reports on the apoptosis-inducing effects of melatonin on cancer cells, unlike the protection of healthy cells [2,43]. Recent studies on canine mammary tumor cell cultures have reported that melatonin reduces cancer cells [34,45]. In apoptosis induction, melatonin and interleukins have been reported to exhibit anti-angiogenic potential in canine mammary tumor cell cultures in addition to a direct anti-tumor effect [17]. Lopes *et al*. [34] determined that melatonin decreased neoplastic mammary cell proliferation and viability and induced apoptosis in canine mammary tumors. Ki-67 is a proliferation marker in canine mammary tumors, and its increased levels have been associated with metastasis, high tumor grade, poor prognosis, and poor survival [29,39].

It was aimed to determine the amounts of melatonin and MTR1A in tumor tissue in bitches with mammary tumors according to the tumor type and histopathological grading of the tumor and to evaluate their relationship with tumor apoptosis and tumor proliferation.

MATERIALS AND METHODS

Sampling

A total of 106 mammary tissues taken from 19 bitches with mammary tumors were evaluated in the study. All animals were living indoors in Istanbul, Turkey (latitude 41°N, longitude 29°E). The ages of the bitches in this study ranged between 8-14 years. The breeds were identified in accordance with the size classification of Salt *et al*. [44]. The study groups consisted of toy, small and medium breed dogs weighing between 5 and 15 kg. Malignant mixed tumors and malignant mesenchymal tumors (sarcomas) detected in histopathological examination were not included in the study. The study groups consisted of malignant epithelial tumors (Group MET; n=36) and hyperplasia & dysplasia (Group H&D; n=23). In order to ensure that the melatonin and MTR1A results were not affected by seasonal differences (daylight exposure time) and to ensure uniformity in the results, the collected samples were evaluated in 2 periods as Spring-Summer and Autumn-Winter. Bilateral mastectomy was performed for all bitches in this study. Tissue samples with a diameter of 0.5-1 cm were taken from the mammary tissue obtained after total mastectomy to be used in ELISA analysis. Histopathological analysis was performed on the remaining mammary tissue.

ELISA analysis

Melatonin and melatonin receptor 1-A (MTR1A) (amounts in mammary tissues were determined by ELISA method[1]. Tissue samples were cut into small pieces, up to 500 mg each. Mammary tissue samples were homogenized with Phosphate Buffered Saline (PBS) in accordance with the kit insert. The homogenization process was carried out in ice using ultraturax[2]. Melatonin (Catalog number: MBS2606798) and MTR1A (Catalog number: MBS7205848) measurements were performed by considering the commercially available canine specific ELISA kits[1] by using MicroQuant ELISA system[3]. The assay was performed according to the manufacturer's instructions and assayed in duplicate. Six samples were measured repeatedly in order to determine the coefficients of variance within and between tests. The sensitivity of Melatonin and MTR1A ELISA kits were >5 pg/mL and 0.1 ng/mL; respectively. Intra assay coefficient of variation (intra CV) values for melatonin and MTR1A were calculated as 4.11% and 6.78%; respectively. Inter assay CV values for melatonin and MTR1A were 7.52% and 9.85%; respectively.

Histopathological analysis

Histopathological examination of the total mastectomy material was performed in the Pathology Department. Mammary tumor tissues were fixed with 10% buffered formalin solution for 48 h. Samples were then subjected to routine tissue processing procedures, paraffin blocks were prepared, 5 µm thick sections were cut and stained with Hematoxylin & Eosin (H&E). Sections were examined under a light

microscope[4] and each case was named according to the canine mammary tumor classification of Goldschmidt *et al*. [18] and graded according to the grading system of Pena *et al*. [40].

Immunohistochemical analysis

Immunohistochemistry was performed to evaluate the proliferation of neoplastic tumor cells with Ki-67, a marker of proliferation in breast tumors [46]. Each case was also labeled with Ki-67 antibody using immunohistochemistry (IHC) technique. Thick sections (5 μm) were cut on positively charged slides and incubated in a 65°C dry oven and then in xylene, deparaffinized, passed through ethyl alcohol from high to low (100%, 96%, 85%, 70%, respectively) and the sections were rehydrated by taking them into distilled water. Then, they were washed 3 times with 0.1 M phosphate buffer solution (PBS; pH7.4). In order to block endogenous peroxidase activity in the tissues, they were kept in 0.3% H_2O_2 prepared in methanol at room temperature for 20 min. The sections washed with PBS 3 times again were heat treated in a microwave oven for 20 min in pH 6.0 citrate buffer solution in accordance with the antibody protocol and the antigen exposure process was completed. After the antigen exposure process, protein blocking was performed on the sections washed with PBS with the serum cocktail included in the kit (Mouse and Rabbit Specific HRP/DAB IHC Detection Kit - ab236466 Micro-polymer) used in IHC staining[5]. Then, Ki-67 antibody[6] (MIB-1 Clone- M7240) was diluted 1/200 and incubated for 90 min at 37°C. After the primary antibody incubation was completed and the sections were washed with PBS, the secondary antibody with HRP was incubated for 10 min according to the protocol of the IHC kit used. Washing was performed again, and the reaction was completed with 3,3'-diaminobenzidine (DAB). After the IHC staining was completed, the sections were then stained with Mayer Hematoxylin for 5 min for background staining and then covered with a coverslip. Visual assessment of the Ki-67 proliferation rate was performed using a light microscope[4] at 40X BA. In each case, a total of 500 tumor cell nuclei were counted in the visually selected hot spot areas, starting from the group of 100 cell nuclei that were seen to have the highest percentage of Ki-67 positive cells. The number of positively stained tumor cell nuclei was recorded [52]. The Ki-67 index was calculated by multiplying the number of positive cells by 100 and then dividing by the total number of cells, which is 500. Brown nuclear staining observed in neoplastic cells was evaluated as immunopositive. The percentage of positive cells was evaluated, and the Ki-67 proliferation index was determined.

Detection of fragmented DNA in situ

To detect the break in the DNA chain, the same paraffin sections prepared and taken according to routine methods were stained with the Terminal deoxynucleotidyl transferase (TdT)-mediated nick end-labeling (TUNEL) method in accordance with the procedure of the kit (HRP-DAB-ab206386)[5] used. The sections previously taken on positively charged slides were deparaffinized and dehydrated and treated with Proteinase K for 20 min. Then, they were incubated in 3% H_2O_2 methanol for 5 min, in TUNEL mixture at 37ºC for 90 min, and in Conjugate and Blocking Buffer for 10 min to complete the reaction and Diamino benzidine (DAB) was dropped. The background stain was made with methyl green dye. Apoptotic index (AI) was determined in microscopic evaluation. For this purpose, the cells on the background and TUNEL positive cells labeled with DAB were counted in 10 separate microscope fields with a 40x magnification objective. After averaging, the number of positive cells was multiplied by 100 and divided by the total number of background cells to determine the AI [52].

Statistical analysis

Total sample size was determined by power analysis with G-Power version 3.1.9.7[7]. Calculations revealed that total sample size of n = 56 would be sufficient to provide 90% power with 0.05 α-error probability. Statistical analysis was performed[8] using SPSS Statistics 25.0 program. Two-way Anova method was used for statistical analysis of Ki67 index, AI, MEL, MTR1A data. Group (H&D and MET), season (summer and winter) and group x season interaction were included as fixed effects in the statistical model. The relationship between the Ki67 index, AI, MEL, MTR1A was evaluated with Pearson correlation test. The values were given as mean and standard error of mean (SEM). The significance was accepted as $P < 0.05$.

RESULTS

A total of 19 intact bitches with mammary tumor were enrolled in this study. All bitches were sexually intact. The mean ages and weights of the bitches were

11.05 ± 0.60 years and 10.38 ± 1.03 kg, respectively. The breeds included in this study were toy size (n=5; Yorkshire terrier, Maltese terrier, Miniature Pinscher), small size (n=4; Jack Russell Terrier, Pekingese), medium size (n=10; Fox Terrier, Pug, Boston Terrier, American Cocker Spaniel, Beagle). Five of 19 dogs had pulmonary metastasis and 4 of 19 dogs had regional lymph node metastases. Totally, 36 masses were constituted Group MET while 23 masses were constituted Group H&D, regardless of the season. Thirty masses were collected during the Spring-Summer season. Twenty-tree of 30 masses were from Group MET and 7 of 30 masses were from Group H&D. Twenty-nine masses were collected at Autumn-Winter season. Thirteen of 29 masses were from Group MET, and 16 of 30 masses were from Group H&D. The mass size in Group MET (n=36) was evaluated in 2 groups as T1 (<3 cm; n=30) and T2+T3 (≥3 cm; n=6), due to the insufficient numbers both in T2 (3-5 cm) and T3 (>5 cm). The corresponding numbers of histological grades in Group MET were grade 1 (G1; n=19) and grade 2+3 (G2+G3; n=17). Comparison of the groups according to Ki-67, AI, MEL, and MTR1A levels regardless of the season was presented at Table 1. Also, relationship between the evaluated parameters was given at Table 2.

Histopathological findings

Histopathological evaluation of a total of 59 mammary tissue samples identified non-neoplastic lesions and various types of mammary tumors. Tumoral lesions were as follows: Complex carcinoma (n=7), simple carcinoma (n=22), carcinoma-mixed type (6), and solid carcinoma (n=1) constituted Group MET. Among the non-neoplastic cases, a total of 23 cases were identified, including, lobular hyperplasia (n=16), fibroadenomatous changes (n=4), and ductal ectasia (n=3); constituted Group H&D.

Lobular hyperplasia, the most common non-neoplastic lesion, was observed in regular or lactational forms. In these lesions, the mammary glands were found to be numerically enlarged, and the gland cells exhibited secretory activity, accumulating proteinaceous secretions in the lumen (Figure 1A & B). In cases with ductal ectasia, the lactiferous ducts were found to be significantly enlarged. In cases with fibroadenomatous changes, histopathological findings revealed mild epithelial hyperplasia of the glands, along with marked fibroplasia around the glands (Figure 1C & D).

The 1st case, with tumoral lesions generally lined by 1 or 2 layers of pleomorphic luminal epithelial cells and tubular formation, was diagnosed as tubular carcinoma (Figure 2A). The nuclei were hypochromic and large, with single and prominent nucleoli. The cells generally had eosinophilic cytoplasm, and the cell borders were relatively distinct. Mitotic activity was low. Occasionally plasma cells, lymphocytes, and macrophages were noted in the intertubular stroma. The 2nd case, which included papillary projections in addition to tubular formation and had other microscopic features similar to the 1st case, was diagnosed as tubulopapillary carcinoma (Figure 2B). Another type of tumor, complex carcinoma cases, exhibited areas of irregular tubular luminal epithelium as well as benign myoepithelial areas. The 2nd component, myoepithelial areas, appeared as irregular fibrillar bundles composed of spindle cells interspersed with basophilic myxoid material (Figure 2C). Mitotic activity was low in both complex carcinomas. In tumors identified as carcinoma-mixed type, malignant luminal epithelium forming irregular tubules, benign myoepithelial areas, and benign cartilage foci were detected (Figure 2D). In the group diagnosed with solid carcinoma, histopathological findings revealed highly pleomorphic tumor cells arranged as areas without tubules, and hyperchromatic nuclei and mitotic figures were frequently observed.

Immunohistochemistry and TUNEL staining findings

A positive Ki-67 reaction is readily identified by the distinct brown nuclear staining produced by DAB. While the mean Ki-67 index was generally lower in non-neoplastic mammary lesions, it was significantly higher in tumor cases (Figure 3). Similarly, apoptotic cells were readily identified by their dark brown to black staining against the light green background (Figure 4). Similar to the Ki-67 reaction, the mean AI was lower in non-neoplastic mammary lesions than in tumor cases in samples collected between Spring-Summer season. However, in samples from Autumn-Winter season, the mean AI values were similar in non-neoplastic breast lesions and tumor cases.

Evaluation of interaction effects and comparison of the groups in terms of Ki-67, AI, MEL, and MTR1A levels

Comparison of the groups between and within the season in terms of Ki-67, AI, MEL, and MTR1A levels were given at Table 3. The effect of group was important in Ki-67 index while season and group vs

season effect were insignificant. Insignificant interaction effect between group and season revealed that the significant difference between H&D and MET will be valid in both seasons. The significance of Ki-67 between the seasons within Group H&D and Group MET were similar due to the insignificant interaction effect. There are no significant changes in Ki-67 index between the seasonal changes within the groups ($P > 0.05$). However, Ki-67 index in Group MET was higher than in Group H&D in both seasons ($P < 0.001$).

The effect of groups tended to be significant in terms of AI but the effect of season was insignificant. The interaction effect of group vs season is also significant. AI value in Group MET tended to be higher than in Group H&D ($P = 0.07$). There were no significant changes in AI values between the seasons ($P > 0.05$). The significance of interaction effect (group vs season interaction effect) indicates that there was a difference in the effect of the group between Spring-Summer and Autumn-Winter seasons. While AI value in Group MET (6.03 ± 0.99) was higher than in Group H&D (1.80 ± 0.13) in Spring-Summer season ($P < 0.05$), this increase was not observed in the Group MET in Autumn-Winter season (3.57 ± 0.36 vs. 4.26 ± 0.64; $P > 0.05$). AI value was higher in Spring-Summer season than in Autumn-Winter season in Group MET, while AI value was lower in Spring-Summer season than in Autumn-Winter season in Group H&D.

The effect of season on MEL levels was not significant. The effect of the group was found to be significant in terms of MEL, and MEL level in Group MET was significantly higher than in Group H&D. Group vs season interaction effect on MEL level was not significant. This result indicates that the insignificant difference between Spring-Summer and Autumn-Winter seasons in terms of MEL levels is valid for both the H&D group and the MET group. At the same time, the significance of the interaction indicates that the significance of the group effect is important in Spring-Summer and Autumn-Winter seasons, that is, the MEL level in the Group MET is higher than the Group H&D in both Spring-Summer and Autumn-Winter seasons.

While the effect of season was found to be insignificant for MTR1A, the interaction effect of group and group vs season was found to be significant. MTR1A levels were higher in Group H&D than in Group MET. The significant interaction effect suggests a difference in the effect of the group across seasons. In Autumn-Winter season, MTR1A level in Group MET was not significantly different from Group H&D ($P > 0.05$). However, in Spring-Summer season, MTR1A level in Group H&D was significantly higher than in Group MET ($P < 0.05$). MTR1A levels in Group H&D were significantly higher in Spring-Summer season compared to Autumn-Winter season ($P < 0.05$). However, MTR1A levels in Group MET were significantly lower in Spring-Summer season compared to Autumn-Winter season ($P < 0.05$).

Interaction effects in Group MET and comparison of mass sizes and histological grades in terms of Ki-67, AI, MEL, and MTR1A levels

Changes in Ki-67, AI, MEL, and MTR1A levels according to mass size and histological grades, regardless of the season, were presented in Table 4. The interaction effect of grade and mass size groups in terms of Ki-67 index was insignificant ($P > 0.05$). The interaction effect of grade on AI tends to be significant ($P = 0.07$), while the interaction effect of mass size is insignificant ($P > 0.05$). However, the interaction effect of grade vs mass size was found to be significant ($P < 0.01$). It is observed that the AI value tends to be higher in the Grade 1 group ($P = 0.07$). No statistical difference was found between the mass size groups in terms of AI values. The significant interaction of grade vs mass size indicates that T1 and T2+T3 groups do not have similar effects in terms of the effect of grade. While there was a significant difference in AI values between grades in the T2+T3 group (G1; 12.20 ± 2.10, G2+G3; 2.92 ± 1.61; $P < 0.05$), G1(4.86 ± 3.97) and G2+G3 (5.88 ± 4.37) appeared to have similar AI values in the T1 group ($P > 0.05$).

The effect of grade tends to be significant in terms of MEL ($P = 0.08$). The interaction effect between mass size and grade vs mass size is significant ($P < 0.05$). The MEL value in the Grade 1 group tends to be higher than in the G2+G3 group ($P = 0.08$). The MEL value in the T2+T3 group was found to be higher than in the T1 group ($P < 0.01$). In Grade 1 tumors, the T2+T3 group (157.78 ± 26.02) had higher MEL values compared to the T1 group (69.20 ± 6.13) ($P < 0.01$), while in the G2+G3 group, MEL values were similar in the T1 (82.55 ± 7.51) and T2+T3 (89.97 ± 11.63) sizes. The interaction effect of grade, mass size and grade vs mass size was insignificant in terms of MTR1A.

Table 1. Comparison of the groups according to evaluated parameters regardless of the season.

Parameters	Group MET	Group H&D	Significance
Ki-67	21.32 ± 2.85[a]	1.6 ± 0.28[b]	< 0.001
AI	5.12 ± 0.67[a]	3.54 ± 0.50[b]	< 0.05
MEL	77.70 ± 4.86[a]	56.13 ± 4.40[b]	< 0.001
MTR1A	0.97 ± 0.12[a]	1.42 ± 0.16[b]	< 0.05

[a,b]Different letters in the same row indicate the significance. AI: Apoptotic index; MEL: Melatonin; MTR1A: Melatonin receptor 1A; MET: Malignant epithelial tumor; H&D: Hyperplasia and dysplasia.

Table 2. Relationship between the parameters (Pearson Correlation test).

Parameters		MEL	MTR1A	Ki-67 Index	Apoptotic Index
MEL	R	1	-.526	.411	.214
	P		.000	.001	.100
MTR1A	R	-.526	1	-.358	-.146
	P	.000		.005	.265
Ki-67 Index	R	.411	-.358	1	.132
	P	.001	.005		.315
Apoptotic Index	R	.214	-.146	.132	1
	P	.100	.265	.315	

MEL: Melatonin; MTR1A: Melatonin receptor 1A; R: Correlation coefficient; *P*: Significance.

Table 3. Comparison of the groups between and within the season in terms of Ki-67, AI, MEL, and MTR1A levels.

Parameters	Groups	Spring-Summer	Autumn-Winter	Significance
Ki-67	H&D	1.11 ± 0.14[a]	1.18 ± 0.39[a]	> 0.05
	MET	22.07 ± 4.12[b]	21.45 ± 3.77[b]	> 0.05
	Significance	< 0.001	< 0.001	
AI	H&D	1.80 ± 0.13[a]	4.26 ± 0.64[b]	< 0.05
	MET	6.03 ± 0.99[c]	3.57 ± 0.36[b]	< 0.05
	Significance	< 0.05	> 0.05	
MEL	H&D	55.54 ± 8.72[a]	56.38 ± 5.24[a]	> 0.05
	MET	86.01 ± 6.16[b]	66.59 ± 7.16[c]	< 0.05
	Significance	< 0.01	< 0.01	
MTR1A	H&D	1.83 ± 0.26[a]	1.25 ± 0.18[b]	< 0.05
	MET	0.81 ± 0.14[c]	1.25 ± 0.23[b]	< 0.05
	Significance	< 0.05	> 0.05	

[a,b,c]Different letters in the same row and column indicate the significance. AI: Apoptotic index; MEL: Melatonin; MTR1A: Melatonin receptor 1A; MET: Malignant epithelial tumor; H&D: Hyperplasia and dysplasia.

Table 4. Changes in evaluated parameters according to mass size and histological grades.

Groups		Ki-67 index	AI	MEL	MTR1A
Grades	G1	19.72 ± 9.10	8.53 ± 2.01	113.49 ± 13.36	0.71 ± 0.38
	G2+G3	23.10 ± 4.71	4.40 ± 1.04	86.26 ± 6.92	0.70 ± 0.19
	Significance	> 0.05	=0.07	=0.08	> 0.05
Mass size (T)	T1	23.51 ± 3.30	5.37 ± 0.72	75.88 ± 4.84[a]	1.04 ± 0.13
	T2+T3	19.32 ± 9.70	7.56 ± 2.14	123.87 ± 14.25[b]	0.46 ± 0.10
	Significance	> 0.05	> 0.05	< 0.01	> 0.05

[a,b]Different letters in the same row and column indicate the significance. AI: Apoptotic index; MEL: Melatonin; MTR1A: Melatonin receptor 1A.

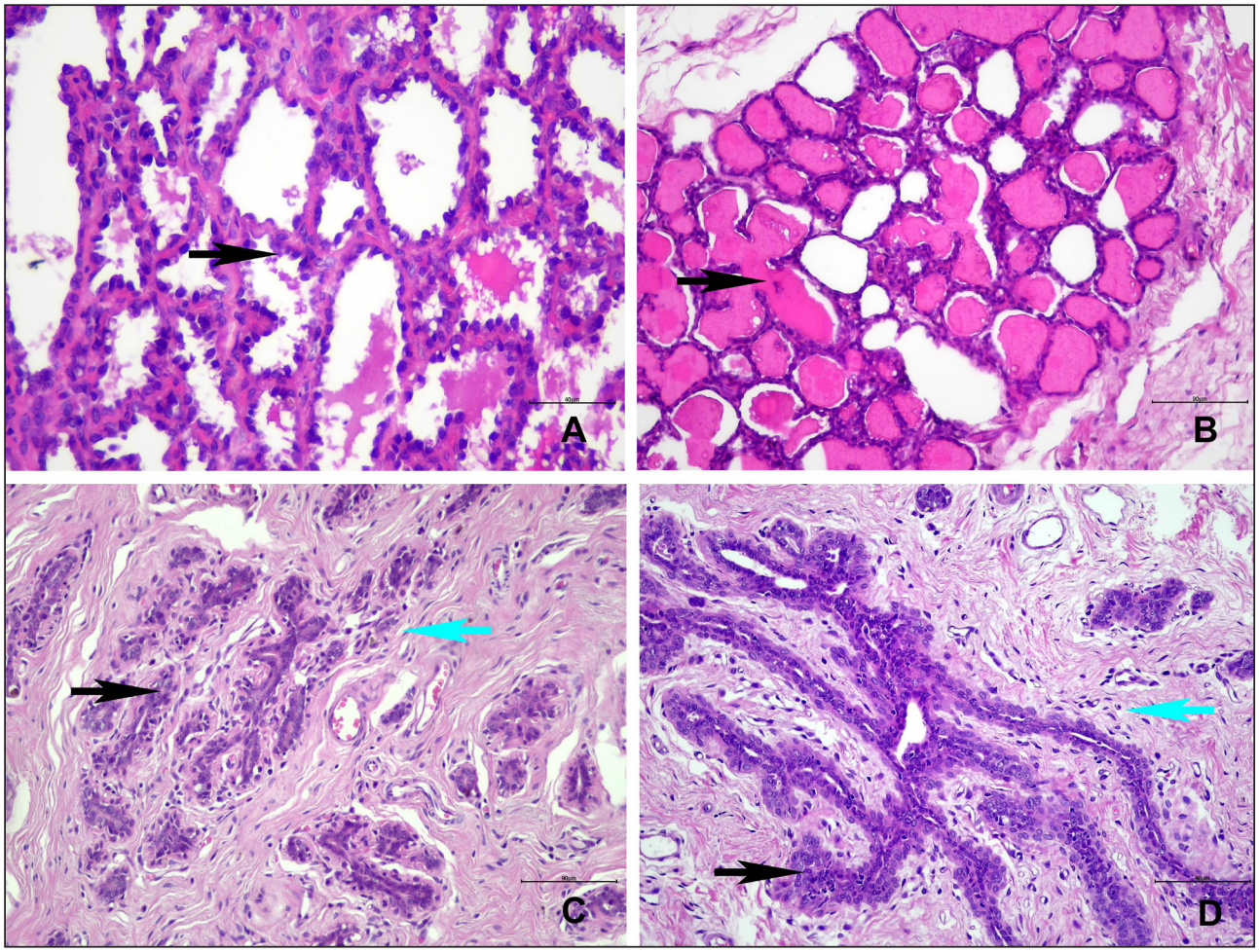

Figure 1. A- Lobular hyperplasia-lactation, mammary gland epithelium contains eosinophilic droplets in the apical portions (arrow). B- Lobular hyperplasia-lactation, mammary alveoli filled with dense proteinaceous material (arrow). C & D- Fibroadenomatous change, mild increase in epithelial (black arrow) and fibrous (blue arrow) tissue in the mammary glands. [Bar= 40 µm (A) and 90 µm (B, C & D); H&E staining].

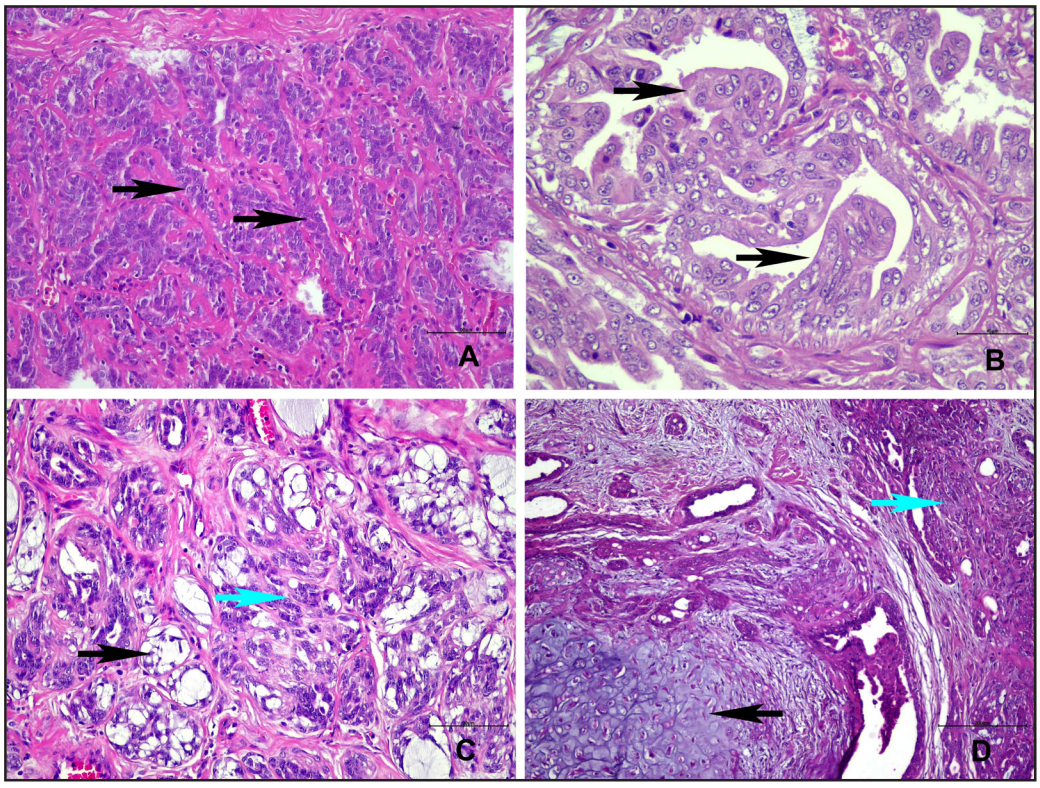

Figure 2. A- Tubular carcinoma case with tubular formations (arrow). B. Tubulopapillary carcinoma case with tubular and papillary formations (arrow). C- Complex carcinoma case with luminal (blue arrow) and myoepithelial (black arrow) components. D- Carcinoma-mixed type case with epithelial (blue arrow) and chondroid (blue arrow) components. [Bar= 40 µm (B), 90 µm (A & C), and 200 µm (D); H&E staining].

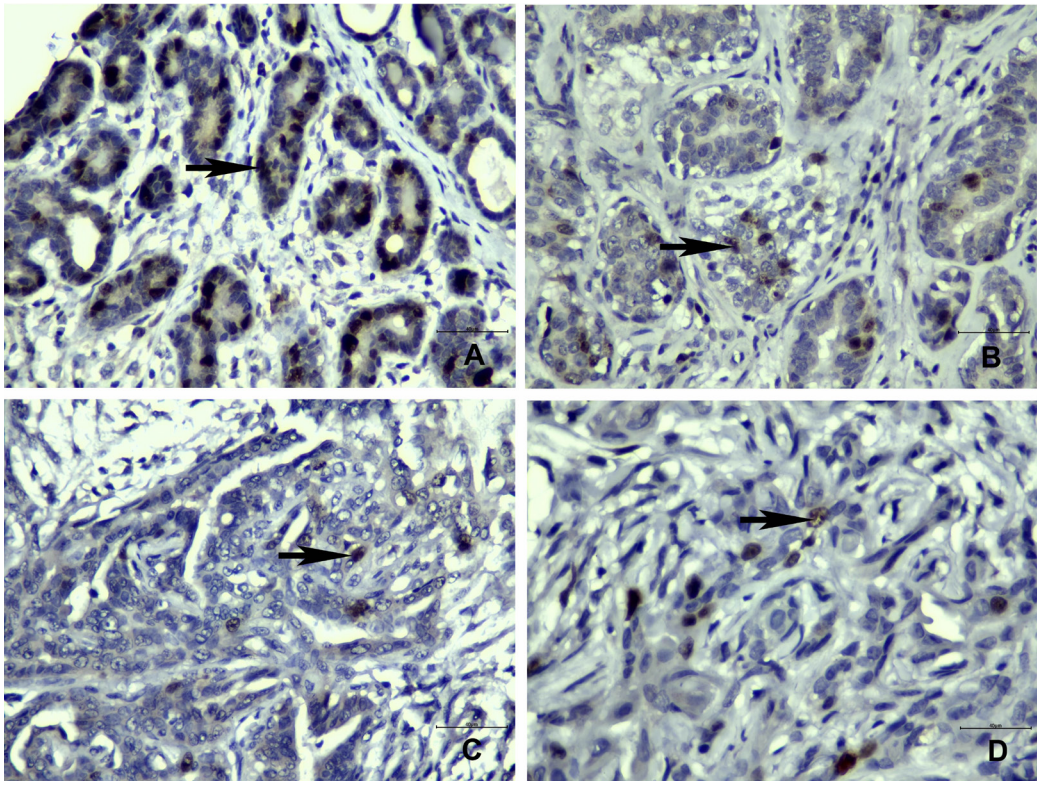

Figure 3. Ki-6 positivity in tubular carcinoma cases A and B, C and D. Ki-67 positivity in carcinoma-complex type cases. [Bar= 40 µm, IHC staining, Chromogen DAB, background stain Mayer hematoxylin].

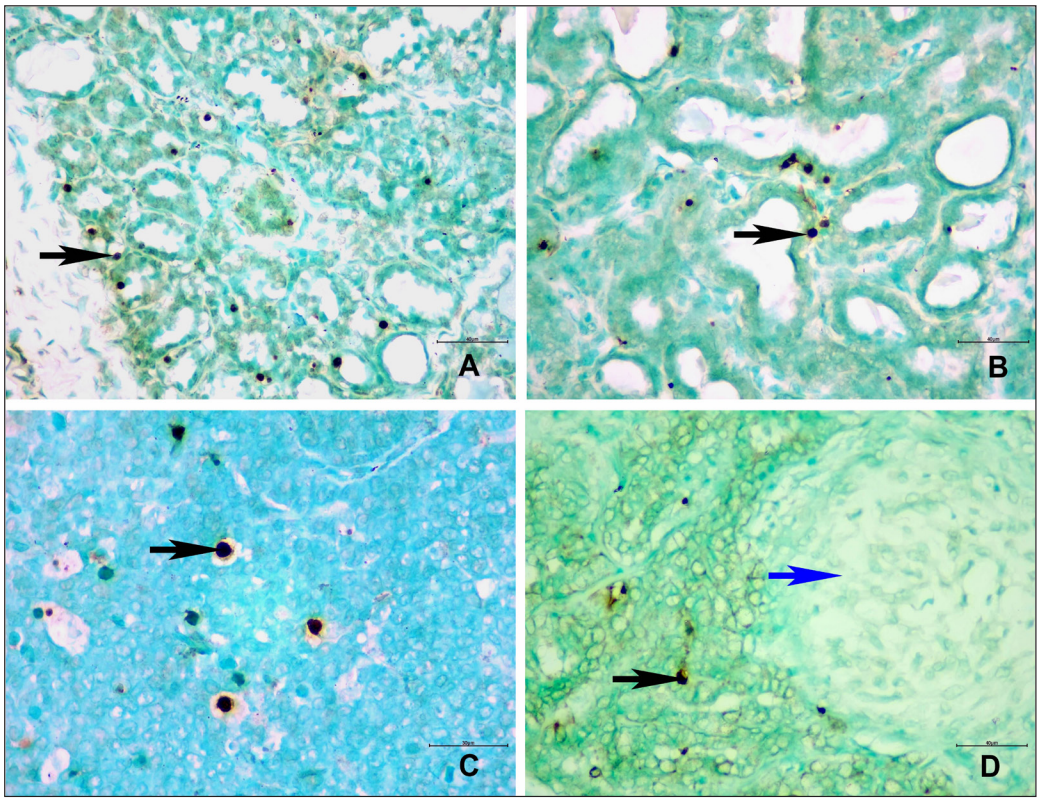

Figure 4. A & B- TUNEL positive reaction showing apoptotic cells in lobular hyperplasia cases (arrow). C- Apoptotic cells in tubulopapillary carcinoma case (arrow). D- Apoptotic cells (black arrow) and mesenchymal component (blue arrow) in Carcinoma-mixed type. [Bar= 40 µm (A, B & D) and 30 µm (C), TUNEL staining, Chromogen DAB, background dye Light green].

DISCUSSION

Mammary tumors, most of which are malignant, are the most common neoplasms in intact bitches, mostly middle-aged and older (8-11 years) [55]. Similarly, malignant epithelial tumors were mostly encountered in bitches with middle-aged (11.05 ± 0.60 years) in this study. Increased incidence of mammary tumors was found in many large as well as smaller breeds of dogs. Poodles, English Cocker Spaniels and Dachshunds have relative risk developing benign and malignant tumors of the mammary gland [55]. In harmony with the previous report, occurrence of mammary neoplasms was dense in medium size breeds.

Evaluation of Ki-67 index

The assessment of tumor-cell proliferation has proved useful for evaluating the biological behavior and potential malignancy of tumors [7]. Proliferation in breast cancer is commonly assessed in routine practice using mitotic counts or the Ki-67 labelling index [24]. High Ki-67 score is associated with poor prognosis [7]. As in humans, tumor proliferative activity, assessed by Ki-67, is a well-recognized prognostic element as demonstrated in several studies on canine mammary carcinomas [3,13,51]. Similarly, proliferative activity of canine mammary tumors was investigated by IHC staining of the Ki-67 antigen, in this study. In fact, a high proliferative index has been associated with disease progression, poor prognosis, and shorter survival times in bitches with mammary carcinomas [4]. The Ki-67 indices for malignant tumors were significantly greater than for either hyperplasia [35] or benign tumors [51] in bitches. Similar with the previous reports, Ki-67 indices in malignant epithelial tumors were significantly higher compared to H&D both in regardless of the season and within the season in this study ($P < 0.001$). This result reflects the efficiency of Ki-67 index for proliferative activity of canine mammary tumors. Proliferative changes within groups were not affected by seasonal changes in either Group MET

or Group H&D ($P > 0.05$). Brunetti et al. [3] investigated immunohistochemical positivity for Ki-67 in relation to histological grade in 170 canine mammary carcinomas. They observed high Ki-67 values were significantly correlated with higher histological grade (Grade 3). However, in present study, Ki-67 index in Grade 2 and Grade 3 tumors were higher than in Grade 1 tumors but it could not reach the significance. This result could be obtained due to the low number of tumors in Grade 2 and Grade 3 tumors. In further studies, it should be investigated with large sample sizes both in Grade 2 and Grade 3 tumors. Estaller et al. [13] stated that tumor volume of neither carcinomas nor sarcomas showed a noticeable influence on serum Ki-67 concentrations when tested by linear regression. Pena et al. [39] indicated that Ki-67 and PCNA were not significantly associated with tumor size, rate of growth, ulceration, or adhesion to underlying tissues in canine mammary tumors. In harmony with the previous reports, amount of Ki-67 index didn't vary in terms of tumor size in this study.

Evaluation of AI

Apoptosis is a strictly controlled programmed cell death with different biochemical and genetic mechanisms that play an important role in tissue homeostasis and maintains the balance between cell survival and cell loss. There is strong evidence that tumor growth is not only related to uncontrolled cell proliferation but also inhibition of apoptosis [38]. Combining both proliferation and apoptosis scores could provide a more precise estimate of tumor growth and hence behavior, and it was reported that they provide better risk stratification in human breast cancer [25]. The apoptotic cell count can be considered as a relation of the percentage of the tumoral cells present, that is, as the number of apoptotic cells per square millimeter of neoplastic tissue, and it is usually designated as the apoptotic index (AI) [41]. TUNEL staining continues to be widely used as a measure of apoptotic cell death [37]. Yildirim *et al*. [52] reported that AI and Bcl-2 protein were not sufficient as dependable markers to estimate biologic behavior and prognosis of canine mammary tumors. As defined many times in previous reports, in this study, AI and Ki-67 evaluated together in canine malignant epithelial tumors and hyperplasia & dysplasia cases, to provide more reliable diagnosis and to estimate biological behaviors of tumor. Also, TUNEL staining was used to measure AI as Mirzayans and Murray [37] reported. Koda *et al*. [31] observed an increased expression of pro-apoptotic Bak in primary tumors of breast cancer compared

with normal breast epithelium and benign dysplasia without intraductal proliferation. De Jong *et al*. [8] found a high AI ductal and medullary tumor types compared to a low AI in the more differentiated tumor types in humans with invasive breast cancer. Kim *et al*. [30] reported that AI is significantly higher in high-grade tumor in comparison to normal mammary gland, adenoma and low-grade tumor in dogs. In present study, AI values in Group MET were significantly higher compared to Group H&D regardless of the season. Our results were supported by Perreira *et al*. [41] who stated that a worse prognosis must be expected when higher number of apoptotic cells are observed present in neoplasia.

Zhang *et al*. [56] detected significantly higher AI in larger tumors with more than 5 cm in diameter than smaller breast tumors in humans. De Jong *et al*. [8] found a high AI to be related to a large tumor size (≥2.5 cm). However, Hadjiloucas *et al*. [23] observed no significant association between AI and tumor size. Funakoshi *et al*. [15] reported that there are no important relationships between AI value of mammary gland tumors and clinical features like metastasis and tumor diameter. Also, Yildirim *et al*. [52] detected no significant association between AI and histopathological diagnosis, grading and staging of the canine mammary tumors. Although AI in T2+T3 sized tumors was higher than in T1 sized tumors in this study, it could not reach the significance as reported previously. This can be explained by the fact that Pereira *et al*. [41] reported that when the AI is high, the tumor grows slower, and when the AI is low, the tumor grows faster.

De Jong *et al*. [8] compared histological grade and AI in invasive breast cancer, and they observed that high histological grade was associated with high amount of apoptosis. The AI in grade 3 tumors was about twice as high as in grade 1 tumors and grade 2 tumors show AI in between these 2 values in invasive breast cancers [8,56]. However, Yildirim *et al*. [52] detected no significant association between AI and histopathological grading of canine mammary tumors. Inconsistent with previous reports [8,56], AI value in G1 tumors tended to be higher compared to G2+G3 tumors ($P = 0.07$) in current study. Also, there was a significant difference in AI values between grades

in the T2+T3 group (G1; 12.20 ± 2.10 and G2+G3; 2.92 ± 1.61; $P < 0.05$), while grades had appeared to have similar AI values in the T1 group ($P > 0.05$). These inconstancies could be related to the levels of initiation, development and progression of the cancers. Epidermal growth factor receptor has an important role in the proliferation in canine mammary tumor cells and epidermal growth factor (EGF) down-regulating its receptor in tumor-growing tissue [48,50]. Therefore, EGF and its receptor may provide a linked role as proliferation and programmed cell death [8]. Also, EGF induces inhibition of cell proliferation and induction of apoptosis [22]. The finding of low degrees of apoptosis (AI) and high levels of proliferation (Ki-67) in highly malignant tumors (G2+G3) can be explained by the role of EGF and its receptor in canine mammary tumorigenesis as previously reported.

Evaluation of MEL

Melatonin is a hormone that is released from the pineal gland into the blood stream and is controlled by nerve impulses from the suprachiasmatic nuclei. Melatonin synthesis, which is inhibited by light on the mammalian retina, peaks in plasma concentrations during the night [11]. Dunlap *et al*. [11] investigated the seasonal and diurnal melatonin production in exercising sled dogs, and they observed that sled dogs in Alaska had lower melatonin levels than sled dogs in New York while winter melatonin levels in both locations were higher than the summer. Highest concentrations of MEL were given at night in winter and were above 15 pg/mL [11]. Zan *et al*. [54] investigated the levels of endogenous melatonin in the blood serum in dogs in different seasons (March, June, September, December) and at different times of day (11:00,12:00, 23:00, 24:00 and 1:00), using immunoassay method. They observed the lowest average concentrations of plasma melatonin during the day in the summer and autumn season, and highest concentrations were during the night at 1:00 in the winter season (12.93 ± 8.27 pg/mL). Considering that melatonin is affected by seasonal changes, our study groups were examined in 2 separate seasonal periods as summer-spring period and autumn-winter period. Additionally, melatonin levels in both groups of the current study were exceedingly higher compared to the highest levels of endogenous melatonin in the blood of healthy dogs in previous reports. The increased levels of melatonin might be obtained due to the presence of pathologies in evaluated mammary tissue samples in this study.

There are neuroprotective, anti-inflammatory, pain-modulating, blood pressure-reducing, retinal, vascular, seasonal reproductive, ovarian physiology, osteoblast differentiation, anti-tumor and antioxidant effects of melatonin [12]. Nowadays, there are several reports on effect of melatonin on cell cultures of canine mammary tumors [5,17,34,45]. Lopes *et al*. [34] analyzed the rate of cell proliferation and apoptosis after treatment with melatonin in primary culture of canine mammary tumors. They suggested that melatonin decreased neoplastic mammary cell proliferation and viability and induced apoptosis with greater efficacy in ER-positive tumors that have a high expression of melatonin receptor (MTR1). It was reported that melatonin decreases in vitro viability and migration of spheres derived from canine mammary carcinoma cells [45]. However, melatonin reduced the degree of breast hyperplasia, reduced the proliferation of hyperplastic breast tissue cells, and promoted cell apoptosis in hyperplastic tissue in humans [57]. To best of our knowledge, it was the 1st study to evaluate the endogenous melatonin concentration in canine mammary tumor tissue samples. Also, mean melatonin concentration in Group MET was significantly higher than in Group H&D both within and regardless of the seasonal changes. The significantly increased levels of melatonin in malignant mammary tumors are thought to be occurred due to the defense mechanism of the body. Although the body accumulates melatonin in tumor tissue to have an oncostatic effect, melatonin could not exhibit an oncostatic effect because it could not bind to its receptor due to the low MTR1 level in malignant tumors. Melatonin has been shown to both positively and negatively regulate its own receptors. Both MTR1 and MTR2 melatonin receptors may become desensitized after melatonin exposure [10]. Radioligand binding studies in the rat suprachiasmatic nucleus have found an inverse relationship between receptor density and serum melatonin levels [16].

Estrogens play a role in tumor growth in mammary cancer [27]. In order to neutralize the effects of estrogens, exogenous melatonin could be utilized to regulate the enzymes responsible for local estrogen synthesis [1]. Melatonin reduces the activity of aromatase, sulfatase, and increases estrogen sulfotransferase expression, followed by declining estrogenic effects

[19]. When melatonin binds to the MTR1 receptor, this melatonin–MTR1 complex reduces ligand–receptor transactivation, interrupting estrogen-ERα binding [53]. In this study, melatonin concentrations T2+T3 size tumors were significantly higher than T1 sized tumors ($P < 0.01$). It was thought that the same mechanism as in malignant epithelial tumors occurred. The rise in endogenous MEL levels in increased tumor growth might occur due to the defense mechanism of the body. However, it could not be effective due to the low MTR1A levels to shape the melatonin–MTR1 complex.

MTR1A

Most of the physiological effects of melatonin are mediated by its interaction with 2 high-affinity G protein coupled rectors, termed melatonin receptors type 1 and type 2 (MTNR1A and MTNR1B) [49]. Melatonin showed antitumor effects on proliferative pathways related to the cell cycle and tumorigenesis, tumor death, angiogenesis, and tumor metastasis, as well as on oxidative stress and immune regulatory pathways. These effects were either dependent or independent of melatonin receptors [14]. Melatonin works through receptors and distinguishes 2nd messenger pathways to reduce cellular proliferation and to induce cellular differentiation [21]. There is a consensus among researchers that the occurrence of breast hyperplasia is closely related to the disruption of hormones and hormone receptors across the body [32]. Zhang *et al*. [57] established a rat model of breast hyperplasia induced by estradiol benzoate and progesterone and then explored the molecular mechanisms underlying the effects of melatonin in the treatment of breast hyperplasia. They indicated that melatonin can significantly reduce cell proliferation in breast hyperplasia. Millanta *et al*. [36] reported that ER expression was significantly higher in healthy tissues, hyperplastic/dysplastic lesions, and benign tumors than in carcinomas. Jablonska *et al*. [26] stated that MTR1 expression was higher in estrogen receptor positive (ER+) and HER2 positive (HER2+) tumors. The expression of MTR1 was found to be higher in less differentiated breast tumors, independent of the expression of ER and of the progesterone receptor (PR) [9]. Similarly, MTR1A levels in Group H&D were significantly higher than in Group MET regardless of the season. However, MTR1A levels were similar between the groups in AW season while it was significantly higher in Group H&D compared to Group MET in SS season, in this study. Dillon *et al*. [9] indicated that MTR1 expression level was inversely proportional to the concentration of melatonin in the organism. Both MTR1 and MTR2 melatonin receptors may become desensitized after melatonin exposure [10]. In accordance with previous reports, decreased levels of MTR1A in Group MET should be obtained due to the downregulatory effect of increased melatonin on MTR1A in SS season.

Jablonska *et al*. [26] reported that G1 tumors manifested statistically insignificant higher levels of MTR1A expression as compared to G2 tumors ($P = 0.068$) or G3 tumors ($P = 0.01$). Moreover, MTR1 expression inversely correlated with increasing malignancy grade (G) of the invasive ductal breast carcinomas [26]. In current study, MTR1A levels in G1 tumors were higher than in G2+G3 tumor but it could not reach the signficance. The distinct results could be obtained due to the presence of all carcinoma types and evaluation of G2+G3 tumors in a group in this study.

Association between the evaluated parameters

Our data is consistent with those from a study indicating that MTR1 receptor expression is correlated inversely with melatonin concentrations and that absence of melatonin increases expression of the MTR1 receptor [9]. Goyal *et al*. [20] could not find any correlation of MTR1 expression with Ki67 index or lymph node status in both the receptor-positive and triple-negative cases. Unlike Goyal *et al*. [20], we observed that MTR1A is inversely and moderately correlated with Ki-67 in canine mammary tissues. Taken together these observations published so far suggest a variable relation between the expression of Ki-67 and melatonin [47]. This relationship depends on the type of cell and tumor involved, as suggested by the authors. No significant correlation was observed between Ki-67 labeling rate and melatonin blood concentrations in breast cancer patients [33]. In contrast with previous report, MEL level in canine mammary tissue was positively and moderately correlated with tissue Ki-67 index, in this study. It is thought that the reason for the different results obtained is due to the difference in the analyzed material.

CONCLUSION

Clinical similarities between human and canine mammary tumors (CMT) include age of onset, hormonal etiology, and disease course. In this respect,

this study, which evaluated the amounts of Ki-67, AI, melatonin, and MTR1A in canine mammary tissue, serves as a role model for human breast cancer studies and provides important data, particularly regarding the amounts of endogenous melatonin and MTR1A in canine mammary tumor tissue. Ki-67 index values and endogenous melatonin concentrations were found to be higher in malignant epithelial tumors than in hyperplasia-dysplasia tumors, while AI values tended to be higher in malignant epithelial tumors. Furthermore, MTR1A concentrations were found to be higher in hyperplasia-dysplasia tumors than in malignant epithelial tumors. Overall, the findings indicate that endogenous melatonin levels and melatonin receptor expression are influenced by both seasonal rhythms and tumor biology; however, the role of melatonin within malignant mammary tissue appears to be a potential yet not fully elucidated modulatory effect.

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