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RESEARCH ARTICLE

Presence and Importance of Oxidative Stress Parameters in Malignant Mammary Gland Tumors in Dogs

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Abstract

This study aimed to evaluate the presence of oxidative stress based on lipid peroxidation and the DNA damage markers malondialdehyde (MDA) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in canine mammary gland carcinomas using immunohistochemistry techniques. A total of ten malignant and six normal canine mammary tissue samples were evaluated. The specimens were fixed in 10% buffered formaldehyde solution, processed routinely, embedded in paraffin wax, sectioned at 5 µm, stained with hematoxylin and eosin, examined under a light microscope, and photographed to detect histopathological changes. For immunohistochemistry, the avidin-biotin-peroxidase method was performed. All canine mammary gland tumors were immunopositive for MDA and 8-OHdG expression. There was a statistically significant increase in MDA and 8-OHdG expressions in the tumor group compared to the control group. Based on this study data, in the context of oxidative stress, it is proposed that lipid peroxidation and reactive oxygen species (ROS)-induced DNA damage are significantly associated with canine mammary gland tumor development. In addition, antioxidants may be useful in the treatment of canine mammary gland tumors.

Keywords: *Canine, Carcinoma, Mammary gland, Oxidative stress*

Köpeklerde Malign Meme Bezi Tümörlerinde Oksidatif Stres Parametrelerinin Varlığı ve Önemi

Öz

Bu çalışmada, kanin meme bezi karsinomlarında lipid peroksidasyonuna dayalı oksidatif stres varlığının ve DNA hasar belirteçleri malondialdehit (MDA) ve 8-hidroksi-2'-deoksiguanozin (8-OHdG)'nin immünohistokimya teknikleri kullanılarak değerlendirilmesi amaçlanmıştır. Toplam on malign ve altı normal köpek meme dokusu örneği değerlendirildi. Örnekler %10'luk tamponlu formaldehit solüsyonunda tespit edildi, rutin olarak işlendi, parafin blok içine gömüldü, 5 µm kalınlığında kesitler alındı, Hematoksilen ve Eozin ile boyandı, ışık mikroskobu altında incelendi ve histopatolojik değişiklikleri saptamak için fotoğraflandı. İmmünhistokimya için Avidin-Biotin-Peroksidaz yöntemi uygulandı. Tüm köpek meme bezi tümörleri, MDA ve 8-OHdG ekspresyonu yönünden immünopozitifti. Kontrol grubuna göre tümör grubunda MDA ve 8-OHdG ekspresyonlarında istatistiksel olarak anlamlı bir artış vardı. Bu çalışma verilerine dayanarak, oksidatif stres bağlamında, lipid peroksidasyonu ve reaktif oksijen türleri (ROT) kaynaklı DNA hasarının köpek meme bezi tümörü gelişimi ile önemli ölçüde ilişkili olduğu önerilmektedir. Ek olarak, köpek meme bezi tümörlerinin tedavisinde antioksidanlar faydalı olabilir.

Anahtar sözcükler: *Köpek, Karsinom, Meme bezi, Oksidatif stres*

INTRODUCTION

Mammary gland tumors are very common in female dogs as well as women; however, the prevalence rate in dogs is three times higher. The majority of these canine mammary tumors are malignant and cause significant clinical problems [1-3]. The incidence of tumors, found primarily in adult female dogs, increases with age (average 8-11 years) [4,5]. Malignant mammary gland tumors have been reported more often in Poodles, English Springer Spaniels, Brittany Spaniels, Cocker Spaniels, English Setters, Pointers, Maltese, Yorkshire Terriers, and Dachshunds

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compared to other breeds [6]. The most common histopathological tumor types found in dogs are tubular, papillary, solid, and complex carcinomas and carcinosarcomas [7,8]; whereas, the most common type of breast tumors seen in humans are invasive ductal carcinomas [9]. Prominent mammary gland regions such as the fourth (caudal abdominal) and fifth (inguinal) glands are more predisposed to tumor formation, with the incidence of tumors in these areas ranging between 65% and 70% [4,6]. Generally, pet owners become aware of tumors when macroscopic changes in the mammary glands become apparent [7]. Canine mammary tumors tend to metastasize to nearby lymph nodes and lungs, while metastases to bones, adrenal glands, kidneys, heart, liver, brain, and skin are extremely rare [4]. Death is primarily attributable to lung metastasis [5]. These tumors have a wide range of clinical behaviors. A definitive diagnosis based on a tumor classification and grade is essential for developing optimal individualized treatment plans [10,11]. In controversial cases, immunohistochemical markers can also be evaluated for a more accurate diagnosis [4]. Risk factors such as ovariohysterectomy performed after 2.5 years of age, an intact reproductive status, treatment with progesterone and estrogen, and early obesity are known to play important roles in tumor formation [12]. The primary treatment for canine mammary tumors is mastectomy, but chemotherapy is also a complementary method for more aggressive or recurrent and metastasizing tumors [1,3].

The etiology of canine mammary tumors is multifactorial. Xeno-estrogens present in water, food, and air are known to accumulate in mammary tissue due to prolonged and continuous exposure [13,14]. Mammary epithelial cells convert xeno-estrogens to highly toxic reactive oxygen species (ROS) [15] known to cause serious structural changes in proteins, lipids, and DNA. These changes can result in cell degeneration and aging [16,17]. In addition, these changes may lead to suppression or activation of some signaling pathways and gene expression, thus leading to cell death (apoptosis) or activation of protooncogenes and/or activation/inactivation of tumor suppressor genes. These events can be important in the initiation and promotion of carcinogenesis [18,19]. An imbalance between oxidative and antioxidative reactions causes excessive ROS production, also called oxidative stress, known to play a significant role in the pathogenesis of many illnesses such as cardiovascular diseases, neuropathies, inflammatory diseases, AIDS, diabetes mellitus, renal disorders, and various cancer types, including breast cancer [17,19-21]. Oxidative stress is also associated with carcinogenesis in dogs [16,22]. The primary target of ROS is polyunsaturated fatty acids in cell membranes, causing lipid peroxidation [23], which in turn cause nuclear damage and consequently mutagenesis and carcinogenesis [24]. Malondialdehyde (MDA) is an end product of lipid peroxidation and an important marker for determining oxidative stress [16,25,26]. Increased lipid peroxidation and MDA-DNA adducts have been found in canine mammary tumors and human breast cancers [21]. An increase in the production rate of ROS leads to various modifications in the nucleotide base of DNA. As a crucial risk factor for many pathological conditions, including breast cancer, the ROS product 8-hydroxy-2′-deoxyguanosine (8-OHdG) is a biomarker widely used to detect DNA damage due to oxidative stress [27-29].

This study aimed to evaluate the presence of oxidative stress parameteres with lipid peroxidation and the DNA damage markers MDA and 8-OHdG in canine mammary gland carcinomas.

Material and Methods

Ethical Approval

The ethics committee approval for this study was obtained from Kafkas University Animal Experimentals Local Ethics Committee (No: KAU-HADYEK-2020/076).

Animals

Malignant mammary gland carcinoma samples taken from ten female dogs (Kangal, n=6; Setter, n=4; average age: 8.3 years) brought to Pathology Department for routine diagnosis, and six normal canine mammary tissues (Crossbreed, n=6, average age: 5.5 years) were evaluated.

Histopathology

Mammary tissue samples were fixed in 10% buffered formaldehyde solution, processed routinely, embedded in paraffin wax, sectioned at 5 µm, stained with hematoxylin & eosin (H&E), examined under a light microscope (Olympus Bx53), and photographed via the Cell^P program (Olympus Soft Imaging Solutions GmbH, 3,4) to detect histopathological changes. Tumor sections were classified according to the modified World Health Organization classification of canine mammary tumors [30]. The malignancy grade of the tumors was determined according to the Nottingham method [31]. Accordingly, tubule formation, nuclear polymorphism, and mitotic cell counts were evaluated and scored from 1-3. Tumor grades were defined as follows: 3-5 points = well-differentiated (Stage 1), 6-7 points = moderately differentiated (Stage 2), 8-9 points = poorly differentiated (Stage 3).

Immunohistochemistry

The Avidin-Biotin-Peroxidase method was used for immunohistochemistry. Slides were deparaffinized and rehydrated in graded alcohols. The sections were treated with 3% hydrogen peroxide solution in phosphate-buffered saline (PBS) for 15 min to prevent endogenous peroxidase activity, then boiled in citrate buffer solution (pH 6) for 25 min in an 800-watt microwave oven for antigen retrieval. The sections were incubated for ten min with non-immune

serum (Thermo Scientific Histostain-Plus IHC Kit, HRP, broad-spectrum, REF: TP-125-HL) at room temperature to prevent nonspecific staining. Diluted antibodies (8-OHdG: Bioss Antibodies, bs-1278R, dilution:1/800; MDA: Abcam, ab6463, dilution:1/250) were incubated overnight in a refrigerator at 4°C after which the sections were washed three times in PBS for three min. The biotinylated secondary antibody (Thermo Scientific, Histostain-Plus IHC Kit, HRP, broad-spectrum, REF: TP-125-HL) was applied at room temperature for ten min. After washing in PBS for three min, all sections were incubated with peroxidase-bound streptavidin (Thermo Scientific, Histostain-Plus IHC Kit, HRP, broad-spectrum, REF: TP-125-HL) for ten min at room temperature. A solution of 3,3'-diaminobenzidine (DAB) tetrahydrochloride (Thermo Scientific, REF: TA-125-HD) was used as a chromogen for 15 min. The sections were treated with Mayer's Hematoxylin for 30 second and washed in running water for 5 min, dehydrated in graded alcohols, cleared in xylene and coated with entellan. Primary antibodies were omitted from the negative control sections and were treated with diluted normal serum. The prepared slides were examined under a light microscope (Olympus Bx53) and photographed via the Cell^P program (Olympus Soft Imaging Solutions GmbH, 3,4). Analyses of the images were accomplished with the Image J Program (1.51j8).

Immunopositivity was evaluated with a 20× objective using a semiquantitative grading scheme based on the determination of 8-OHdG and MDA markers in five representative fields as (+) mild labeling of 1%-10% of cells, (++) moderate labeling of 11%-59% of cells, or (+++) severe labeling of >60% of cells [32].

Statistical Analysis

Before the study, a power analysis was performed using G-Power 3.1.9.7. As a result, the sample size was based on a test power of 0.8 and a significance level of 0.05. A Mann-Whitney U test was used to compare mammary tumor and control groups according to immune-positive cell scoring. The obtained results were given as mean ± standard error (SE) and median. Statistical analyses were performed using the SPSS® program (Version 26.0, Chicago, IL, USA). Differences between groups were considered significant at the P<0.05 level.

Results

Macroscopic Results

Lobular single or multiple tumor masses with hemorrhagic and ulcerative surfaces were observed. The generally round and oval-shaped masses were grayish-white in color. While the incision faces of some masses had soft and spongy areas, others were quite hard and difficult to cut due to the formation of bone and cartilage tissue *(Fig. 1-a,b)*. Metastases to regional lymph nodes and lungs were observed in only two cases.

Microscopic Results

Histological classification and grade information of all cases are provided in *Table 1*. In the histopathological examination of 10 mammary gland tumor samples, 1 tubular carcinoma (10%), 1 solid carcinoma (10%), 4 intraductal papillary carcinomas (25%), and 4 mix carcinoma variants (25%) were identified. Of the 10 cases, 5 were Grade 1 (50%), 3 were Grade 2 (30%), and 2 were Grade 3 (20%). In the tubular carcinoma variant, one or two cell thick tubular formations, pleomorphism, pronounced hyperchromasia, and an increase in mitotic figures were remarkable. In the solid carcinoma variant, neoplastic cells in the form of solid layers/clumps separated by thin fibrous capsules were detected. The increase in the ratio of nuclei to cytoplasm in these neoplastic cells in favor of the nucleus was remarkable. Mitotic figures were rare in some areas and quite numerous in others. In the intraductal papillary carcinoma variant, finger-like projections, formed by neoplastic cells extending towards the lumen and supported by a fibrovascular layer, were prominent. In addition to these findings, nuclear pleomorphism and increased mitotic activity were detected similar to the other variants. In the mixed carcinoma variant, neoplastic epithelial cells, spindle-shaped myoepithelial cells, as well as cartilage and bone tissue formations in the tumor area were observed. Other significant histopathological findings included disorganized glandular structures formed by neoplastic epithelial cells, mitotic figures, and pleomorphism *(Fig. 2-ab,c,d,e,f,g,h)*.

Immunohistochemical Results

All canine mammary gland tumors (CMGT) were immunopositive for MDA and 8-OHdG expression. Mean ± SE values

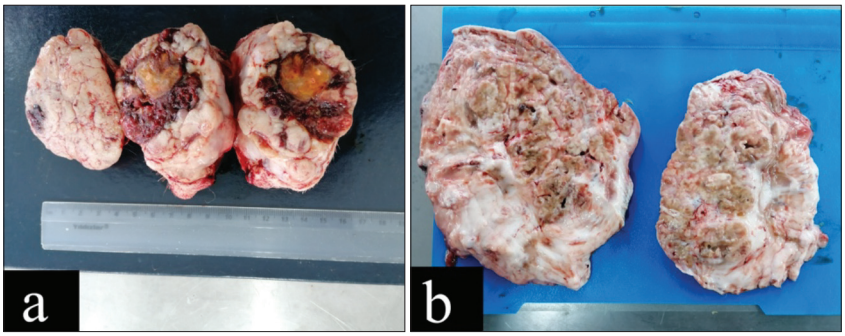

Fig 1. a-b: Macroscopic view of tumor masses

Table 1. *Breed, age information and tumor characteristics of dogs in CMGT and control groups*

Groups	Case No	Breed	Age	Histological Classification	Grade
CMGT	1	Kangal	4 years	Mix carcinoma	1
CMGT	2	Kangal	15 years	Intraductal papillary carcinoma	3
CMGT	3	Setter	4 years	Intraductal papillary carcinoma	2
CMGT	4	Setter	17 years	Solid carcinoma	3
CMGT	5	Kangal	6 years	Tubular carcinoma	2
CMGT	6	Kangal	8 years	Mix carcinoma	3
CMGT	7	Kangal	7 years	Mix carcinoma	2
CMGT	8	Setter	5 years	Intraductal papillary carcinoma	1
CMGT	9	Setter	7 years	Intraductal papillary carcinoma	1
CMGT	10	Kangal	10 years	Mix carcinoma	1
Control	11	Cross breed	4 years	-	-
Control	12	Cross breed	6 years	-	-
Control	13	Cross breed	5 years	-	-
Control	14	Cross breed	6 years	-	-
Control	15	Cross breed	4 years	-	-
Control	16	Cross breed	8 years	-	-

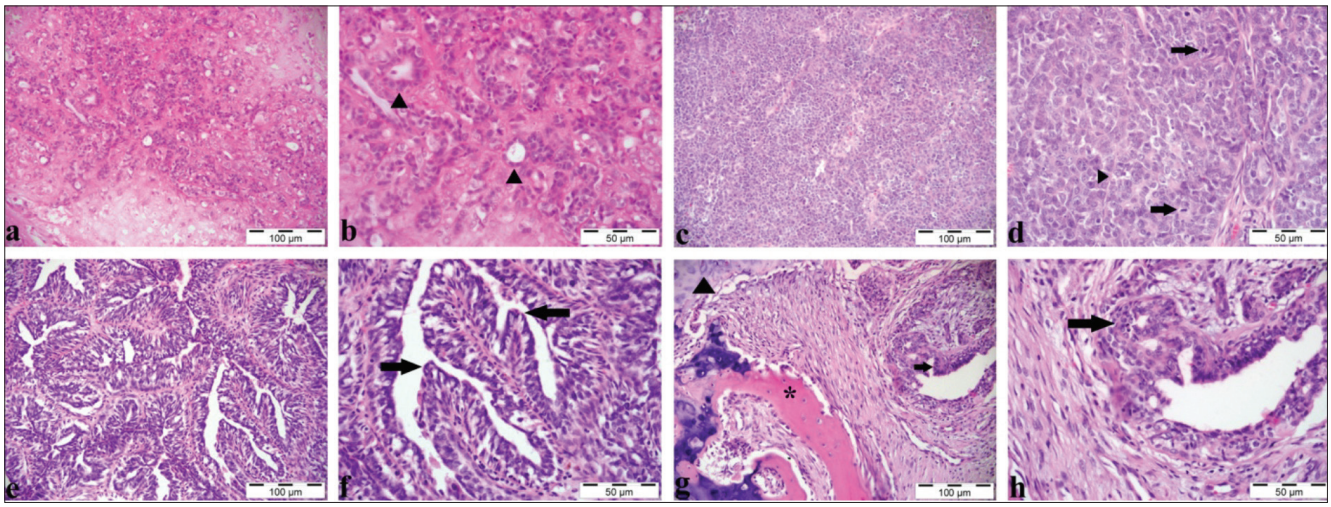

Fig 2. Histological classifications, H&E staining, **a-b:** Tubular carcinoma, tubular formations *(arrowheads);* **c-d:** Solid carcinoma, clusters of solid cells separated by a thin fibrous capsule *(arrowhead),* mitotic figures *(arrows);* **e-f:** Intraductal papillary carcinoma, finger-like extensions towards the lumen *(arrows);* **g-h:** Mixed carcinoma, epithelial tumor cells *(arrows),* bone *(star),* and cartilage formations *(arrowhead)*

Table 2. *Mean ± SE values of all groups*

Groups	MDA Mean±SE (Median)	8-OHDG Mean±SE (Median)
CMGT	2.00±0.30[a] (2)	2.10±0.23[a] (2)
Control	0±0[b] (0)	0±0[b] (0)

[a,b] Different letters in each column show the statistical differences of the groups, P<0.001; Mean ± standard error and median values of cell scoring are given; ***MDA:*** *Malondialdehyde,* ***8-OHDG:*** *8-Hydroxy-2′-deoxyguanosine*

of all groups are provided in *Table 2*. No MDA or 8-OHdG immunoreactivity was found in the healthy mammary gland tissues of control animals. It was found that a statistically significant increase in MDA and 8-OHdG expressions in the tumor group compared to the control group. MDA positive reactions were particularly strong in intraductal papillary carcinomas and tubular carcinomas. The MDA expression intensity increased in Grades 2 and 3 compared to Grade 1. Intracytoplasmic yellow-brownish MDA immune reactivity was apparent in tubular carcinomas, especially in tubular structures formed by tumor cells and in areas where pleomorphism was evident. Intracytoplasmic MDA expression

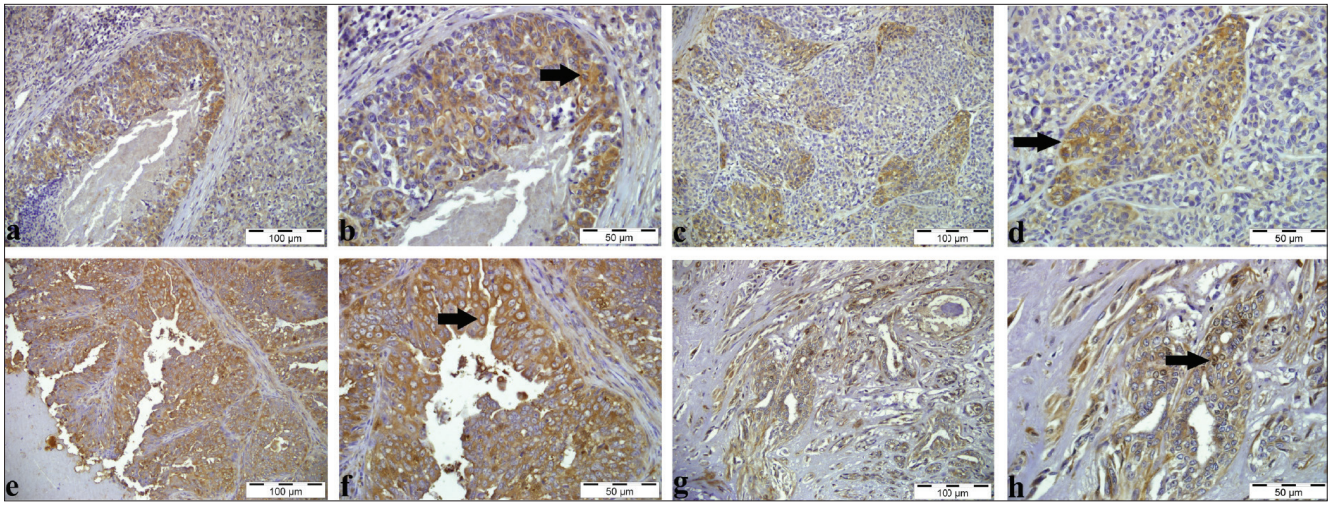

Fig 3. MDA, IHC, **a-b:** Tubular carcinoma, intracytoplasmic MDA immunopositive expressions *(arrow);* **c-d:** Solid carcinoma, MDA expressions in the cytoplasm of tumor cells *(arrow);* **e-f:** Intraductal papillary carcinoma, severe MDA reactions in the cytoplasm of neoplastic cells forming finger-like extensions *(arrow);* **g-h:** Mixed carcinoma, intracytoplasmic MDA immunoreactivity in tumor cells forming glandular structures *(arrow)*

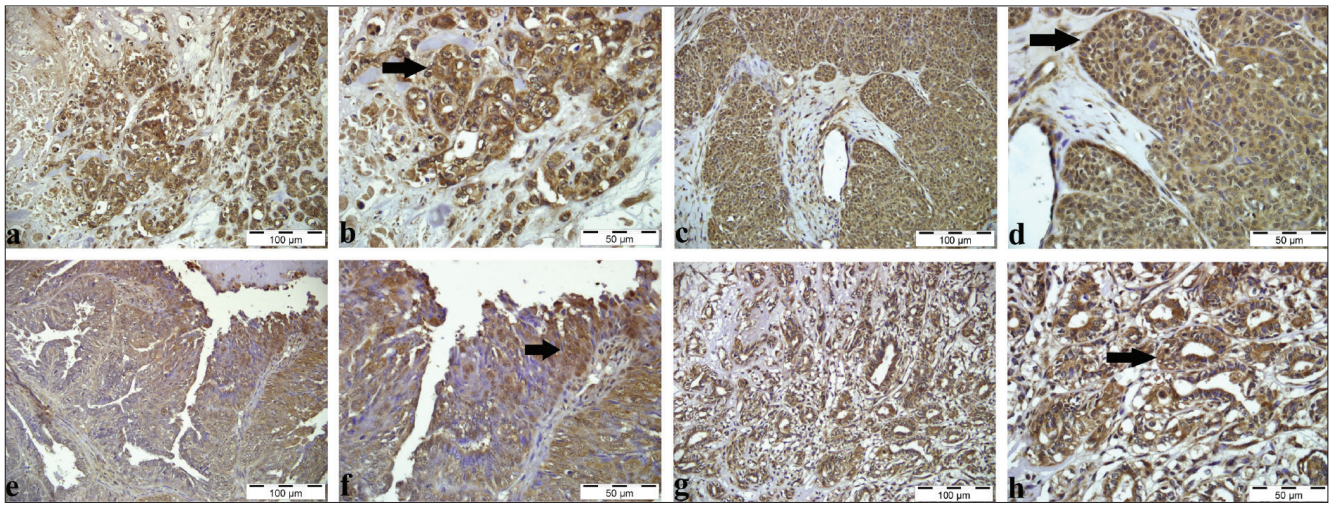

Fig 4. 8-OHdG, IHC, **a-b:** Tubular carcinoma, intranuclear and intracytoplasmic 8-OHdG immunopositive reactions *(arrow);* **c-d:** Solid carcinoma, 8-OHdG expression in both the cytoplasm and nucleus of tumor cells *(arrow);* **e-f:** Intraductal papillary carcinoma, intracytoplasmic and intranuclear dark-brown 8-OHdG positive reactions in cells located at the periphery of the finger-like projections extending towards the lumen *(arrow);* **g-h:** Mixed carcinoma, 8-OHdG immunopositive expression in the cytoplasm of neoplastic epithelial cells forming glandular structures *(arrow)*

occurred especially in tumor cells localized as clusters in solid carcinomas. The reaction was much more severe in the cells located in the periphery of these clusters. Intracytoplasmic dark-brown MDA positive reactions were apparent in papillary structures extending towards the lumen in intraductal papillary carcinomas. MDA immunoreactivity was determined in the cytoplasm of tumor cells forming glandular structures in mixed carcinomas. There was no positive reaction in bone or cartilage tissue formations *(Fig. 3-a,b,c,d,e,f,g,h)*. Solid carcinomas and tubular carcinomas had particularly strong 8-OHdG immune positive reactions. The intensity of 8-OHdG expressions was increased in Grades 2 and 3 compared to Grade 1. Expression of 8-OHdG was also localized similarly to foci where MDA expression was observed in the different tumor variants. In addition to dark-brown intracytoplasmic reactions, positive immune reactions in the nucleus were also observed *(Fig. 4-a,b,c,d,e,h,g,h)*.

Discussion

High ROS levels or failure to remove ROS results in oxidative stress, which causes severe metabolic disturbances and damage to biological macromolecules such as lipids, proteins, and DNA [18,33]. Excessive ROS production causes cytotoxicity, membrane damage, lipid peroxidation, and mutagenesis, as well as initiation and promotion of multistage carcinogenesis [13,14]. Lipids are macromolecules most susceptible to the toxic effects of ROS. Products such as MDA are formed as a result of ROS-induced lipid peroxidation [21]. The determination of the amount of MDA in biological systems is an important parameter used to

evaluate cellular oxidative stress [16,25]. Various researchers have concluded that MDA is highly cytotoxic and genotoxic and that oxidative damage should be seen as more than a biomarker due to its interaction with DNA and other proteins [26]. Controversial results exist as to whether there is any significant difference in lipid peroxidation values between clinically healthy dogs and dogs with malignant tumors. Some researchers noted that there was no significant difference when comparing lipid peroxidation levels (MDA, thiobarbituric acid reactive substances [TBARS]) between healthy animals and those with tumors [15,20]. Contrary to these reports, there are also those that indicate significant differences in lipid peroxidation values between normal tissues and canine mammary tumors [13,14,16,17,21,24]. Present study revealed a significant increase in lipid peroxidation values between dogs with malignant tumors and dogs in the control group. It is attributed that the increase in MDA expression in the tumor group to the overproduction of ROS.

Reactive oxygen species production can lead to DNA damage, double-strand breaks, rearrangements resulting in point mutations and deletions, and gene amplification in the early stage of carcinogenesis [17]. Oxidative damage of DNA induced by ROS causes the production of 8-OHdG, an oxidized form of deoxyguanosine nucleoside [34,35]. Although there are more than 20 oxidative DNA damage products, 8-OHdG has been concentrated on due to its sensitivity and mutagenicity potential. A serious association between 8-OHdG and carcinogenesis has been reported [20,29], and 8-OHdG is known to cause GC to TA transversions. Measurement of 8-OHdG levels is used to detect oxidative stress-mediated DNA damage [28,36]. Various researchers have noted that 8-OHdG levels are significantly increased in various types of cancer, such as gastric cancer, epithelial ovarian carcinoma, colorectal carcinoma, and esophageal cancer, and may be associated with a poor prognosis [37]. A literature search failed to find any studies in which 8-OHdG levels were used to evaluate canine mammary tumors or different types of cancer. However, similar to human cancers, it was found that 8-OHdG expression was more severe in tubular and solid carcinomas, which have a worse prognosis among canine mammary tumors and advanced Grades 2 and 3 [11,37].

In conclusion, based on these results, in the context of oxidative stress, lipid peroxidation and ROS-induced DNA damage are significantly associated with tumor development. The use of antioxidants in the treatment of these tumors may be beneficial. Since there are no reports in the literature detailing the evaluation of oxidative stress markers MDA and 8-OHdG in canine mammary tumors by immunohistochemical methods, this study represents novel data. Additional studies are needed to determine the value of incorporating oxidative markers in the grading and prognosis of CGMTS.

Author Contributions

Surgical operation and sample collection: MK, HO, Histopathological and immunohistochemical stainings: HN, AY, Tumor classification and staging: SD, EB, EK, Statistical analyses: MK, EK, Idea, concept and writing the article: EK.

Conflicts of Interest

The authors declared that there is no conflict of interest.

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