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

Emin KARAKURT [1,a (*)] Mushap KURU [2,b] Serpil DAĞ [1,c] Enver BEYTUT [1,d]
Hasan ORAL [2,e] Hilmi NUHOĞLU [1,f] Ayfer YILDIZ [1,g]

[1] Department of Pathology, Faculty of Veterinary Medicine, Kafkas University, TR-36100 Kars - TURKEY
[2] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Kafkas University, TR-36100 Kars - TURKEY
ORCIDs: [a] 0000-0003-2019-3690; [b] 0000-0003-4409-251X; [c] 0000-0001-7667-689X; [d] 0000-0003-3360-2940; [e] 0000-0002-4366-4988
[f] 0000-0003-2530-2542; [g] 0000-0002-6569-5435

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

compared to other breeds [6]. The most common histo-pathological tumor types found in dogs are tubular, papillary, solid, and complex carcinomas and carcino-sarcomas [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 ovario-hysterectomy 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 muta-genesis 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 histo-pathological 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 immuno-histochemistry. 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 neo-plastic 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 immuno-positive 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*

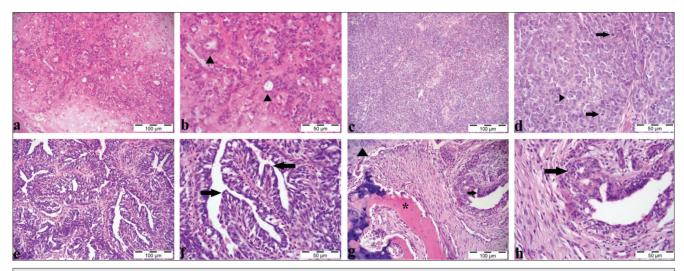| Groups	Case No	Breed	Age	Histological Classification	Grade
CMGT	1	Kangal	4 years	Mix carcinoma	1
CMGT	2	Kangal	15 years	Intraductal papillary carcinoma	3
CMGT	3	Setter	4 years	Intraductal papillary carcinoma	2
CMGT	4	Setter	17 years	Solid carcinoma	3
CMGT	5	Kangal	6 years	Tubular carcinoma	2
CMGT	6	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 pleo-morphism was evident. Intracytoplasmic MDA expression
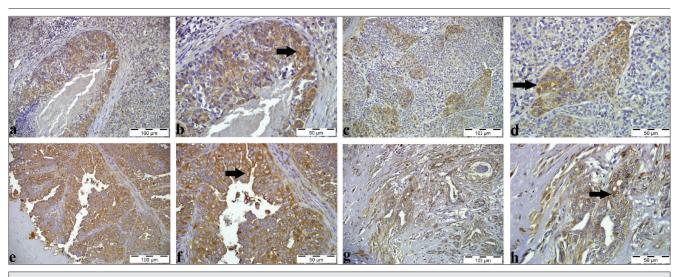
Fig 3. MDA, IHC, **a-b:** Tubular carcinoma, intracytoplasmic MDA immunopositive expressions *(arrow);* **c-d:** Solid carcinoma, MDA expressions in the cytoplasm of tumor cells *(arrow);* **e-f:** Intraductal papillary carcinoma, severe MDA reactions in the cytoplasm of neoplastic cells forming finger-like extensions *(arrow);* **g-h:** Mixed carcinoma, intracytoplasmic MDA immunoreactivity in tumor cells forming glandular structures *(arrow)*
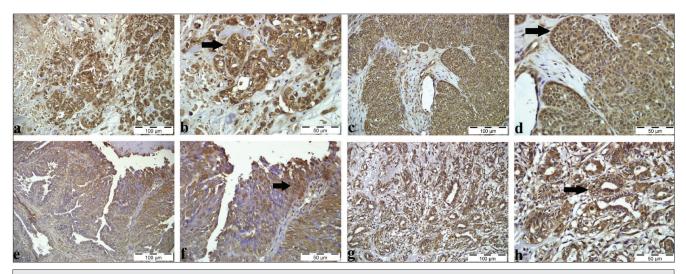

Fig 4. 8-OHdG, IHC, **a-b:** Tubular carcinoma, intranuclear and intracytoplasmic 8-OHdG immunopositive reactions *(arrow);* **c-d:** Solid carcinoma, 8-OHdG expression in both the cytoplasm and nucleus of tumor cells *(arrow);* **e-f:** Intraductal papillary carcinoma, intracytoplasmic and intranuclear 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. Intra-cytoplasmic dark-brown MDA positive reactions were apparent in papillary structures extending towards the lumen in intraductal papillary carcinomas. MDA immun-oreactivity 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 multi-stage 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 per-oxidation [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, Histo-pathological 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.

REFERENCES

1. Benavente MA, Bianchi CP, Aba MA: Expression of oxytocin receptors in canine mammary tumours. *J Comp Pathol*, 170, 26-33, 2019. DOI: 10.1016/j.jcpa.2019.05.005

2. Borghesi J, Caceres S, Mario LC, Alonso-Diez A, Silveira Rabelo AC, Illera MJ, Silvan G, Miglino MA, Favaron PO, Carreira ACO, Illera JC: Effects of doxorubicin associated with amniotic membrane stem cells in the treatment of canine inflammatory breast carcinoma (IPC-366) cells. *BMC Vet Res*, 16:353, 2020. DOI: 10.1186/s12917-020-02576-0

3. Lainetti PF, Leis-Filho AF, Kobayashi PE, de Camargo LS, Laufer-Amorim R, Fonseca-Alves CE, Souza FF: Proteomics approach of rapamycin anti-tumoral effect on primary and metastatic canine mammary tumor cells in vitro. *Molecules*, 26 (5): 1213, 2021. DOI: 10.3390/molecules26051213

4. Borecka P, Ratajczak-Wielgomas K, Ciaputa R, Kandefer-Gola M, Janus I, Piotrowska A, Kmiecik A, Podhorska-Okolów M, Dzięgiel P, Nowak M: Expression of periostin in cancer-associated fibroblasts in mammary cancer in female dogs. *In Vivo*, 34 (3): 1017-1026, 2020. DOI: 10.21873/invivo.11870

5. Diessler ME, Castellano MC, Portiansky EL, Burns S, Idiart JR: Canine mammary carcinomas: Influence of histological grade, vascular invasion, proliferation, microvessel density and VEGFR2 expression on lymph node status and survival time. *Vet Comp Oncol*, 15 (2): 450-461, 2017. DOI: 10.1111/vco.12189

6. Litterine-Kaufman J, Casale SA, Mouser PJ: Prevalence of malignancy in masses from the mammary gland region of dogs with single or multiple masses. *J Am Vet Med Assoc*, 255 (7): 817-820, 2019. DOI: 10.2460/javma.255.7.817

7. Kaszak I, Ruszczak A, Kanafa S, Kacprzak K, Król M, Jurka P: Current biomarkers of canine mammary tumors. *Acta Vet Scand*, 60:66, 2018. DOI: 10.1186/s13028-018-0417-1

8. Dong H, Diao H, Zhao Y, Xu H, Pei S, Gao J, Wang J, Hussain T, Zhao D, Zhou X, Lin D: Overexpression of matrix metalloproteinase-9 in breast cancer cell lines remarkably increases the cell malignancy largely via activation of transforming growth factor beta/SMAD signalling. *Cell Prolif*, 52 (5): e12633, 2019. DOI: 10.1111/cpr.12633

9. Bulkowska M, Rybicka A, Senses KM, Ulewicz K, Witt K, Szymanska J, Taciak B, Klopfleisch R, Hellmén E, Dolka I, Gure AO, Mucha J, Mikow M, Gizinski S, Krol M: MicroRNA expression patterns in canine mammary cancer show significant differences between metastatic and non-metastatic tumours. *BMC Cancer*, 17:728, 2017. DOI: 10.1186/s12885-017-3751-1

10. Ariyarathna H, Thomson N, Aberdein D, Munday JS: Low stromal mast cell density in canine mammary gland tumours predicts a poor prognosis. *J Comp Pathol*, 175, 29-38, 2020. DOI: 10.1016/j.jcpa.2019.12.004

11. Borghesi J, Giancoli Kato Cano da Silva M, de Oliveira Pimenta Guimarães K, Mario LC, de Almeida da Anunciação AR, Silveira Rabelo AC, Gonçalves Hayashi R, Lima MF, Miglino MA, Oliveira Favaron P, Oliveira Carreira AC: Evaluation of immunohistopathological profile of tubular and solid canine mammary carcinomas. *Res Vet Sci*, 136, 119-126, 2021. DOI: 10.1016/j.rvsc.2021.02.004

12. Torres CG, Iturriaga MP, Cruz P: Hormonal carcinogenesis in canine mammary cancer: Molecular mechanisms of estradiol involved in malignant progression. *Animals*, 11 (3): 608, 2021. DOI: 10.3390/ani11030608

13. Kumaraguruparan R, Balachandran C, Manohar BM, Nagini S: Altered oxidant-antioxidant profile in canine mammary tumours. *Vet Res Commun*, 29 (4): 287-296, 2005. DOI: 10.1023/b:verc.0000048499.38049.4b

14. Jayasri K, Padmaja K, Saibaba M: Altered oxidative stress and carbohydrate metabolism in canine mammary tumors. *Vet World*, 9 (12): 1489-1492, 2016. DOI: 10.14202/vetworld.2016.1489-1492

15. Karayannopoulou M, Fytianou A, Assaloumidis N, Psalla D, Constantinidis TC, Kaldrymidou E, Koutinas AF: Markers of lipid peroxidation and α-tocopherol levels in the blood and neoplastic tissue of dogs with malignant mammary gland tumors. *Vet Clin Pathol*, 42 (3): 323-328, 2013. DOI: 10.1111/vcp.12064

16. Macotpet A, Suksawat F, Sukon P, Pimpakdee K, Pattarapanwichien E, Tangrassameeprasert R, Boonsiri P: Oxidative stress in cancer-bearing dogs assessed by measuring serum malondialdehyde. *BMC Vet Res*, 9:101, 2013. DOI: 10.1186/1746-6148-9-101

17. Chandravathi T, Anand Kumar A: Investigation of the antioxidant status in canine tumours. *Int J Livest Res*, 10 (7): 21-25, 2020. DOI: 10.5455/ijlr.20200519031736

18. Tanja P, Alenka N, Butinar J, Natasa T, Marija P, Bettina K, Kessler M: Antioxidant status in canine cancer patients. *Acta Vet-Beograd*, 58, 275-286, 2008. DOI: 10.2298/AVB0803275P

19. Leonel C, Gelaleti GB, Jardim BV, Moschetta MG, Regiani VR, Oliveira JG, Zuccari DA: Expression of glutathione, glutathione peroxidase and glutathione S-transferase pi in canine mammary tumors. *BMC Vet Res*, 10:49, 2014. DOI: 10.1186/1746-6148-10-49

20. Szczubiał M, Kankofer M, Łopuszyński W, Dabrowski R, Lipko J: Oxidative stress parameters in bitches with mammary gland tumours. *J Vet Med A Physiol Pathol Clin Med*, 51 (7-8): 336-340, 2004. DOI: 10.1111/j.1439-0442.2004.00647.x

21. Askar TK, Salmanoglu B, Salmanoglu R, Erkal N, Beskaya A: Changes in the oxidative status and serum trace element levels in dogs with mammary tumours. *Acta Veterinaria-beograd*, 59 (4): 405-411, 2009. DOI: 10.2298/AVB0904405A

22. Machado VS, Crivellenti LZ, Bottari NB, Tonin AA, Pelinson LP, Borin-Crivellenti S, Santana AE, Torbitz VD, Moresco RN, Duarte T, Duarte MM, Schetinger MR, Morsch VM, Jaques JA, Tinucci-Costa M, Da Silva AS: Oxidative stress and inflammatory response biomarkers in dogs with mammary carcinoma. *Pathol Res Pract*, 211 (9): 677-681, 2015. DOI: 10.1016/j.prp.2015.06.011

23. Karayannopoulou M, Fytianou A, Assaloumidis N, Psalla D, Savvas I, Kaldrymidou E: Lipid peroxidation in neoplastic tissue of dogs with mammary cancer fed with different kinds of diet. *Turk J Vet Anim Sci*, 37 (4): 449-453, 2013. DOI: 10.3906/vet-1211-7

24. Souza IB, Cardoso CV, Poppe SC, Bondan EF: Lipid peroxidation in female dogs bearing mammary gland carcinomas. *Arq Bras Med Vet Zootec*, 69 (5): 1335-1338, 2017. DOI: 10.1590/1678-4162-9751

25. Enginler SO, Toydemir TSF, Ates A, Ozturk B, Erdogan O, Ozdemir S, Kirsan I, Or ME, Arun SS, Barutcu UB: Examination of oxidative/antioxidative status and trace element levels in dogs with mammary tumors. *Bulg J Agric Sci*, 21 (5): 1086-1091, 2015.

26. Russo C, Bracarense APFRL: Oxidative stress in dogs. *Semina: Ciênc Agrár*, 37 (3): 1431-1440, 2016. DOI: 10.5433/1679-0359.2016v37n3p1431

27. Lee JD, Cai Q, Shu XO, Nechuta SJ: The role of biomarkers of oxidative stress in breast cancer risk and prognosis: a systematic review of the epidemiologic literature. *J Womens Health (Larchmt)*, 26 (5): 467-482, 2017. DOI: 10.1089/jwh.2016.5973

28. Nour Eldin EEM, El-Readi MZ, Nour Eldein MM, Alfalki AA, Althubiti MA, Mohamed Kamel HF, Eid SY, Al-Amodi HS, Mirza AA: 8-Hydroxy-2'-deoxyguanosine as a discriminatory biomarker for early detection of breast cancer. *Clin Breast Cancer*, 19 (2): E385-E393, 2019. DOI: 10.1016/j.clbc.2018.12.013

29. Ercan N, Yüksel M, Koçkaya M: Determination of 8-hydroxy-2'deoxyguanosine, malondialdehyde levels and antioxidant enzyme activities in Kangal dogs with venereal tumour. *Ankara Üniv Vet Fak Derg*, 67 (2): 121-125, 2020. DOI: 10.33988/auvfd.492765

30. Goldschmidt M, Peña L, Rasotto R, Zappulli V: Classification and grading of canine mammary tumors. *Vet Pathol*, 48 (1): 117-131, 2011. DOI: 10.1177/0300985810393258

31. Elston EW, Ellis IO: Method for grading breast cancer. *J Clin Pathol*, 46 (2): 189-190, 1993. DOI: 10.1136/jcp.46.2.189-b

32. Beytut E: Pathological and immunohistochemical evaluation of skin and teat papillomas in cattle. *Turk J Vet Anim Sci*, 41 (2): 204-212, 2017. DOI: 10.3906/vet-1609-65

33. Çenesiz S: The role of oxidant and antioxidant parameters in the infectious diseases: A systematic literature review. *Kafkas Univ Vet Fak Derg*, 26 (6): 849-858, 2020. DOI: 10.9775/kvfd.2020.24618

34. Kubo N, Morita M, Nakashima Y, Kitao H, Egashira A, Saeki H, Oki E, Kakeji Y, Oda Y, Maehara Y: Oxidative DNA damage in human esophageal cancer: Clinicopathological analysis of 8-hydroxydeoxyguanosine and its repair enzyme. *Dis Esophagus*, 27 (3): 285-293, 2014. DOI: 10.1111/dote.12107

35. An AR, Kim KM, Park HS, Jang KY, Moon WS, Kang MJ, Lee YC, Kim JH, Chae HJ, Chung MJ: Association between expression of 8-OHdG and cigarette smoking in non-small cell lung cancer. *J Pathol Transl Med*, 53 (4): 217-224, 2019. DOI: 10.4132/jptm.2019.02.20

36. Chatterjee A, Ronghe A, Padhye SB, Spade DA, Bhat NK, Bhat HK: Antioxidant activities of novel resveratrol analogs in breast cancer. *J Biochem Mol Toxicol*, 32 (1): e21925, 2018. DOI: 10.1002/jbt.21925

37. Jelic MD, Mandic AD, Maricic SM, Srdjenovic BU: Oxidative stress and its role in cancer. *J Cancer Res Ther*, 17 (1): 22-28, 2021. DOI: 10.4103/jcrt.JCRT_862_16