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The role of oxidative and nitrosative stress in bovine ocular squamous cell carcinomas

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ABSTRACT

Oxidative stress is strongly linked to carcinogenesis, especially head, neck and oral SCCs in humans. In this study, we aimed to evaluate the role of oxidative and nitrosative stress in Bovine ocular squamous cell carcinomas (BOSCCs) using immunohistochemical markers such as Nitrotyrosine (NT), Malondialdehyde (MDA) and 8-hydroxy-2' -deoxyguanosine (8-OHdG). Tissues were collected from 24 cattle brought to the Pathology Department for routine diagnosis. Tumour samples were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin wax. Tissue sections were cut into 5 **µm** thickness and stained with Hematoxylin-Eosin to detect histopathological changes. The sections were examined under a light microscope and photographed. The avidin-biotin-peroxidase method was used as immunohistochemical staining. It was observed that the cauliflower-like tumoral masses were mostly located on the upper and lower eyelids, the third eyelid, cornea, limbus, or covering the entire eyeball. NT, MDA and 8-OHdG expressions were statistically increased in poorly-differentiated (PD) cases compared to well-differentiated (WD) and moderately-differentiated (MD) cases. On the basis of the results of the increase in these markers, we concluded that nitrosative and oxidative stress may have an important role in the carcinogenesis of BOSCCs.

Key words: bovine; nitrosative stress; oxidative stress; squamous cell carcinoma

Introduction

Bovine ocular squamous cell carcinoma (BOSCC), also called 'cancer eye', is the most common tumour seen in cattle, and causes significant economic losses (SÖZMEN et al., 2019; VALA et al., 2020). Hereditary factors and environmental factors, such as latitude, altitude, exposure to sunlight (solar UV radiation), deficiencies in eyelid pigmentation, age, dietary habits and various viral agents (e.g., bovine papillomavirus and bovine herpesvirus type 1-5), have been reported to play a significant role in the etiopathogenesis of BOSCCs (PUGLIESE et al., 2014; PODARALA et al., 2020). BOSCC is a malignant tumour of epithelial origin, mostly formed in various ocular

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and periocular regions, such as the junction of the cornea, the sclera, eyelids, third eyelid, cornea, conjunctiva, and palpebral skin in aged Holstein, Hereford or derived breeds (CARVALHO et al., 2005; FORNAZARI et al., 2017; SÖZMEN et al., 2019; VALA et al., 2020). In all species, the tumour undergoes a series of pre-malignant developmental stages, called epidermal plaques and papillomas, before progressing to carcinoma in situ, and to invasive carcinoma over months or years (GHARAGOZLOU et al., 2007).

The UVA and UVB spectra, which play a role in the etiopathogenesis of BOSCCs, cause the production of reactive oxygen species (ROS), deterioration of antioxidant defence and, thus, oxidative damage (CARRARA et al., 2019). Oxidative damage caused by ROS and reactive nitrogen species (RNS) is a key factor in carcinogenesis, as in chronic inflammation (BENTZ et al., 2000; CHAIYARIT et al., 2005). During the inflammatory process, large amounts of nitric oxide (NO) are produced by inducible nitric oxide synthase (iNOS), which is usually expressed in inflamed tissues (SEPEHR et al., 2001). Peroxynitrite ($ONOO^-$), the reaction product of superoxide (O_2^-) and NO, reacts with tyrosine to form nitrotyrosine (NT), which is a strong nitrating and oxidising agent (KATO et al., 2000). NT, a stable end-product of the nitration of a tyrosine residue, can be used as a marker of cell damage, inflammation, and protein damage resulting from excessive production of NO, peroxynitrite and other nitrating species (AKSOY and KURNAZ, 2019; SILVA SERVATO et al., 2019; KATO et al., 2000).

The lipid peroxidation of polyunsaturated fatty acids (PUFAs) is initiated due to the formation of ROS, resulting in the production of reactive carbonyl compounds, and it causes significant changes in the structural integrity, as well as the functions of the cell membrane (POLANIAK et al., 2010; MALIK et al., 2014). As a representative lipid peroxidation indicator and a reliable oxidative stress marker, Malondialdehyde (MDA) is highly mutagenic and carcinogenic (SINGH et al., 2015; YOSHIFUKU et al., 2018). Various researchers have reported that serum concentrations of MDA increase even in the early stages of various cancer types (SHETTY et al., 2014).

ROS can cause various forms of genotoxic damage, such as single and double-strand breaks, DNA-protein crosslinks, abasic sites and modified bases (ZANNONI et al., 2006). 8-Hydroxy-2'-deoxyguanine (8-OHdG) is induced by guanine oxidation due to the interaction of oxygen-free radicals with nucleobases of the DNA strand, so this product is a useful indicator of oxidative damage to DNA (KUBO et al., 2014; SOARES et al., 2018). Increased levels of 8-OHdG are associated with many pathological conditions, including cancer, and this increase is particularly associated with poor prognosis (KUMAR et al., 2012; AN et al., 2019).

In this study, we aimed to evaluate the role of oxidative and nitrosative stress in BOSCCs using immunohistochemical markers such as NT, MDA and 8-OHdG.

Materials and methods

Animals. The biopsy samples were collected from 24 cattle brought to the Pathology Department from the Surgery Department for routine histopathological diagnosis.

Surgical operation. Systematic clinical examinations were performed of patients with eye discharge and swelling complaints brought to the Department of Surgery of the Faculty of Veterinary Medicine of Kafkas University. During the anamnesis, it was learned that the lesions in the eyes of the animals started initally with tear discharge, then a lentil-sized swelling appeared on the eyelid and around it, and this swelling gradually grew, while becoming infective. In the inspection and palpation examination, it was observed that the mass was generally in a necrotic state and had a soft consistency, along with an infective tear discharge. After the clinical examinations were completed, the necessary preparations for removing the mass in the eye and sending it for pathological examination were started.

Following the shaving of the operation area, using the method of Reuff, accompanied by xylazine sedation, the animal was placed in the lateral position leaving the area to be operated on top. Then, after providing asepsis-antisepsis, local

anaesthesia (lidocaine) was applied. After surgical extirpation of the mass, it was sent to the pathology department for histopathological examination. The patient was discharged after the administration of postoperative antibiotics.

Histopathological examinations. Tumour samples were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin wax. The tissue sections were cut into sections of 5 μm thickness and stained with Hematoxylin and Eosin (H&E) in order to detect histopathological changes. Sections were examined under a light microscope and photographed. The degrees of differentiation of the BOSCCs were determined on the basis of the size and number of keratin pearls, the formation and width of the tumoral islands, and the squamous differentiation (CARVALHO et al., 2005; GHARAGOZLOU et al., 2007; SÖZMEN et al., 2019).

Immunohistochemical examinations. The avidin-biotin-peroxidase method was used as immunohistochemical (IHC) staining. Serial sections of 4 μm in thickness were deparaffinised and rehydrated in graded alcohols. The sections were incubated in 3% H2O2 for 15 min at room temperature to block endogenous peroxidase activity. After the sections were washed for 5 min in Phosphate Buffered Saline (PBS), they were boiled in citrate buffer solution (pH 6) for 25 min in a microwave oven (at 800 watts) to induce antigen release. All the sections were stained with Thermo Scientific Histostain-Plus IHC Kit using NT (Santa Cruz, sc-32731, Dilution Ratio: 1:250), MDA (Abcam, ab6463, Dilution Ratio: 1:1500) and 8-OHdG (Bioss Antibodies, bs-1278R, Dilution Ratio: 1/800) primary antibodies, according to the manufacturer's instructions. For immunolabeling, 3,3'-diaminobenzidine (DAB) was used as the chromogen. Mayer›s hematoxylin was used as the counterstain. Negative control sections were incubated with PBS instead of the primary antibodies. The slides prepared after covering were examined under a light microscope (Olympus Bx53) and photographed using the Cell^P program (Olympus Soft Imaging Solutions GmbH, 3,4). Analyses of the images were performed using the Image J Program (1.51j8).

Analysis of NT, MDA and 8-OHdG expressions were scored on the basis of the number of positive cells in the areas that best reflected the character of the staining. For quantification of the immunostaining in the tissue, the analysis started on the basis of high-intensity reaction areas. For each tumour sample, 5 different areas were examined at a total magnification of 200x. The number of immunopositive cells stained in each area was recorded, and the average of these 5 sites was taken as the data for that animal.

Statistical analysis. It was determined that WD, MD and PD groups did not show normal distribution according to the Shapiro-Wilk test and histogram graph. In group comparison, the Kruskal-Wallis H test was used. Mann-Whitney U test was used for paired comparison of the groups. Statistical analyses were performed using the SPSS® (Version 20.0, Chicago, IL, USA) program. Differences obtained between groups after the statistical analysis were considered significant at the $P<0.05$ level.

Results

Macroscopic findings. Tumoral masses were mostly located in the upper and lower eyelids, the third eyelid, cornea, limbus or covering the entire eyeball. In macroscopical examinations, it was noted that the surfaces of the tumoral tissues showing irregular, cauliflower-like growths that were highly hemorrhagic and ulcerative (Fig. 1).

Histopathological findings. In the microscopical examinations, we determined that the keratin pearls were quite large in number and size in well-differentiated (WD) BOSCCs compared to moderately differentiated (MD) and poorly differentiated (PD) BOSCCs. We determined that the number of keratin pearls in the MD cases was very low, and their sizes were small to medium, while in the PD cases, only cell-level keratinisation was present. We determined that the cases with the largest tumoral island formation were the WD cases. In MD cases, we found that the size of the tumoral islands was smaller compared to WD cases. In PD cases, we found that the tumoral island formations were very low in number and almost absent. Pleomorphic tumoral areas were more marked in PD cases. We

noted that in these cases the presence of abnormal mitotic figures, dyskeratotic cells, anisonucleosis, anisocytosis, an increase in the nucleus/cytoplasm ratio in favour of nuclei, apoptotic bodies, an increase in the number of nucleoli, hyperchromasia, and bizarre giant cells was more pronounced than in other cases. In MD cases, pleomorphism was of a moderate degree, and we observed a significant increase in the number of poorly differentiated tumoral cells compared to the WD group. In contrast to other cases, squamous differentiation was quite evident in WD cases (Fig. 2).

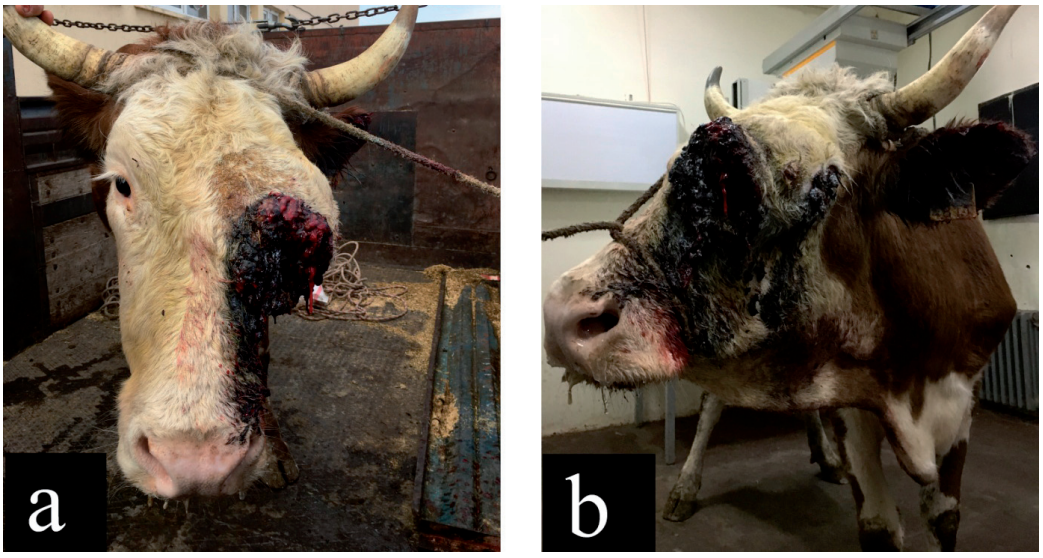

Fig. 1. (a-b) View from different angles of a bleeding cauliflower-like tumoral mass covering the entire eyeball

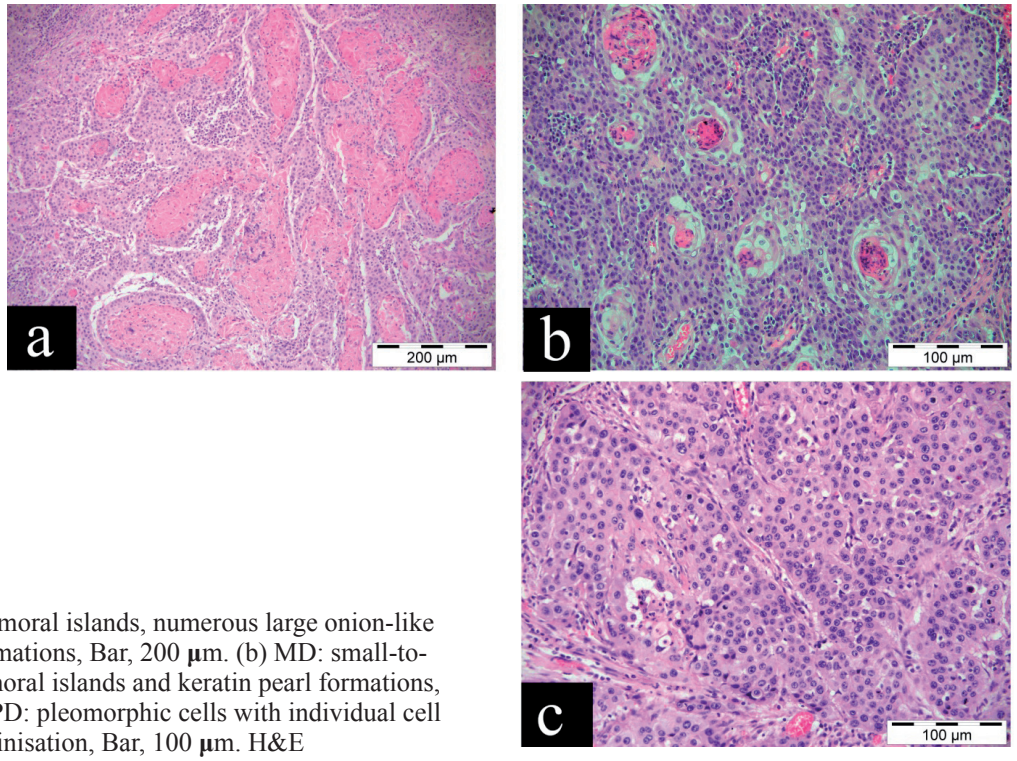

Fig 2. (a) WD: tumoral islands, numerous large onion-like keratin pearl formations, Bar, 200 µm. (b) MD: small-to-medium sized tumoral islands and keratin pearl formations, Bar, 100 µm. (c) PD: pleomorphic cells with individual cell keratinisation, Bar, 100 µm. H&E

Immunohistochemical findings. The mean±SE values of WD, MD and PD cases are given in Table 1. WD, MD and PD cases were all immuno-positive for NT, MDA and 8-OHdG expression. We detected a statistically significant difference in NT expressions between WD, MD and PD groups (P<0.001). However, we found no statistically significant difference in mean NT positive cell numbers between the MD and PD groups. We found dark brown NT-positive reactions in the WD group, especially in the cytoplasm of neoplastic cells in the periphery of the tumoral islands. Similar to WD cases, we detected dark brown NT expressions in the cytoplasm of neoplastic cells in the periphery of the tumoral islands in MD cases. In addition, we observed that the severity of NT-positive reactions increased in pleomorphic/atypical areas. In PD cases, we detected dark brown positive NT immunoreactivity, especially in the cytoplasm of neoplastic tumoral cells. Unlike WD and MD cases, the reaction was much more pronounced in pleomorphic areas than in tumoral islands. While there was no statistically significant difference between the WD and MD cases in terms of mean NT positive cell counts, in contrast, we found a notable increase between PD cases and these two cases (P=0.008). Similar to NT reactions, we observed intracytoplasmic yellow-brown MDA positive reactions in tumoral cells in the periphery of the tumoral islands in both WD and MD cases. In PD cases, the severity and intensity of the reaction spread to all areas of the tumoral mass rather than in a specific pattern. Dark-brown NT immunoreactivity in the cytoplasm of atypical cells was stronger than in other cases. There was no statistical difference between WD and MD cases in terms of 8-OHdG expression. We found that the increase in PD cases was statistically significant compared to WD and MD cases (P=0.009). 8-OHdG-positive expressions were concentrated, especially in the cytoplasm of the neoplastic cells forming the tumoral islands in the WD and MD groups. In the PD group, reactions in the cytoplasm of atypical cells were sometimes granular (Fig. 3).

Table 1. Pairwise comparisons of all cases with mean ± SE values

Parameters	Cases			P value	Pairwise comparison P value of the groups		
	WD (n=8)	MD (n=8)	PD (n=8)		WD-MD	WD-PD	MD-PD
NT	319.88 ± 13.16	405.18 ± 10.89	449.50 ± 17.66	<0.001	0.002	0.001	0.103
MDA	338.75 ± 14.53	349.25 ± 9.14	420.38 ± 17.12	0.008	0.40	0.016	0.004
8-OHdG	418.63 ± 17.76	448.00 ± 14.17	508.50 ± 15.16	0.009	0.372	0.006	0.016

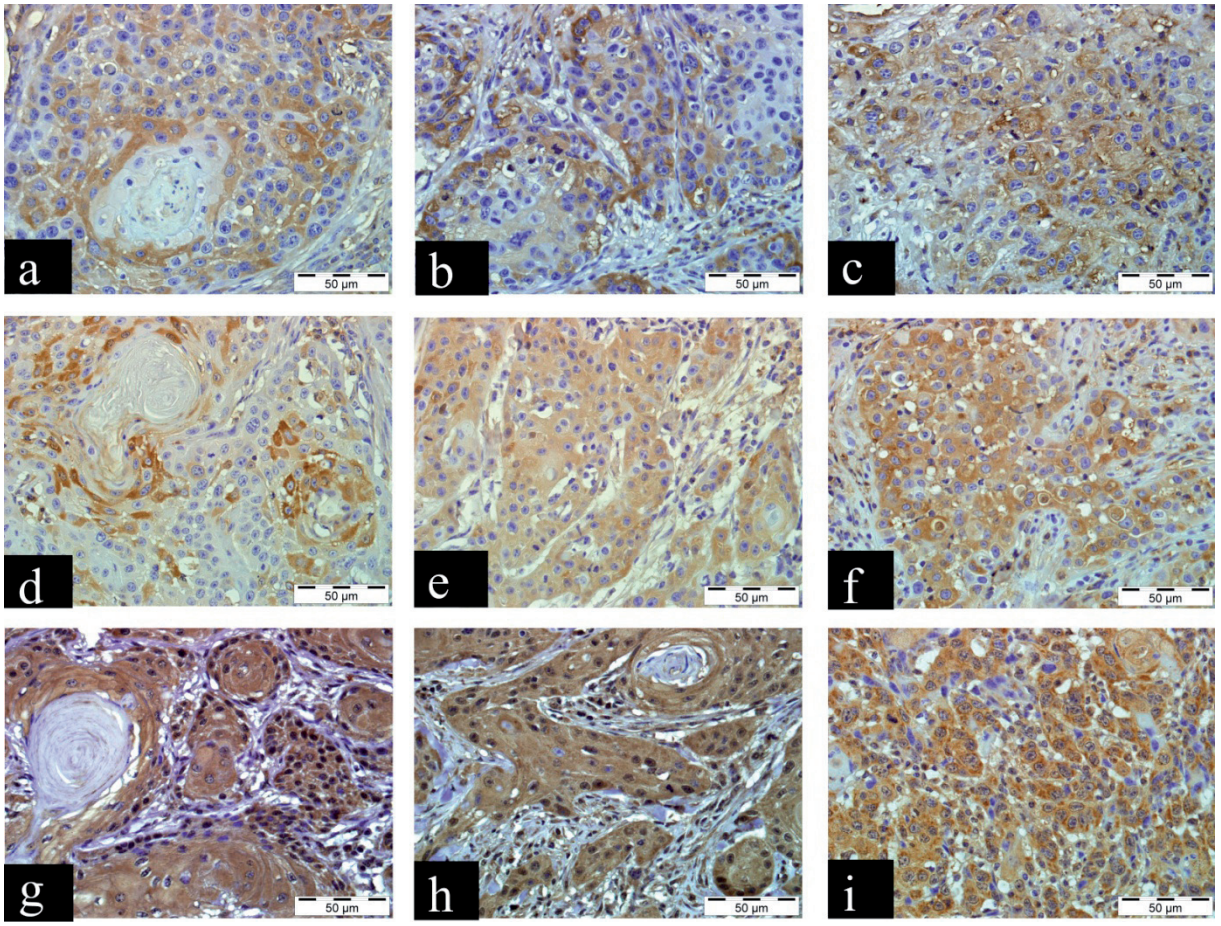

Fig. 3. (a) WD: intracytoplasmic NT reactions in cells located in the periphery of the tumoral islands. (b) MD: Dark brown NT expressions in the cytoplasm located in the periphery of tumoral islands and neoplastic cells. (c) PD: Severe cytoplasmic NT immunoreactivity in atypical cells. (d) WD: Cytoplasmic MDA reactions in tumoral cells around the keratin pearl. (e) MD: intracytoplasmic NT reactions in cells located in the periphery of tumoral islands and atypical cells. (f) PD: Intracytoplasmic dark brown MDA positive expressions in pleomorphic cells. (g) WD: 8-OHdG expressions in the cytoplasm of cells located outside the tumour islands. (h) MD: 8-OHdG immunoreactivity in the cytoplasm of pleomorphic cells forming the tumoral islands. (i) Intracytoplasmic granular 8-OHdG immune positive reactions in atypical cells, Bar= 50 µm. IHC.

Discussion

NO participates in numerous intracellular pathways and the generation of RNS. During oxidative stress, NT can be produced by post-translational nitrification of tyrosine amino acid residues, impairing protein folding and functions (SILVA SERVATO et al., 2019). For this reason, the detection of NT is a useful nitrosative stress marker for detecting protein damage caused by high levels of NO production (SEPEHR et al., 2001; CARRARA et al., 2019). It is also known that NO causes structural changes in the p53 tumour suppressor gene, which plays an important role in the etiopathogenesis of BOSCCs (BENTZ et al., 2000; CARVALHO et al., 2005). We could not find any literature data that evaluated by immunohistochemical methods the expression of NT in OSCCs of humans and cattle. In humans, SCC is significantly linked to cumulative solar

exposure and photooxidative skin damage, and photooxidative stress leads to oxidative modifications in biomolecules such as lipid peroxide and protein carbonylation, along with DNA oxidative damage (CARRARA et al., 2019). It has been reported by various researchers that, compared to healthy individuals, in cancer patients, nitrooxidative stress increases while antioxidant activity decreases (AKSOY and KURNAZ, 2019; CARRARA et al., 2019; SILVA SERVATO et al., 2019; KATO et al., 2000). In our study, similar to SCCs in humans (head and neck, oral etc.), a significant increase was observed in the number of NT-positive cells in PD cases compared to WD cases (AKSOY and KURNAZ, 2019; SILVA SERVATO et al., 2019; BENTZ et al., 2000; KATO et al., 2000). We interpreted that this increase in NT expression may be related to protein damage caused by solar UV radiation, which has an important role in the formation of BOSCCs. Within the scope of the data obtained from our study, we concluded that NT expression, which increases in parallel with the development of tumour differentiation, and nitrosative stress may be a serious parameter in the pathogenesis of BOSCCs.

Oxidative stress is strongly linked to carcinogenesis (YOSHIFUKU et al., 2018). Cancer is basically a multi-step process that involves initiation, promotion and progression, and all these stages can be triggered through ROS, thus promoting tumour growth (MALIK et al., 2014). Free radicals can create lipid peroxidation, and MDA, a highly toxic aldehyde molecule, is a reliable marker for detecting lipid peroxidation and oxidative stress (POLANIAK et al., 2010; SHETTY et al., 2014; SINGH et al., 2015). It has been reported in previous studies that MDA levels increased significantly in patients with oral SCC and head and neck SCC compared to healthy individuals (MALIK et al., 2014; SHETTY et al., 2014; YOSHIFUKU et al., 2018). Similar to the case of NT, we could not find any literature data evaluating MDA immunoreactivity in BOSCCs and HOSCCs. As expected, we found a remarkable increase in the number of MDA-positive cells in PD cases compared to WD and MD cases. We interpreted this to mean that, from the perspective of increased MDA expression, lipid peroxidation may have an important role in the growth of BOSCC.

Oxidative damage of DNA by ROS and RNS leads to the production of 8-OHdG, an oxidised form of deoxyguanosine nucleoside (KUBO et al., 2014; AN et al., 2019). 8-OHdG can cause the transversion of GC to TA, and the levels of 8-OHdG have been reported to increase in many human cancers (breast cancer, lung cancer, bladder cancer, colorectal cancer, renal cell carcinoma, prostate cancer and gastric cell adenocarcinoma) and animal experimental models (KUMAR et al., 2012; SOARES et al., 2018; AN et al., 2019). We could not find any literature data evaluating by immunohistochemical methods using 8-OHdG expressions the oxidative DNA damage in both bovine and human ocular squamous cell carcinomas. Various studies have shown that overexpression of 8-OHdG is associated with poor prognosis (AN et al., 2019). In our study, similar to the literature data (ZANNONI et al., 2006; KUMAR et al., 2012; KUBO et al., 2014; SOARES et al., 2018; AN et al., 2019), we found that the number of 8-OHdG positive cells in PD cases increased statistically significantly compared to WD and MD cases.

In conclusion, NT, MDA and 8-OHdG expressions were found to be statistically increased in PD cases compared to WD and MD cases. We interpreted this increase to mean that nitrosative and oxidative stress may play an important role in the carcinogenesis of BOSCCs.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Ethical statment

This study was approved by the Kafka University Animal Experiments Local Ethics Committee (KAU-HADYEK-2020/116).

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SAŽETAK

Oksidacijski je stres snažno povezan s karcinogenezom, osobito kad je riječ o skvamocelularnom karcinomu (SCC) u području glave, vrata i usne šupljine u ljudi. Cilj je istraživanja bio procijeniti ulogu oksidacijskog i nitrozativnog stresa kod skvamocelularnih karcinoma oka u goveda (BOSCCs) upotrebom imunohistokemijskih biljega kao **što** su nitrotirozin (NT), malondialdehid (MDA) i 8-hidroksi-2›-deoksigvanozin (8-OHdG). Prikupljena su tkiva 24 goveda i dopremljena na Odjel za patologiju radi rutinske dijagnostike. Uzorci tumora fiksirani su u 10%-tnom neutralnom puferiranom formalinu, rutinski obrađeni i ugrađeni u parafinski vosak. Dijelovi tkiva rezani su na debljinu od 5 µm i obojeni hematoksilin-eozin bojenjem kako bi se otkrile histopatološke promjene. Rezovi su pregledani svjetlosnim mikroskopom i fotografirani. Za imunohistokemijsko bojenje primijenjena je metoda avidin-biotinske peroksidaze. Uočeno je da su tumorske tvorbe nalik na cvjetaču većinom smještene na gornjem i donjem kapku, na trećem kapku, rožnici i limbusu, ili su prekrivale cijelu očnu jabučicu. Izražaji NT, MDA i 8-OHdG bili su statistički znakovito povećani u slabo diferenciranim (PD) slučajevima u usporedbi s dobro diferenciranim (WD) i umjereno diferenciranim (MD) slučajevima. Na temelju povećanih vrijednosti ovih biljega zaključili smo da nitrozativni i oksidacijski stres mogu imati važnu ulogu u karcinogenezi skvamoznih stanica oka u goveda.

Ključne riječi: govedo; nitrozativni stres; oksidacijski stres; skvamocelularni karcinom