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Immunohistochemical Investigation of Oxidative Stress-induced DNA Damage and Lipid Peroxidation in Bovine Papillomas and Fibropapillomas

Emin KARAKURT

Kafkas University, Faculty of Veterinary Medicine, Department of Pathology, Kars, 36100, Turkey

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ABSTRACT In this study, it was aimed to evaluate the expressions of 8-OHdG and MDA immunohistochemically in order to determine the oxidative stress-induced DNA damage and lipid peroxidation in 16 papillomas and 14 fibropapillomas from 30 cattle brought to our department between 2013-2020. Biopsy samples taken after surgery were fixed in buffered 10% formaldehyde solution. Sections of 5 µm thickness were taken from the paraffin blocks prepared after routine tissue follow-up procedures and Hematoxylin & Eosin staining was applied to the sections in order to detect histopathological changes. Avidin Biotin Peroxidase method was used for immunohistochemical staining. In papilloma cases, severe hyperkeratosis, retepects extending from the epidermis to the dermis, spongiosis and balloon-like degeneration in squamous epithelial cells, basophilic inclusion bodies in granular cells, ulcerations in the epidermis layer, hemorrhagic areas and an increase in kerato hyaline granules were observed. In addition to these findings, dense connective tissue increases were detected in fibropapilloma cases. In immunohistochemical evaluations, positive reactions for BPV were observed in the nuclei of cells in stratum granulosum. 8-OHdG positive reactions were detected in the cytoplasm and nucleus of the epidermal cells in papilloma cases, whereas in cases of fibropapilloma, reactions in these cells were observed in the cytoplasm of fibrocytes and fibroblasts in the dermis. Membranous MDA positive reactions were observed in the epidermal cells in papilloma cases, whereas MDA expressions were detected in the cytoplasm of fibrocytes and fibroblasts in the dermis of fibropapilloma cases. As a result of the literature reviews, no study data was found in which DNA damage due to oxidative stres and lipid peroxidation was detected by means of 8-OHdG and MDA expressions in papilloma and fibropapilloma cases of cattle, it is thought that the findings obtained in this study will contribute to the literature. In addition, it has been concluded that oxidative stress plays an important role in the pathogenesis of this tumor.

Keywords: *DNA damage, Lipid peroxidation, Oxidative stress, Papilloma*

ÖZ

Sığır Papillom ve Fibropapillomlarda Oksidatif Stres Kaynaklı DNA Hasarı ve Lipid Peroksidasyonun İmmunohistokimyasal Olarak Araştırılması

Bu çalışmada 2013-2020 yılları arasında anabilim dalımıza getirilen toplamda 30 adet sığırlara ait 16 adet papillom ve 14 adet fibropapillom örneğinde oksidatif stres kaynaklı DNA hasarı ve lipid peroksidasyonu belirlemek amacıyla immunohistokimyasal olarak 8-OHdG ve MDA ekspresyonlarının değerlendirilmesi amaçlandı. Cerrahi operasyon sonrası alınan biyopsi örnekleri tamponlu %10'luk formaldehit solüsyonunda fikze edildi. Rutin doku takip işlemleri sonrası hazırlanan parafin bloklardan 5 µm kalınlığında kesitler alındı ve histopatolojik değişikliklerin saptanabilmesi amacıyla kesitlere Hematoksilen & Eozin boyaması uygulandı. İmmunohistokimyasal boyamalarda Avidin Biotin Peroksidaz metodu uygulandı. Papillom vakalarında şiddetli hiperkeratoz, epidermisten dermise doğru uzanan retepektler, skuamöz epitel hücrelerinde spongiyozis ve balonumsu dejenerasyon, granüler hücrelerde bazofilik inklüzyon cisimcikleri, epidermis katmanında ülserasyonlar ve kanama alanları ile kerato hiyalin granüllerinde artış gözlendi. Fibropapillom vakalarında ise bu bulgulara ek olarak girdap tarzında bağ doku artışları tespit edildi. İmmunohistokimyasal değerlendirmelerde ise BPV pozitif reaksiyonlar stratum granulozumdaki hücrelerin çekirdeğinde gözlendi. 8-OHdG pozitif reaksiyonlar papillom vakalarında epidermal hücrelerin sitoplazmasında ve çekirdeğinde saptanırken, fibropapillom vakalarında ise bu hücrelerdeki reaksiyonlara ek olarak dermisteki fibrosit ve fibroblastların sitoplazmasında rastlanıldı. Papilloma olgularında epidermal hücrelerde membranöz MDA pozitif reaksiyonlar görülürken, fibropapilloma vakalarının dermisinde fibrosit ve fibroblastların sitoplazmasında MDA ekspresyonları tespit edildi. Yapılan literatür taramaları sonucunda sığırlara ait papillom ve fibropapillom vakalarında oksidatif strese bağlı DNA hasarı ile lipid peroksidasyonun 8-OHdG ve MDA ekspresyonları vasıtasıyla tespit edildiği herhangi bir çalışma verisine rastlanmamış olup ve bu yönüyle bu çalışmada elden edilen bulguların literatüre katkı sunacağı düşünülmektedir. Bunlara ek olarak oksidatif stresin bu tümörün patogenezinde önemli bir rol oynadığı kanaatine varılmıştır.

Anahtar Kelimeler: *DNA hasarı, Lipid peroksidasyon, Oksidatif stres, Papillom*

✉ Corresponding author: mehmeteminkarakurt@hotmail.com

INTRODUCTION

Bovine papillomatosis is usually a benign tumor characterized by the development of multiple warts (Atasever et al. 2005; Beytut 2017). Bovine papilloma viruses (BPV), the etiological agent of bovine papillomatosis, has a double-stranded circular, 8-kb DNA genome belonging to Papillomaviridae family (Grindatto et al. 2015; Araldi et al. 2015; Timurkan and Alcigir 2017). There is currently 24 known BPV types (Bianchi et al. 2020). Viral replication takes place in the nucleus, as infected cells are lysed and new virions are released (Hong and Kim 2015). These viruses show an affinity for mucosal tissues and squamous epithelial as well as mesenchymal tissue (Araldi et al. 2014; Hamad et al. 2016). Although these viruses show tropism to the squamous epithelium of warm-blooded animals, particulary cattle, cross-species infection has been reported only in horses and other equines (AL-Salihi et al. 2020). BPV, which is highly species specific and has been detected in water buffaloes, bison, giraffes, zebras, antelopes, yak, horses, donkeys, tapirs, and other species (da Silva et al. 2015; Rojas-Anaya et al. 2016). Although the tumor is seen at all ages, it is mostly detected in young animals and regresses spontaneously with the development of immune systems (Atasever et al. 2005; Özsoy et al. 2011). Sometimes this process progresses towards cancer (Tozato et al. 2013; Hamad et al. 2017). Warts caused by papillomaviruses, known as epitheliotropic, are mostly seen on the scalp, tongue, breasts, penis, vulva, oral cavity and upper digestive tract (Munday 2014; Rojas-Anaya et al. 2016). BPV cause significant economic losses (growth reduction, weight loss, decreased milk production) by causing benign and malignant tumors such as cutaneous papillomas, fibropapillomas, bladder and esophageal cancers in cattle (Carvalho et al. 2013; Hamad et al. 2017; Timurkan and Alcigir 2017). This viral disease is distributed in many region of the world such as America, Europe and Asia (Ata et al. 2018). BPV can be scattered through contaminated milking and care equipment, contact with infected animals, and have also been detected in epithelial tissue and blood interpreted as infection reservoirs in studies. In addition to these, there are serious findings that it can be transmitted by lymphocytes, milk, urine, oocyte, ovaries and uterus (Araldi et al. 2015; Rojas-Anaya et al. 2016; Dörttaş and Bilge Dağalp 2020). Heredity, hormonal and nutritional disorders, sunlight and the suppressed immune system play a serious role in the pathogenesis of the disease (Atasever et al. 2005; Özsoy et al. 2011).

Free radicals are highly reactive chemical products that occur during metabolism in the body, and they interact with macromolecules such as lipids, carbohydrates, proteins and nucleic acids, causing oxidative damage (Atmaca and Aksoy 2009; Özcan et al. 2015). Under normal conditions, there is a balance in the production of free oxygen radicals and radical toxicity and antioxidant system that has a scavenging effect against them. The disruption of this balance between antioxidants and oxidants in favor of oxidants is called oxidative stress (Tabakoğlu and Durgut 2013; Özcan et al. 2015). Reactive oxygen species (ROS) cause more than 20 oxidative base damage products in DNA. Among these damaged bases, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is the most used marker in determining oxidative DNA damage (Atmaca and Aksoy 2009; Özcan et al. 2015). The severity of oxidative damage can be determined by measuring the levels in blood and tissues of the end products that are accepted as specific indicators such as Malondialdehyde (MDA) resulting from lipid peroxidation (Sasmaz et al. 2005; Aslan and Saraç 2011).

In this study, it is aimed to evaluate the expressions of 8-OHdG and MDA immunohistochemically in order to determine the oxidative stress-induced DNA damage and lipid peroxidation in bovine papilloma and fibropapilloma samples brought to our department between 2013-2020.

MATERIALS and METHODS

Animals

The material of this study consisted of tissue samples of 16 papilloma, 14 fibropapilloma and 6 normal bovine skin tissues taken from cattle brought to our department between 2012-2020.

Ethical Approval

The ethics committee report of this study was obtained from Kafkas University Animal Experimentals Local Ethics Committee (Authorization number: KAU-HADYEK-2020/164).

Histopathological Investigations

Tissue samples taken after surgery were fixed in 10% buffered formalin solution. After routine procedures paraffin blocks were cut to 5 µm thickness and Hematoxylin & Eosin (H&E) staining was applied to the sections in order to detect histopathological changes. Sections were examined and photographed under a light microscope.

Immunohistochemical Investigations

Avidin-Biotin Peroxidase method was used as immunohistochemical method. For immunohistochemical staining, the sections of 4 µm in thickness taken to poly-L-lysine coated slides were deparaffinized and rehydrated in graded alcohols. In order to prevent endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide solution in Phosphate Buffered Saline (PBS) for 15 minutes. For antigen retrieval, the sections were boiled in Citrat Buffer Solution (pH 6) for 25 min in the microwave oven (at 800 watt). In order to prevent nonspecific staining, the sections were incubated for 10 min with non-immune serum (Thermo Scientific Histostain-Plus IHC Kit, HRP, broad spectrum, REF: TP-125-HL) at room temperature. Diluted antibodies (BPV: MyBioSource, MBS320197, Dilution Ratio: 1/100; 8-OHdG: Bioss Antibodies, bs-1278R, Dilution Rate: 1/800, MDA: Abcam, ab6463, Dilution Rate: 1/250) were incubated for overnight (+ 4 °C in refrigerator). The sections were washed 3 times in PBS solution for 5 minutes, and the biotinylated secondary antibody (Thermo Scientific, Histostain-Plus IHC Kit, HRP, broad spectrum, REF: TP-125-HL) was applied to them at room temperature for 10 minutes. After washing in PBS (3-5 min), all sections were incubated with peroxidase-bound Streptavidin (Thermo Scientific, Histostain-Plus IHC Kit, HRP, broad spectrum, REF: TP-125-HL) for 10 minutes at room temperature. A solution of 3.3-diaminobenzidine tetra hydrochloride (DAB) (Thermo Scientific, REF: TA-125-HD) was used as a chromogen for 15 minutes. 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 (goat). The slides prepared after the covering were examined under a light microscope (Olympus Bx53) and photographed via the Cell^P program (Olympus Soft Imaging Solutions GmbH,

3,4). Analyzes of the images were done with Image J Program. Results were evaluated as negative (-), mild (+), moderate (++) and severe (+++).

Statistical Analysis

The significance of the difference between the histopathologically scored data between the groups was evaluated with the Mann-Whitney U test. All analyzes were performed on the SPSS® (Version 18.0, Chicago, IL, USA) program. Differences obtained between groups after statistical analysis were considered significant at the $P<0.05$ level.

RESULTS

Macroscopical Results

In macroscopic examination of the masses, wart-like growths with or without a stalk in the form of cauliflower were observed (Figure 1).

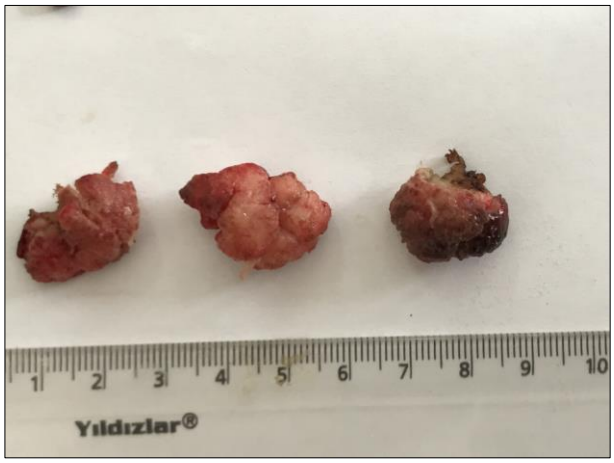

Figure 1. Macroscopic appearance of papilloma.

Microscopical Results

In papilloma cases, severe hyperkeratosis in the stratum corneum layer (Figure 2 a), acanthosis characterized by hyperplasia of stratum spinosum cells (Figure 2 a), and finger-like structures (rete pegs) extending from the epidermis to the dermis were detected. In addition to these, severe degeneration in keratinocytes in the stratum spinosum layer (Figure 2 b), increase in large and small keratohyaline granules in the stratum granulosum layer (Figure 2 c), and coilocytes with transparent cytoplasm with eccentric localized pycnotic nuclei in the stratum spinosum and granulosum layer were observed (Figure 2 d).

Few mitotic figures, areas of hemorrhage and ulceration, and inflammatory cell infiltration were other important histopathological findings. In the fibropapilloma cases, in addition to these histopathological changes, dense connective tissue bundles were determined in different directions between the rete peg structures (Figure 2 e). There were no any pathological changes found in normal bovine skin tissue (Figure 2 f).

Immunohistochemical Results

In immunohistochemical evaluations, BPV immune positive reactions were detected in the nucleus of cells in the stratum granulosum layer of the epidermis all papilloma and fibropapilloma cases (Figure 3 a-b). 8-OHdG positive reactions were determined in the cytoplasm and nucleus of the epidermal cells in all papilloma cases (Figure 3 d), whereas in all cases of fibropapilloma, reactions in these cells were observed in the cytoplasm of fibrocytes and fibroblasts in the dermis (Figure 3 e). Membranous MDA positive reactions were observed in the epidermal cells in all papilloma cases (Figure 3 g), whereas MDA expressions were detected in the cytoplasm of fibrocytes and fibroblasts in the dermis of all fibropapilloma cases (Figure 3 h). Normal bovine skin tissue was negative for BPV, 8-OHdG and MDA expressions (Figure 3 c-f-i).

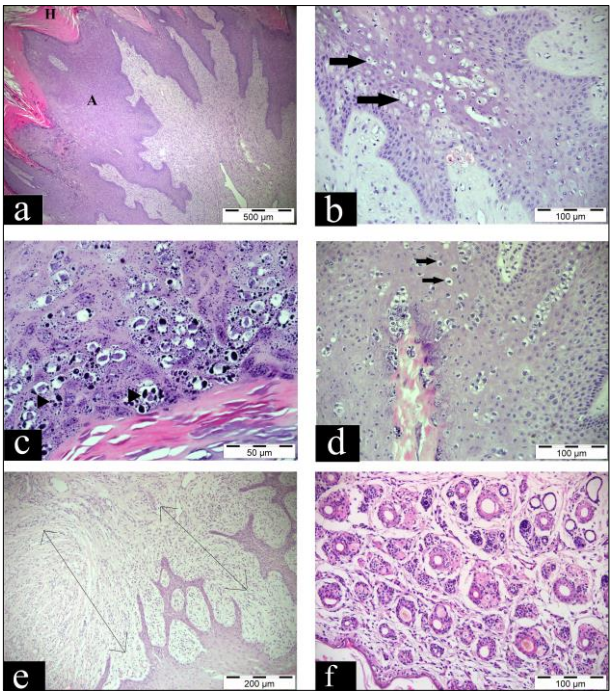

Figure 2. Hyperkeratosis (H), acanthosis (A), H&E, Bar= 500 µm (a), Hydropic degeneration in keratinocytes (arrows), H&E, Bar = 100 µm (b), Keratohyaline granules (arrowheads), H&E, Bar = 50 µm (c), Koilocytosis, H&E, Bar= 100 µm (d), Dense bundles of connective tissue in different directions between rete ridge structures (lines), H&E, Bar = 200 µm (e), Normal bovine skin tissue, H&E, Bar= 100 µm (f).

Statistical Results

Mean ± SE mean and median values of all groups are given in Table 1. A statistically significant increase found in the papilloma and fibropapilloma groups in terms of BPV reactions compared to the control group. In terms of 8-OHdG immune positive expressions, a significant increase found in papilloma and fibropapilloma groups compared to the control group. In addition, the increase in 8-OHdG immune positive reactions was statistically more significant in papilloma groups than in fibropapilloma groups. In terms of MDA expressions, a significant increase found in papilloma and fibropapilloma groups compared to the control group. However, any statistically significant difference found in MDA expression between papilloma and fibropapilloma groups.

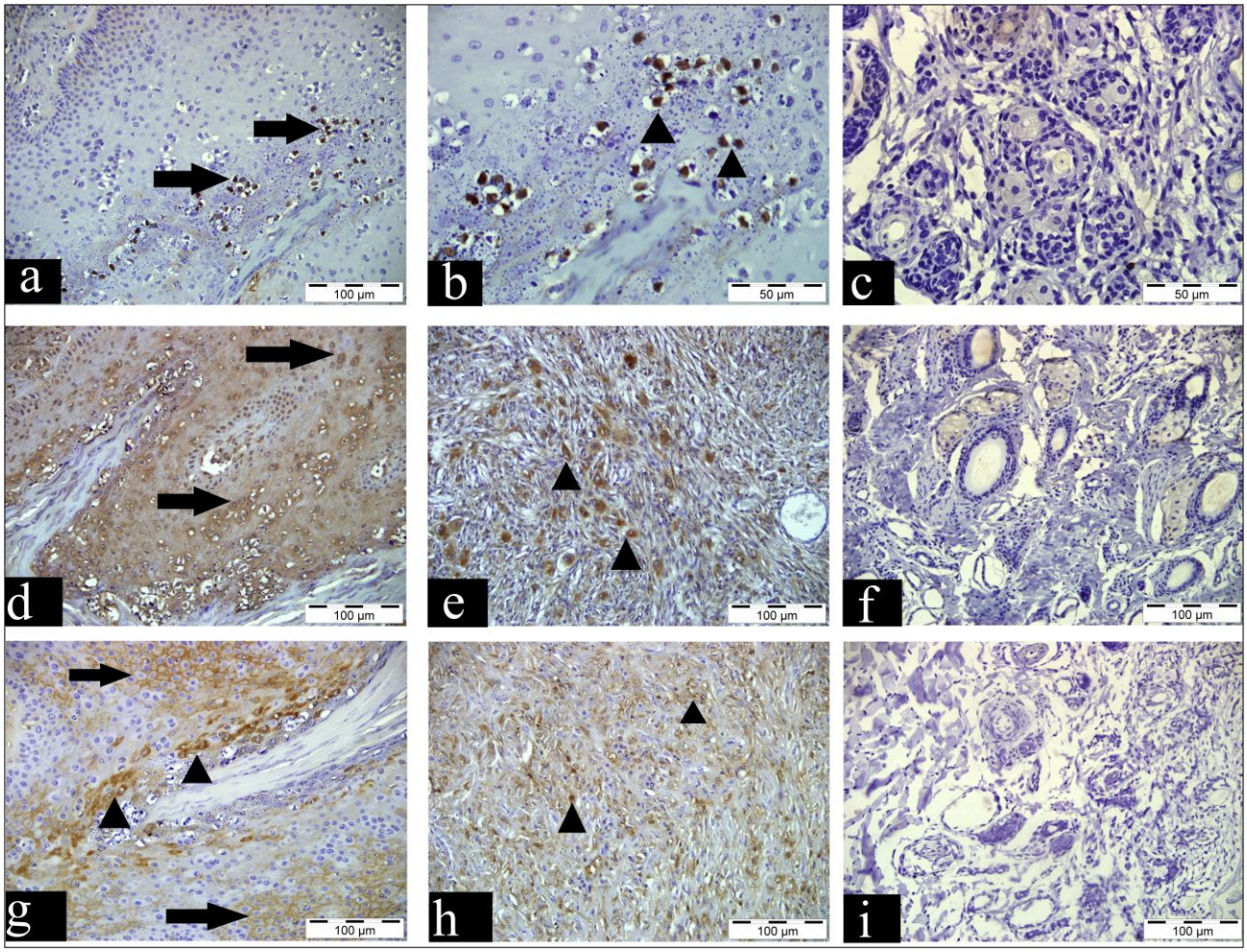

Figure 3. BPV immune positive reactions in the nucleus of cells (arrows) in the stratum granulosum layer of the epidermis, IHC, Bar = 100 µm (a), Higher magnification, intranuclear BPV expressions (arrowheads), IHC, Bar= 50 µm (b), Normal bovine skin tissue, negative BPV immunoreactivity, IHC, Bar= 50 µm (c), Papilloma, 8-OHdG positive reactions in the cytoplasm and nucleus of the epidermal cells (arrows), IHC, Bar = 100 µm (d), Fibropapilloma, 8-OHdG positive reactions in the cytoplasm of fibrocytes and fibroblasts (arrowheads) in the dermis, IHC, Bar = 100 µm (e), Normal bovine skin tissue, negative 8-OHdG immunoreactivity, IHC, Bar= 100 µm (f), Papilloma, membranous (arrows) and cytoplasmic (arrowheads) MDA positive reactions in the of the epidermal cells, IHC, Bar = 100 µm (g), Fibropapilloma, MDA positive reactions in the cytoplasm of fibrocytes and fibroblasts (arrowheads) in the dermis, IHC, Bar = 100 µm (h), Normal bovine skin tissue, negative MDA immunoreactivity, IHC, Bar= 50 µm (i).

Table 1. Mean ± SE mean and median values of all groups.

Groups		BPV	8-OHdG	MDA
Control	Mean ± SEM	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]
	(Median)	(0)	(0)	(0)
Papilloma	Mean ± SEM	3 ± 0 [b]	2.69 ± 0.12 [c]	1.69 ± 0.18 [b]
	(Median)	(3)	(3)	(3)
Fibropapilloma	Mean ± SEM	3 ± 0 [b]	1.93 ± 0.20 [b]	1.36 ±0.13 [b]
	(Median)	(3)	(2)	(1)

[a,b,c]: Expresses the statistical differences between groups in each column. [a-b]: P<0.001, [a-c]: P<0.001, [b-c]: P=0.004.

DISCUSSION and CONCLUSION

In accordance with the findings of the literature (Atasever et al. 2005; Özsoy et al. 2011; Carvalho et al. 2013; Hong and Kim 2015; Rojas Anaya et al. 2016; Beytut 2017; Hamad et al. 2017), in this study, a cauliflower-like wart-like growth with or without a stem was detected on the skin of animals with papillomas and fibropapillomas. In cattle, papillomas and fibropapillomas are mostly seen on the head (Atasever et al. 2005; Özsoy et al. 2011; Hong and Kim 2015), neck (Atasever et al. 2005; Beytut et al. 2017; Timurkan and Alcigir 2017), shoulder (Batista et al. 2013; Hong and Kim 2015; Beytut et al. 2017), abdomen (Hamad et al. 2016; Timurkan and Alcigir 2017) teat (Beytut et al. 2017; Timurkan and Alcigir 2017; Branchi et al. 2020), foot (Rojas-Anaya et al. 2016), vulva (Yamashita-Kawanishi et al. 2019) and penis (Dörttaş and Bilge Dağalp 2020) have been reported in various studies. Similar to these data, wart-like growths in various regions such as

head, neck, shoulder, abdomen, breast, foot, vulva and penis were detected in this study.

Hyperkeratosis (Atasever et al. 2005; Hong and Kim 2015; Branci et al. 2020), acanthosis (Özsoy et al. 2011, Araldi et al. 2015; Al-Salihi et al. 2020), rete pegs (Beytut et al. 2017; Hamad et al. 2017; Ata et al. 2018), koilocytosis (Carvalho et al. 2013; Araldi et al. 2014; Hamad et al. 2017), degeneration in keratinocytes (Atasever et al. 2005; Tozato et al. 2013; Timurkan and Alcigir 2017), keratohyaline granules (da Silva et al.2015; Beytut et al.2017, Timurkan and Alcigir 2017), dermal proliferation (Grindatto et al. 2015; Hamad et al. 2016; Yamashita-Kawanishi et al. 2019), the histopathological changes observed in papillomas and fibropapillomas were also recorded in this study.

In the immunohistochemical investigations of these cases, which were defined as papilloma and fibropapilloma as a result of macroscopic and microscopic examinations, BPV immune positive reactions were detected in the nucleus of cells in the stratum spinosum layer of the epidermis, as reported in previous studies (Abdouslam et al. 1997; Jelínek and Tachezy 2005; Maeda et al. 2007; Hatama et al. 2009; Tan et al. 2012; Yamashita-Kawanishi et al. 2019).

Free radicals are formed in cells due to endogenous and exogenous factors. Exogenous factors can be briefly summarized as follows; stress, viruses, infectious agents, drug intoxications, ionizing and ultraviolet radiation (Atmaca and Aksoy 2009). ROS, radicals formed from oxygen, lead to the disruption of nucleic acid function by causing mutations or cancer, irreversible DNA damage, changes in enzyme activities, and the formation of new immunological structures by damaging proteins (Tabakoğlu and Durgut 2013). ROS causes the formation of more than 20 oxidative base damage products in DNA and oxidatively modified DNA in the 8-OHdG form is a highly sensitive marker used in determining the amount of DNA damage (Atmaca and Aksoy 2009; Özcan et al. 2015). The most serious pathological process associated with DNA damage is carcinogenesis, and oxidative damage is thought to play an important role in the initiation, progression and promotion phases of carcinogenesis (Özcan et al. 2015). There are studies evaluating 8-OHdG levels in various cancer types such as dysplastic cervical cells infected with human papilloma virus (Romano et al. 2000), squamous cell carcinomas of the head and shoulder (Kumar et al. 2012), recalcitrant warts (Erturan et al. 2019), solid tumors (Qing et al. 2019), breast cancer and endometrial cancer with and without diabetes mellitus (Berstein et al. 2016) and colorectal adenocarcinomas (Płachetka et al. 2013). In these studies, it was determined by the researchers that 8-OHdG expressions increased significantly in tumoral tissues compared to healthy-control groups. In this study, all papilloma and fibropapilloma cases were immune positive in terms of 8-OHdG expressions. A significant increase found in papilloma and fibropapilloma groups compared to the control group. It was concluded that this increase in 8-OHdG expression was seriously related to DNA damage caused by free radicals caused by oncogenic BPVs (Bocanetti et al. 2015).

ROS create lipid peroxidation by especially acting on unsaturated fatty acids in the cell membrane (Tabakoğlu and Durgut 2013). Lipid peroxides formed as a result of lipid peroxidation reactions eventually turn into aldehydes named MDA, 4-hydroxynonenal (HNE) and hexanol, which are secondary or end products (Özcan et al. 2015). It is known that free radicals and lipid peroxidation lead to the initiation and promotion of carcinogenesis. It has been determined that lipid peroxidation increases in carcinogenic processes and MDA, which is a lipid peroxidation product, has mutagenic and carcinogenic effects by damaging proteins and DNA (Das and Saha 2009; Georgescu et al. 2018). In a study, Sasmaz et al. 2005 found that plasma MDA levels increased in patients with non-genital warts. In a different study Aslan and Saraç, 2011 found a statistically significant increase in plasma MDA level in the bovine group with papillomatosis compared to the healthy control group. In another study, Erturan et al. 2019 detected that compared with the controls, patients with recalcitrant warts had significantly higher levels of MDA. In this study, a significant MDA expression increase found in papilloma and fibropapilloma groups compared to the control group as reported by Aslan and Saraç, 2011. This increase in MDA immune positive expressions was interpreted as the excessive production of free radicals during the formation process of both papilloma and fibropapilloma tumors and these free radicals caused lipid peroxidation.

As a result of the literature review, no study data was found in which DNA damage due to oxidative stress and lipid peroxidation was detected by means of 8-OHdG and MDA expressions in papilloma and fibropapilloma cases of cattle, and it was thought that the findings obtained from this study would contribute to the literature data. Additionally, it has been interpreted that oxidative stress plays an important role in the pathogenesis of papillomas and fibropapillomas.

CONFLICT of INTEREST

The author declares that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

Idea / Concept: EK
Design: EK
Supervision / Consultancy: EK
Data Collection and / or Processing: EK
Analysis and / or Interpretation: EK
Literature Review: EK
Writing the Article: EK
Critical Review: EK

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