## RESEARCH ARTICLE

# Bovine Papillomatosis: A Serological, Hematobiochemical, Ultrastructural and Immunohistochemical Investigation in Cattle

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## ABSTRACT

Bovine papillomatosis (BP) is one of the imperative cutaneous oncogenic viral diseases resulting in leather depreciation and economic losses. This study aimed to evaluate oxidative stress, immunohistochemistry and ultrastructural alterations in BP and its impact on cowhide. Ninety skin tissue specimens and blood samples were collected from cattle. Hematological parameters including complete blood picture, copper and zinc concentration in sera and oxidative stress biomarkers in skin were evaluated. Sera were tested for bovine papillomatosis antibodies. Histopathology of all skin samples, immunohistochemistry of collagen 1 and gamma catenin were performed. Moreover, BP virus was identified by transmission electron microscope in papilloma lesions. Bovine sera tested were positive for bovine papillomatosis antibodies (28.8%). In diseased cases, a significant reduction in Cu and hemoglobin were recorded compared to normal animals. Protein oxidation and MDA were elevated whereas GSH and SOD were reduced in diseased cases. Histopathology of bovine skin lesions of papilloma infected cases showed finger-like projecting papillae and papilloma with overlying stratum corneum and acanthosis. Collagen I fibers were altered in papilloma's and fibropapillomas. Gamma catenin expression was translocated to the nucleus in many epidermal cell layers indicating desmosomes damage. In conclusion, BPV altered the host hematological parameters, oxidant/antioxidant balance toward oxidative damage, collagen, γ-catenin content, trace elements, especially copper, and the tensile strength of the skin. Therefore, BP decreased the quality of animal hide.

## INTRODUCTION

Bovine papillomavirus (BPV) is a non-enveloped icosahedral double-stranded DNA virus (van Doorslaer, 2013). BP viruses are classified into 4 subgroups based on phylogenetic analysis; Delta papillomaviruses (BPV-1, 2, 13 and 14) causing fibropapillomas, Xi-papillomaviruses (pure epitheliotropic BPV-3, 4, 6, 9, 10, 11 and 12), Epsilon-papillomavirus (BPV-5 and 8) resulting in fibropapillomas and epithelial papilloma's and Dyoxipapillomavirus (BPV 7) (Hamad *et al.*, 2016). Recently in Egypt, four different isolates of Delta-papillomavirus were recorded (Ata *et al.*, 2021).

Bovine papillomatosis (BP) is one of the imperative cutaneous oncogenic viral diseases associated with leather depreciation in addition to teat obstruction (Borzacchiello and Roperto, 2008; Khan *et al.*, 2022). Skin lesions appear as warts or papilloma accompanied with hyperplasia of epidermal layers and mucosal epithelium which may sometimes regress or progress to neoplasia (Borzacchiello and Roperto, 2008). The relative proportion of epithelial and connective tissues determines whether it's a papilloma or a fibropapillomas. The site of lesions may vary with each pathotype, BPV-1 for instance prefers teat and penile skin causing fibropapillomas. BPV-1 and BPV-2 also cause skin lesions in the ventral and anterior parts comprising the forehead, neck, and back. Moreover, BPV-3 results in cutaneous papilloma's whereas BPV-4 affects the upper alimentary tract (Radostits *et al.*, 2006).

Delta-papillomaviruses affect the subepithelial fibroblasts initially then the epithelium causing acanthosis which ends with papillomatosis whereas Xi-

papillomaviruses induce epithelial papilloma's without fibroblast involvement. Macroscopically, proliferative neoplasms of fibromas, papilloma's, or fibropapillomas appear as exophytic or endophytic plaque-like or papillary lesions. They vary in appearance in which solitary to multiple grains of rice or cauliflower masses of variable sizes occur in the skin of animals at different parts (Al-Salihi *et al.*, 2020).

Tissues experiencing mechanical stress like the skin have an adhesive intercellular junction which is mediated by a complex structure known as desmosomes (Acehan *et al.*, 2008). Plakoglobin (γ-catenin) is one of the armadillo proteins well recognized in the desmosome (Delva *et al.*, 2009). It interacts with desmoglein and desmocollin in desmosomes (Harrison *et al.*, 2016). γ-catenin (plakoglobin) with β-Catenin occurs in adherents' junctions, connecting E-cadherin through α-catenin to the actin cytoskeleton (Yin *et al.*, 2005). The tensile strength of the skin is maintained mainly by collagen fibrils which are formed to a great extent of collagen I and a lower extent by collagen III (Bruckner, 2010). This study aimed to evaluate the effect of bovine papillomatosis on biochemical parameters, oxidant /antioxidant balance mechanism, immunohistochemistry and histopathological alterations, with special reference to tensile strength of the skin by determination of collagen I and γ-catenin expression in BP infected cases.

## MATERIALS AND METHODS

**Animals and study design:** The study was conducted during the period from 2019 until 2021. Ninety native breed cattle weighed approximately 400kg of both sexes, aged about 2 years raised in cattle farms were examined for possible skin lesions. Thirty cattle showed gross lesions of papillomatosis. Animals were divided into 2 groups; group 1 animals clinically healthy with negative serology and group 2 animals affected by papilloma with positive serology.

**Blood samples:** Blood samples were collected on EDTA from the jugular vein to estimate the hematological parameters including hemoglobin (Hb), packed cell volume (PCV), and Total erythrocyte count (TEC). Total Leucocyte Count (TLC) by an automatic cell counter (Hospitex Hemascreen 18, Italy) and differential leucocyte count (DLC) in thin blood smears stained with diff-3 stain were performed. Sera separated from coagulated blood samples were stored at -20°C to be used for serology and assessing zinc and copper concentration. Copper was determined calorimetrically using specified test kit according to manufacturer instructions (Spectrum Diagnostics, Egypt). Briefly, a chelate complex is formed by 4-(3,5-dibromo-2-pyridylazo)-N-ethylsulfopropylaniline and copper. The absorption of the complex is determined and is relative to total copper concentration in sample (Abe *et al.*, 1989).

Zinc was determined calorimetrically using specified test kit according to manufacturer instructions (Spectrum Diagnostics, Egypt). Briefly, a red chelated complex formed between 2-(5-Bromo-2-pyridylazo)-5-(N-propyl-Nsulfopropylamino)-phenol and zinc and the absorption of the complex is determined and is relative to total copper concentration in sample (Johnsen and Eliasson, 1987).

**Elisa:** Sera were tested using Elisa Kits for anti-BPV antibodies (Catalog no. BZEK1334, Chongqing Biospes Co. Ltd, China) according to the manufacturer protocol. The ELISA plates were read by an ELISA reader at wavelength 450nm (Chromate, Awareness Technology INC). The cut-off value was calculated from the optical density of negative samples. Serum samples with optical density above the cut-off value were considered positive.

**Histopathology examination:** Tissues were divided into two parts; the first one was kept in 10% neutral buffered formalin for histopathology and the other one was stored at − 80°C for oxidant/antioxidant biomarkers analysis. Fixed skin specimens (n= 90 cattle) were processed by paraffin embedding technique and sectioned (3-4μm thick) using microtome and stained by routine hematoxylin and eosin stain and Masson's Trichrome stain. The tissue sections were examined by light microscopy equipped with a digital camera (Olympus XC30, Tokyo, Japan).

**Immunohistochemistry analysis:** IHC was performed on paraffin embedded tissues of control (n=3) and diseased skin (n=10). After deparaffinization, antigen retrieval was performed in citrate buffer pH 6. Primary antibodies against collagen 1 (COLI) (1:100, ab34710, Abcam, UK) and gamma catenin (0.2μg/mL, ab218437, Abcam, UK) were then applied to sections followed by the application of horseradish peroxidase-conjugated antibodies (Abcam, Cambridge, UK). DAB was used as a substrate and Mayer's hematoxylin was used as a counterstain.

**Electron microscopy:** Specimens from warts were fixed in 2% glutaraldehyde in phosphate buffer and were then washed for 90-minute in osmium tetroxide, thoroughly dehydrated in a graded series of ethanol, and then placed in propylene oxide. Infiltration began with a 60-minute wash in 50% propylene oxide/50% fresh agar 100 epoxy resin with agitation. After another 60 minutes in pure resin, samples were placed into beam capsules and polymerized at 60°C overnight. Ultrathin sections (70 nm) were made using Leica UC6 ultramicrotome, loaded on Grids and examined in the Electron Microscope Unit (Research Park, Faculty of Agriculture, Cairo University, Giza, Egypt).

**Antioxidant biomarkers evaluation:** Reduced glutathione (GSH) content in addition to superoxide dismutase (SOD) activity were assessed according to Ellman (1959), using commercial kits (Biodiagnostics, Cairo, Egypt).

**Assessment of oxidative stress biomarkers:** Lipid peroxide content (MDA) of skin homogenate was assessed using commercial kits (Biodiagnostics, Cairo, Egypt). Protein carbonyl content (PCC) concentration (mM/gm protein) was determined as an index of protein oxidation (Reznick and Packer, 1994). Phosphate homogenizing buffer pH 7.4 having a mixture of antiproteases were added to skin samples (200mg). Then PCC was estimated by derivatization of the carbonyl group with dinitrophenylhydrazine which forms stable dinitrophenylhydrazone that was measured at 370nm by a UNICO-UV-2100 spectrophotometer.

**Statistical analysis:** The comparison of hematology and serum biochemistry data between control and the diseased groups was made using SPSS statistic program version 16.0 (independent-samples T test).

## RESULTS

**Bovine papillomatosis antibodies:** Elisa Kits for BPV antibodies revealed 26 (28.8%) from 90 bovine sera tested were positive for BPV antibodies and 64 (71%) from 90 bovine sera tested were negative for BPV antibodies.

**Haematological alterations:** Hematologic alterations associated with papilloma infection are presented in Table 1. Clinically infected cows showed a significant reduction in RBCs, HB, MCHC and lymphocytes associated with significant elevation in neutrophils compared to normal control animals. There was a significant reduction in Cu in diseased cases compared to normal control animals (Table 2).

**Gross findings in bovine papillomatosis infection:** Exophytic papillomatous growth or warts appearing as a cauliflower and endophytic growth were observed on the skin of infected cattle (n=30). Their distribution varied in which they occurred on the neck, limbs and back. The lesions were firm and pedunculated with a rough and dense surface, and no hair growth. They appeared as multiple irregulars raised growths of different sizes and grayish/blackish color on the skin.

**Histopathological findings:** Microscopy of clinically infected animals displayed finger-like projecting papillae and papilloma with overlying stratum corneum and acanthosis (n=21) (Fig. 1a, b). A moderate to severe degree of ortho or parakeratotic hyperkeratosis was observed. The dermis was infiltrated with mononuclear cells especially perivascular and peri-glandular. The epidermis showed also increased basal melanin pigmentation and melanocyte migration in the dermis. Some cases showed fibropapillomas lesions (n=9) which appeared as severe parakeratosis, acanthosis and hyperkeratosis with basketweave picture. Hyperplasia, mitotic activity, and hyperchromatic nuclei were observed in the basal cell layer. Koilocytes with eosinophilic and vacuolated cytoplasm and condensed hyperchromatic, centrally, or eccentrically placed or crescent-shaped nuclei were observed in the epidermis. In the stratum, numerous variably sized keratohyalin granules were observed in granulosa cells (Fig. 1 c, d).

The collagen content of the skin dermis as demonstrated by Masson's trichrome stain showed moderate edema and separation of the collagen bundles due to mononuclear cell infiltration. In skin areas of fibropapillomas, myxomatous degeneration with excessive mucoid substance in-between fibroblasts were observed (Fig. 1e, f).

**Immunohistochemistry findings:** Collagen I was separated and disorganized in the dermis of clinically infected cases, papilloma's and fibropapillomas compared to negative control (Fig. 2a, b). The epidermocytes showed nuclear translocation of gamma catenin expression in the epidermocytes folded into microscopic finger-like papillae (n=10) (Fig. 2 c, d).

**Table 1:** Hematological alterations associated with bovine papillomatosis virus.

| Parameters/Units | Control group | Clinical diseased group |
|---|---|---|
| RBCs ($10^{12}$/L) | 7.43±0.22 | 5.50±0.74* |
| HB (g/dl) | 12.06±0.40 | 9.92±0.73* |
| PCV (%) | 32.20±2.39 | 30.01±1.53 |
| MCV(fl) | 43.04±3.75 | 53.02±3.81 |
| MCHC (g/dl) | 37.50±0.68 | 33.00±0.35* |
| Platelets ($10^9$/L) | 273±28.14 | 228.39±34.70 |
| WBCs ($10^9$/L) | 7.07±0.28 | 6.42±0.85 |
| Neutrophils (%) | 32.25±1.11 | 50.40±5.63* |
| Lymphocytes (%) | 54.75±1.70 | 38.8±4.87* |
| Monocytes (%) | 6.56±0.65 | 5.80±0.80 |
| Eosinophils (%) | 5.50±0.79 | 5.0±0.71 |

Data were represented as mean±standard error. * P value ≤0.05 considered significant.

**Table 2:** Trace element alterations associated with bovine papillomatosis virus.

| Parameters/Units | Control group | Clinical diseased group |
|---|---|---|
| Cu (Mmol/L) | 18.220±0.79 | 11.52±2.34* |
| Zn (Mmol/L) | 18.06±1.12 | 18.54±1.57 |

Data were represented as mean±standard error. * P value ≤0.05 considered significant.

**Table 3:** Antioxidant changes in diseased cattle compared with control group.

| | GSH (Mg/g. tissue) | SOD (U/g. tissue) |
|---|---|---|
| Control group | 57.3±2.09 | 159.6±3.4 |
| Diseased group | 28.3±1.5* | 144.4±7.1* |

All data were expressed as mean±SE, * P value ≤0.05 considered significant.

**Table 4:** Protein oxidation and lipid peroxidation marker of Skin in the infected cattle with BP compared with control group.

| | PCC (mM/g. tissue) | MDA (Nmol/g. tissue) |
|---|---|---|
| Control | 0.5±0.06 | 61.9±4.3 |
| Diseased | 3.3 ±0.2* | 120.04±7.8* |

All data were expressed as mean±SE, * P value ≤0.05 considered significant.

**Electron microscopy finding:** The nuclear chromatin of keratinocytes was partially marginated and nucleoli were prominent. An indented nuclear membrane by a small amount of fibrillar material and keratohyalin granules which appeared to have been pushed aside was noticed. Osmiophilic particles of virus-like particles were seen in the keratinized layers. The virus-like particles appeared to be confined within plasma membranes and were found in pools that were entrapped by bands of keratin. Viral-like particles were detected between tonofibrillar material (Fig. 3).

**Oxidant -antioxidant biomarkers findings:** The activity of SOD and the level of GSH were significantly reduced in diseased animals compared with normal control group (Table 3).

The levels of MDA were significantly elevated in diseased animals compared with normal control group. The concentration of protein oxidation of skin homogenate recorded a significant increase of 3.3 (mM/gm protein) in the skin infected with BP compared to control animals (Table 4).

## DISCUSSION

Bovine papillomatosis is an important veterinary viral infection that has a great impact on the leather industry and is linked with economic losses (Ugochukwu *et al.*, 2018). The host immune response plays a main role in the course of infection; therefore, it may not be clinically manifested or may produce various benign or malignant lesions (Georgescu *et al.*, 2018). Sometimes the activated immune
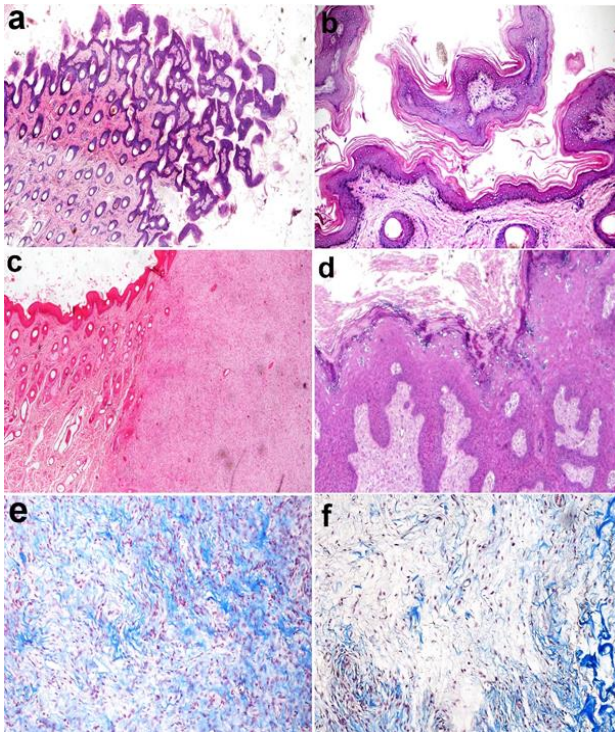
**Fig. 1:** Histopathology of bovine skin clinically infected with BPV. (a, b) Papillomas on the surface of the skin with fibrous connective tissue core (40x, 100x). (c) Endophytic fibropapilloma (40x), (d) Pseudocarcinomatous growth of the epidermis in a fibropapilloma (x100). (Hematoxylin and eosin stain). (e) Disorganized fragmented collagen bundles in fibropapilloma, (f) Myxomatous degeneration in fibropapilloma. (MTC stain X 200).
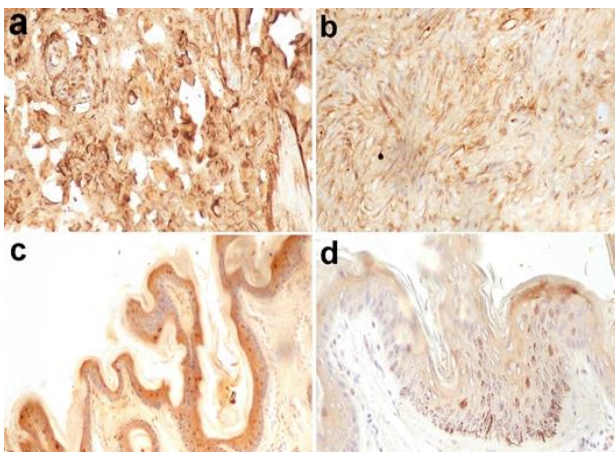


**Fig. 2:** Bovine skin infected with BPV showing (a) Separation and disorganization of collagen I in the dermis (X200). (b) collagen I in fibropapilloma (X400 collagen I immunoperoxidase). (c) nuclear translocation of gamma catenin expression in the epidermis having microscopic finger-like papillomas (200 x), (d) moderate nuclear translocation and weak staining of cytoplasm of gamma catenin in hyperplastic epithelial cells in a subclinical case (Gamma catenin immunoperoxidase 400X).



**Fig. 3:** Transmission electron micrograph of bovine skin showing: (a) Intended nuclear membrane and pool of osmiophilic particles of virus-like particles (b) virus-like particles appeared to be confined within plasma membranes (c & d) viral-like particles in between tonofillars material

who reported a reduction in haemoglobin and CD4+/CD8+ ratio and elevation in natural killer cells. However, these findings contradicted those reported by Panjaitan *et al.* (2021) who indicated an elevation in lymphocytes count in clinically infected cattle. Accordingly, its believed that DLC may vary with the stage of the infection. In stress conditions, the release of endogenous glucocorticoids could be implicated (Palanivel *et al.* 2017). BP causes weakness of animal with subsequent reduction in appetite, and anaemia which makes the animal under stress condition (Bassi *et al.*, 2019) and that lead to alteration in haematological parameters by decrease or increase compared with normal non-infected animals.

In this study, there was a copper reduction observed in infected animals. Copper is an integral component of numerous enzymes and despite it being bound to protein, it could be released to catalyze hydroxyl radicals that play a role in oxidative stress damage (Chaturvedi *et al.*, 2004), causing its depletion. In prolonged infection, the virulence of BPV increases, and chronic inflammation ensues. There is a strong positive correlation between chronic inflammation and oxidative stress damage (Georgescu *et al.*, 2018) which, finally leads to the release of copper from its binding protein to scavenge hydroxyl radicals.

The current study revealed successful detection of BPV antibodies using ELISA with a percentage comparable to a previous study (Ghim *et al.*, 1996). Our ELISA results were confirmed by electron microscopy of the skin lesions which revealed the presence of negatively stained rounded, non-enveloped virus particles with a size of 60 nm in diameter as previously reported (Ata *et al.*, 2021).

Few studies reported the blood antioxidant and lipid peroxidation status and its relation with the cutaneous diseases of cattle (El-Mandrawy and Alam, 2018). In the present study, oxidant/antioxidant balance was tilted toward the oxidative damage through the decrement of reduced glutathione and superoxide dismutase and elevation of lipid and protein oxidation in infected cases coinciding with previous research (Karakurt, 2021). These lipid peroxidation products resulting from ROS attacking polyunsaturated fatty acids the main cell membrane component have carcinogenic and mutagenic impacts through damaging proteins and DNA (Georgescu *et al.*, 2018). Consequently, the irreversible DNA damage

system may fail to provide a proper response to BPV in cattle (Ugochukwu *et al.*, 2019). BPV can elude from the host immune system resulting in the progression of benign warts but, some warts fail to progress and regress under immune-mediated effect (Nicholls and Stanley, 2000).

In this study, the haematological parameters of clinically infected cows revealed a reduction in RBCs, HB, MCHC and lymphocytes with neutrophilia is in harmony with Palanivel *et al.* (2017) and Bassi *et al.* (2019),
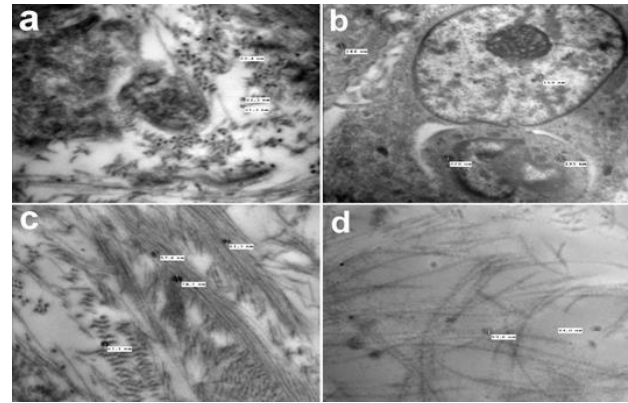
impairs enzyme activity through the translation and expression of abnormal proteins that produce new immunological cell structures (Tabakoğlu and Durgut, 2013). An imbalance between prooxidants and antioxidants could be induced by chronic inflammation favoured by persistent infection (Mangino *et al*., 2016). So, the imbalance between oxidant/antioxidant mechanisms toward oxidative damage in this study could be attributed to chronic inflammatory response initiated by BPV infection as confirmed by histopathological findings which showed mononuclear inflammatory cells infiltration in the skin.

The histopathological lesions observed in infected cases in this study were in harmony with previous findings (Al-Salihi *et al*., 2020). BP may progress to skin neoplasm (Ugochukwu *et al*., 2019). Many factors may interplay to initiate carcinogenesis in BP including free radicals, lipid peroxidation, and oncoproteins encoded by the viral genome. Oncoproteins and oxidative damage may deactivate tumor suppressor genes like p53 favoring uncontrolled growth of cells (Bocaneti *et al*., 2015). Therefore, the lesions induced by BPV infection could be attributed to its oncoproteins which, suppress P53 and finally, lead to warts, papilloma, and fibropapillomas. Also, Koilocytes formation occurred due to the cytopathic effect of BPV oncoproteins (Krawczyk *et al*., 2008). In our results, skin areas of fibropapillomas showed myxomatous degeneration in the connective tissue and were replaced by a mucoid substance between fibroblasts. These results are in accordance with Movassaghi *et al*. (2013) as it is a subsequent fate of fibroma and fibropapillomas.

In this study, collagen I was disorganized in papilloma's and fibropapillomas in agreement with Movassaghi *et al*. (2013). Collagen is the main architecture, functional and abundant component of extracellular matrix (ECM) which consisted of many micro and macro filaments and provide its tensile strength (Sampaio *et al*., 2021). The proliferation of cancer cells leads to several changes in the surrounding matrix (a dynamic interaction between cells and the microenvironment), such as increased fibronectin secretion, collagen type I, II, and IV. Increased matrix proteins deposition enhances tumor progression as it restricts the cell-cell adhesion, and cell polarity (Walker *et al*., 2018).

The epidermocytes showed desmosomes damage and disorganization of cytokeratin filaments as demonstrated by gamma catenin expression. Our results are in parallel to Kolligs *et al*. (2000). γ-catenin is a major protein in cell-cell adhesion at the desmosome which can bind to both classic and desmosomal cadherins maintaining normal epithelial tissue architecture (Acehan *et al*., 2008). The transformation of epithelial cells towards malignancy includes alteration of cell-cell adhesion which, makes the malignant cells assume a phenotype of migration with subsequent metastasis and invasion of the tumour cells. In addition, the E6 and E7 oncoproteins in the BPV genome regulates the Wnt/β-catenin pathway which, affects γ-catenin content (decrease its content and signal confusion) (Acehan *et al*., 2008).

**Conclusions:** BPV altered the host hematological parameters, oxidant/antioxidant balance toward oxidative damage, collagen, γ-catenin content, trace elements, especially copper, and the tensile strength of the skin. BP decreased the quality of animal hide.

**Ethical approval:** This study was approved by the Institutional Animal Care and Use Committee and allotted number (Vet CU 2009 2022524), Faculty of Veterinary Medicine, Cairo University, Egypt.

**Conflict of interest:** The authors declare that they have no conflicts of interest.

**Authors contribution:** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by MK, AO, HF, RA, HO, ER and AA. All authors read and approved the final manuscript.

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