B-4683
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RESEARCH ARTICLE Indian Journal of Animal Research

Evaluation of Oxidative Stress and Histiocytic Behaviour of Canine TVT using Alpha Antitrypsin

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10.18805/IJAR.B-4683

ABSTRACT

Background: There is scanty literature on evaluation of oxidative stress and alpha antitrypsin marker expression in canine TVT to know its histiocytic behaviour. Since venereal transmissible tumor is one of most important tumor affecting large population of canine, thus, study was conducted to investigate the above based on clinicopathology.

Methods: The study was conducted on dogs suffering from transmissible venereal tumor (TVT) for evaluation of clinicopathological alterations, oxidative stress and expression pattern of alpha antitrypsin. Ten bitches and five male dogs with tumour masses seen on the external genitalia were used for the study. Detailed alterations due to TVT with respect to haematobiochemistry, oxidative stress, pathomorphology, proliferation and expression pattern of alpha antitrypsin were assessed.

Result: Grossly tumors on the genital area were either pedunculated or sessile and of various shape such as cauliflower, filiform and irregular. Hematological alterations such as normocytic normochromic type of anaemia, thrombocytopenia, neutropenia and lymphocytopenia in TVT cases indicated immunosuppression. Significant alterations in liver enzymes, hypercalcemia and hypoglycaemia were also observed in TVT affected dogs. Increased level of MDA and decreased in concentration of antioxidant enzymes indicated association of oxidative stress in proliferation of tumor and reduction in number of T lymphocytes. Cytological examination revealed characteristic punctuate basophilia and vacuolation in tumour cells. Histopathological examination showed characteristic round cells with a little fibrous septa in a sheet like manner. AgNOR staining indicated moderate proliferative capacity as evidenced by scattered AgNOR dots. TVT cells showed moderate type of immunoreactivity with alpha anti-trypsin confirming its histiocytic origin upon immunohistochemistry.

Key words: Alpha antitrypsin, Canine, Clinicopathology, External genitalia, Oxidative stress, Transmissible venereal tumor.

INTRODUCTION

Cancer arises when a single cell lineage acquires somatic mutations that promote it towards a program of continued proliferation. Moreover the excessive use of drugs in treatment of infectious diseases is predisposing factor for causing cancer in dogs. Vaginal and vulvar tumors are the second most common canine female reproductive tumors after those of the mammary gland. They constitute 2.4 - 3.0 per cent of canine neoplasia (Rizk *et al.* 2015).

Transmissible venereal tumour (TVT) also known as infectious sarcoma, venereal granuloma, transmissible lymphosarcoma or Sticker tumor, is a reticuloendothelial (histiocytic) tumour of the dog that mainly affects the external genitalia. The tumour occurs naturally on the genital area of both male and female dogs. Although dogs over one year of age are at high risk in endemic areas, most common in dogs 2 to 5 years old (Birhan and Chanie, 2015). TVT has cauliflower-like shape and it could be pendular, nodous, papilar, or multilobular. Females are more susceptible than males (Das and Das, 2000; Aydin *et al.* 2009). Immune response against the tumor plays a major role in determining course of tumor. TVT evokes both cellular and humoral immune response as well as transplantation immunity in immunological competent host which prevent subsequent tumors. Keeping this in mind authors planned their first objective to study hematobiochemical alterations and quantify the T and B lymphocyte in blood affected by TVT through ANAE staining. At the same time a critical factor for the assessment of tumor aggressiveness is an inflammatory reaction generated as a response to tumor (Birhan and Chanie, 2015). Commonly associated with inflammation is increase in free radicals called oxidative stress (Rubio *et al.* 2017). Free radicals can seriously alter the structure of biomolecules. These alterations may result in cell degeneration and death and play a significant role in the pathogenesis of many diseases, such as inflammatory diseases and mammalian cancers. In dogs, oxidative stress has been associated with carcinogenesis (Macotpet *et al.* 2013). Although there is single report about venereal tumors and oxidative status relationship, but there are reports on

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How to cite this article: Soni, S., Jakhar, K.K., Chandratre, G.A., Singh, S. (2021). Evaluation of Oxidative Stress and Histiocytic Behaviour of Canine TVT using Alpha Antitrypsin. Indian Journal of Animal Research. DOI: 10.18805/IJAR.B-4683.

Submitted: 21-06-2021 **Accepted:** 06-09-2021 **Online:** 06-10-2021

role of oxidative stress in pathogenesis of canine mammary tumors (Mukaratirwa and Gruys, 2003; Kumaraguruparan *et al.* 2005). Therefore the second objective of present study is to assess the role of free radicals and status of antioxidant enzymes in blood of TVT bearing dogs. Venereal tumors cause economic losses due to negative impact on health and production. Therefore, its diagnosis is very important. Cytological examination can be used for diagnosis of tumors as pen side test since it is time saving. Differentiation between benign and malignant tumors is often made morphologically however the histopathological examination remains gold standard. Further, in case of generalized metastasis to carry out differential diagnosis with other tumors and to know the origin of neoplasm immunohistochemistry plays a major role in differentiating and determining the origin of tumor (Mascarenhas *et al.* 2014). To know the origin of TVT, immunohistochemical studies with a panel of several antibodies have carried out suggesting its histiocytic origin (Das and Das, 2000; Mascarenhas *et al.* 2014) but still studies are on to know the exact origin due to atypical behaviour of tumor. Alpha antitrypsin is reported to be present in the cytoplasm of histiocytes (Mascarenhas *et al.* 2014) however there very few reports in canine TVT. Hence, authors decided to investigate role of alpha antitrypsin in TVT under third objective of present study.

MATERIALS AND METHODS

Source of animals

The present study was conducted on 15 (Ten bitches and five male) cases of canine venereal tumors collected from the Teaching Veterinary Clinical Complex, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar and from other private veterinary clinics located in Delhi and Hisar during September 2017 to April 18. Samples from 6 tumor free dogs were also taken which served as control. Breed, age sex, location of tumor, its shape, size, colour and consistency were recorded.

Haematology

Blood samples were analyzed using automatic haematological analyser MS4se - Melet Schloesing Laboratories- France: for estimation of haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total leukocyte count (TLC) and total thrombocyte count (TTC). Differential leucocyte count (DLC) and erythrocyte sedimentation rate (ESR) was analysed by blood smear examination stained with field stain and wintrobe tube method respectively.

Alpha naphthyl acetate esterase (ANAE) staining

ANAE Staining was done in blood smears for T and B lymphocytes differentiation using single step reagent kit (Acid phosphatase kit- 181A-1KT, Sigma-Aldrich). At least 100 lymphocytic cells were counted. Positivity was expressed as the percentage of counted cells.

Serum biochemistry

Serum samples were analyzed for the estimation of biochemical parameters *viz*. total protein, albumin, globulin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase, blood urea nitrogen, creatinine, calcium, phosphorus and glucose using single step reagent kit ERBA diagnostics Mannheim GmbH (Transasia Bio-Medicals Ltd.) employing Semiautomatic Biochemistry Analyzer (Erba Mannheim Chem-5 Plus, Transasia).

Assessment of oxidative stress

Preparation of hemolysate

Blood samples were centrifuged to harvest the erythrocytes. Erythrocytes were washed thrice with normal saline solution and finally, 10 per cent hemolysate was prepared by adding chilled distilled water. Haemoglobin concentration of hemolysate was measured by method of Benjamin (1985).

Superoxide dismutase

Superoxide dismutase (SOD) activity in hemolysate was determined as per the method of Marklund and Marklund (1974) and activity was expressed as SOD units/mg of hemoglobin. This involved generation of superoxide by pyrogallol auto-oxidation and the inhibition of superoxide dependent decrease of the tetrazolium dye MTT [3-(4-5dimethyl thiazol 2-xl) 2,5-diphenyl tetrazolium bromide] to its formazan, which was then measured at 420 nm.

Catalase

The activity of catalase (CAT) in hemolysate was determined using hydrogen peroxide as substrate by the method of Aebi (1974). Results were expressed as µmol of H_2O_2 decomposed/min/mg of protein. The use of H_2O_2 by catalase was measured spectrophotometrically as decrease in optical density at 240 nm.

Lipid peroxidation

The extent of lipid peroxidation was evaluated in terms of MDA (malondialdehyde) production, determined by thiobarbituric acid (TBA) method (Shafiq-U-Rehman, 1984). The absorbance was read at 535 nm using double beam UV-VIS spectrophotometer (2R/UVevisible; Shimadzu, Tokyo, Japan).

Reduced glutathione (GSH)

GSH was estimated by estimating free -SH groups, using DTNB (5, 5'- dithiobis 2-nitrobenzoic acid) method of Sedlak and Lindsay (1968). Calculation was done by using the extinction co-efficient (EC, 13000 $M^{-1}.cm^{-1}$) and the results were expressed in mM GSH per ml of sample.

Statistical analysis

The quantitative data derived were statistically analyzed using analysis of variance and paired Student t test. The criterion for statistical significance was set at $p<0.05$.

Pathomorphology

Cytological examination

Impression smears were made from the biopsy samples, dried rapidly, stained with Field stain (Himedia India) and examined under oil immersion objective.

Histopathological examination

Biopsy samples were fixed in 10% neutral buffered formalin. Samples were processed as per procedure of Luna (1968). Paraffin sections were stained with hematoxylin and eosin (HE).

Special staining - Masson's trichrome (MST)

MST was done for the estimation of connective tissue proliferation specially collagen fibers (Calvi *et al.* 2012). Collagen fibers were identified by blue colour in tissue section.

Argyrophilic nucleolar organizing region (AgNOR) staining

AgNOR Staining was done in formalin fixed tumor biopsies as per the method of Santos *et al.* 2011. All AgNOR dots scattered in 100 consecutive nuclei were counted and mean number of AgNOR dots per nucleus was calculated for each sample.

Immunohistochemical staining for Alpha anti-trypsin

Immunohistochemistry was performed using a rabbit polyclonal antibody. After paraffin removal and hydration, antigen retrieval was performed by subjecting the tissue sections to microwave irradiation. The slides were placed in 3 per cent hydrogen peroxide (H_2O_2) solution to quench the endogenous peroxidise. Sections were covered with 100-150 µl of primary monoclonal antibody (1:25 rabbit polyclonal alpha antitrypsin) (Thermoscientific Chemicals, USA) in PBS containing 1 per cent BSA (Sigma Chemicals, USA). Biotinylated goat anti-rabbit IgGs (Sigma Chemicals, USA) were applied to cover the moist sections and incubated for 30 minutes in humidified chamber. The sections were incubated with ExtrAvidin® peroxidase (Sigma Chemicals, USA) diluted at 1:20 concentration in PBS containing 1 per cent BSA for 30 minutes in a humidified chamber. The sections were then counterstained lightly for 3-5 min with Mayer's hematoxylin (Sigma, MHS-16). Slides were rinsed for 5 min in running tap water and mounted in aqueous mounting medium CC/MountTM (Sigma Chemicals, USA).

RESULTS AND DISCUSSION

TVT is big threat to canine population and nowadays it is getting metastasized in other visceral organs (Cantone *et al.* 2003; Purohit, 2008). It has been reported that free radicals plays important role in proliferation of tumor (Lykkesfeldt and Svendsen, 2007; Aydin *et al.* 2009). Further oxidative stress is also correlated with immunosuppression in human cancers (Chen *et al.* 2016). In the similar fashion, present study was planned to investigate the oxidative stress and antioxidants in blood of TVT affected dogs as well as immunosuppression in terms of quantification of T and B lymphocytes in blood so that proper use of antioxidants and immunity booster should be initiated in therapeutic management of TVT. Further, transmissible venereal tumour is one of the important histiocytic round cell tumours, its histiocytic behavior was studied using alpha antitrypsin in tissue sections by immunohistochemistry.

History and clinical examination

Clinical examination of the 15 tumor affected dogs revealed that the most of the tumors were bright red/ reddish pink in colour due to high vascularisation. Out of 15, 4 bitches showed secondary lesions such as necrosis, ulcerations and bleeding in tumor (Purohit *et al.* 2008; Aydin *et al.* 2009). Dogs having venereal tumors showed clinical signs such as anorexia, continuous bloody discharge from external genitalia, soiling of floor, foul smelling, swelling and ulceration. The size of tumor varied in length from 4 to 25 cm. Consistency of the tumors varied from soft to hard. Tumors were either pedunculated or sessile and of various shape such as cauliflower, filiform *etc* (Fig 1 A and B). The cross section of tumor was grayish black, brown, whitish and pinkish *etc* (Fig C and D). Age of animal, sex and macroscopic features of tumors observed in different animals are summarized in Table 1. Age wise distribution of venereal tumors revealed that out of 15 cases, 2 cases (13.33 per cent) were evidenced in 0-2 years age group, 7 cases (46.67 per cent) were evidenced in 2-4 years age group, 4 cases (26.67 per cent) were evidenced in 4-6 years age group and 2 cases (13.33 per cent) were evidenced in 6-11 years age group.

Haematology and ANAE staining

There was a drastic decrease in hematological parameters, particularly in values of TLC indicating leucopenia and lymphopenia. Hb, PCV, TEC and TTC were decreased significantly ($p<0.05$) in the tumor affected dogs (Table 2)

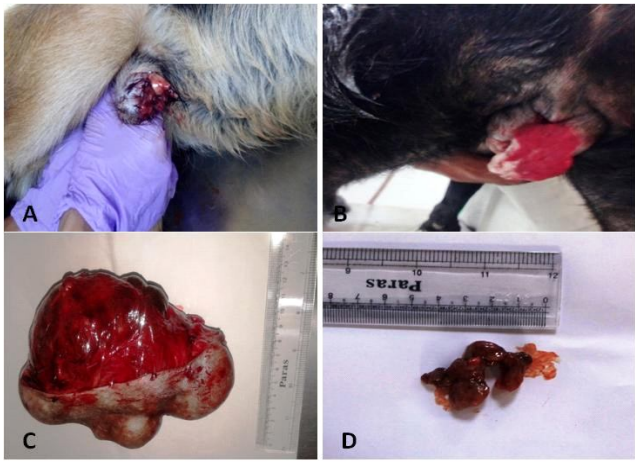

Fig 1: Gross pathology of transmissible venereal tumor (TVT) in dog. A: Showing cauliflower like reddish tumor mass attached to penis; B: Bitch showing cauliflower like reddish tumor mass attached to vagina; C: Photograph showing cut surface of TVT with soft consistency; D: Photograph showing small irregular growth of TVT.

whereas Erythrocyte sedimentation rate was significantly ($p<0.05$) increased. Blood smear examination revealed normocytic normochromic anaemia, rouleaux formation (Fig 2 A) and toxic changes in leucocytes (Fig 2 B). Anaemia which might be due to bleeding and inappetence leading to nutritional deficiencies causing proportionate decrease in number of erythrocytes (Birhan and Chanie, 2015). Significant increase in ESR in tumor affected dogs might be due to tissue injury, infection, anaemia or inflammation (Birhan and Chanie, 2015). Thrombocytopenia observed is related to loss of platelets in oozed out blood or due to immunosuppression. (Benjamin, 1979). It revealed leucopenia, lymphopenia and neutropenia in tumor affected cases. The ANAE stained T lymphocytes showed brown color while B lymphocytes stained blue (Fig 2 C). Percentage of T lymphocytes in blood smear was significantly decreased

Table 1: Clinical signs and gross findings of transmissible venereal tumor (TVT) from different cases.

Sex	Age (yr)	Site of the growth	Shape	Tumor size (cm^2) approx	Consistency	Colour	Ulceration
M	3	Penile region	Cauliflower	25×8	Hard	Greyish pink	Yes
F	4	Vaginal region	Cauliflower/lobulated	6×8	Soft	Reddish pink	No
F	2.5	Vulvar region	Irregular	9×8	Soft	Reddish pink	No
M	5	Penis	lobulated/irregular	24×8	Hard	Reddish pink	No
F	2	Vaginal region	Cauliflower	10×3	Moderately hard	Reddish pink	Yes
F	4	vaginal region	Irregular	6×5	Moderately soft	Brownish pink	Yes
F	6	Vulvar region	Cauliflower	4×5	Soft	Reddish pink	No
F	2.5	Vulvar region	Cauliflower	4×5	Soft	Reddish pink	No
F	2	Vaginal region	Nodular	9×5	Moderately hard	Brownish pink	No
M	2.5	Penile region	Cauliflower	10×8	Hard	Greyish white to brown	No
F	4	Vaginal region	Cauliflower	6×8	Moderately soft	Reddish	Yes
M	5	Penis	Irregular	7×5	Soft	Reddish pink	No
F	5	Vulvar region	Irregular	6×5	Soft	Whitish grey	No
M	7	Penile region	Irregular/nodular	6×5	Moderately hard	Brownish pink	No
F	11	Vaginal region	Cauliflower	10×8	Soft	Redish pink	No

M- Male, F- Female, TVT- Transmissibble venereal tumor.

Table 2: Alterations in haematological parameters in TVT affected dogs.

Parameters	Tumour affected cases	Tumour free cases
Hb (g/dl)	11.30 ± 0.87^a	14.11 ± 0.59^b
PCV (%)	33.95 ± 2.64^a	43.00 ± 1.84^b
TEC ($\times10^6$/µl)	5.62 ± 0.44^a	7.16 ± 0.30^b
MCV (fl)	60.93 ± 2.63^a	60.16 ± 0.17^a
MCH (pg)	20.26 ± 0.87^a	19.80 ± 0.14^a
MCHC (%)	32.72 ± 0.72^a	33.17 ± 0.13^a
TLC ($\times10^3$ µl)	11.30 ± 1.17^a	15.86 ± 0.99^b
Absolute neutrophil count ($\times10^3$ µl)	$8.789.3\pm960.6^a$	10.173 ± 789.4^a
Absolute lymphocyte count ($\times10^3$ µl)	$1.574.2\pm308.3^a$	$4.704.3\pm967.6^b$
Absolute eosinophil count ($\times10^3$ µl)	0.4057 ± 99.9^a	0.392 ± 114.7^a
Absolute monocyte count ($\times10^3$ µl)	0.4482 ± 98.9^a	0.5123 ± 108.9^a
Absolute basophil count ($\times10^3$ µl)	0.0513 ± 19.1^a	0.0850 ± 38.3^a
N (%)	77.92 ± 2.28^a	65.00 ± 5.14^b
L (%)	14.33 ± 2.43^a	29.00 ± 4.70^b
E (%)	3.58 ± 0.67^a	2.33 ± 0.67^a
M (%)	3.75 ± 0.60^a	3.17 ± 0.60^a
B (%)	0.42 ± 0.15^a	0.50 ± 0.22^a
Thrombocytes ($\times10^5$/µl)	2.92 ± 45.52^a	4.27 ± 33.67^b
ESR (mm/hr)	27.17 ± 2.60^a	11.00 ± 1.18^b
T lymphocytes in blood smear	35.08 ± 1.69^a	78.50 ± 1.09^b
B lymphocytes in blood smear	64.92 ± 1.69^a	21.50 ± 1.09^b

Data are expressed as mean ± SE. Means bearing different superscript letters differ significantly between groups at $p<0.05$; TVT- Transmissibble venereal tumor.

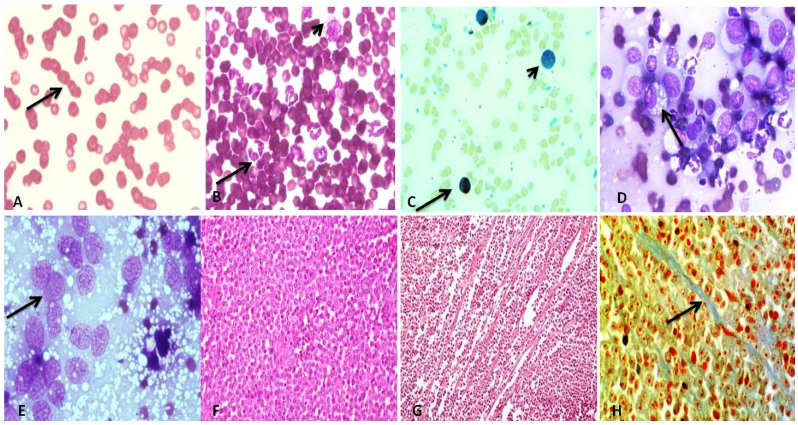

Fig 2: Clinicopathological alterations in Transmissible Venereal Tumor TVT in dog.

A: Blood smear showing rouleaux formation. (arrow) (Field's stain, 200X); B: blood smear showing nuclear swelling in neutrophils (arrow) and vacuolation in cytoplasm of monocyte (arrowhead) (Field's stain, 200X); C: Blood smear showing ANAE positive T lymphocytes (brown colored, arrowhead) and negative B lymphocytes (blue colored). (ANAE, 1000X); D: TVT showing tumor cells with coarse to reticulate chromatin and punctate basophilic cytoplasm (arrow), high vacuolation and neutrophils. (Field's stain, 1000 X) E: Tumor cells showing hyperchromatic nuclei with prominent nucleolus. (arrow) (Sertoli cell tumor) (Field's stain, 1000 X); F: TVT showing sheet of round cells with vesicular nuclei, scanty stroma and mitotic Figs. (H & E, 200 X); G: TVT showing sheet of round cells with vesicular nuclei, scanty stroma, high nucleocytoplasmic ratio and mitotic Figs (arrow). TVT (HandE, 200 X); H: TVT showing proliferation of collagen fibres (arrow). (Masson's trichrome stain ×1000).

Table 3: Alterations in biochemical parameters in TVT affected dogs.

Parameters	Tumor affected cases	Tumor free cases
Aspartate transaminase (IU/L)	50.30±5.41[a]	14.32±0.49[b]
Alanine transaminase (IU/L)	56.28±4.67[a]	7.37±0.79[b]
Total protein (g/dl)	6.96±0.47[a]	5.96±0.11[a]
Albumin (g/dl)	2.24±0.12[a]	2.77±1.33[a]
Globulin (g/dl)	4.50±0.51[a]	3.19±0.14[b]
Alkaline phosphtase (g/dl)	71.68±5.95[a]	59.00±7.19[a]
Creatinine (mg/dl)	3.02±0.29[a]	0.98±0.70[b]
Blood urea Nitrogen (mg/dl)	10.16±1.62[a]	6.33±0.47[a]
Calcium (mg/dl)	15.72±1.03[a]	5.57±0.53[b]
Phosphorus (mg/dl)	3.88±0.29[a]	4.70±0.45[a]
Glucose (mg/dl)	69.44±10.95[a]	113.00±1.59[b]

Data are expressed as mean ± SE. Means bearing different superscript letters differ significantly between groups at p<0.05; TVT- Transmissibble venereal tumor.

(p<0.05) in tumor affected animal (Table 2). Lymphocytopenia might be due to suppression of lymphocytes (mainly T lymphocytes) by tumor growth factor (TGF) which is released by tumor mass during progressive growth phase. These findings are in accordance with the study of Siddle and Kaufman (2014) who studied immunological behaviour of naturally transmissible tumors. This also support the decrease in T lymphocytes observed in blood upon ANAE staining (Bayraktaroglu *et al.* 2015).

Serum biochemistry

Data for serum biochemical evaluation is listed in Table 3. The concentration of aspartate transaminase, alanine transaminase, globulin, creatinine and calcium were significantly increased in tumor affected animal (p<0.05) whereas blood glucose level was significantly decreased (p<0.05). Significant increase in the activities of serum aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphtase in tumor affected cases might be due to cellular injury to cardiac muscles and hepatocytes as a part of paraneoplastic syndrome (Birhan and Chanie, 2015). Hypercalcemia observed in present study is also associated with paraneoplastic syndrome. It might be due to production of certain humoral factors which have an effect on the bones, kidneys and gastrointestinal tract (Bergman, 2001).

Assessment of oxidative stress

In tumor affected animals, oxidative stress was observed as indicated by alteration in the levels of antioxidant enzymes such as superoxide dismutase, catalase, reduced glutathione and lipid peroxidation. The values of oxidative stress parameters are presented in Table 4. A significant increase in levels of MDA in hemolysate, with a significant decrease in the activity of antioxidant enzymes, *i.e.* catalase, SOD and reduced glutathione in tumor affected cases was observed. The imbalance between the rates of reactive oxygen species (ROS) production and removal is known as oxidative stress, which leads to the degradation of lipids, proteins and nucleic acids. Lipid peroxidation products, including MDA, are often used as biomarkers for oxidative stress status. Lipid peroxidase (LPO) causes peroxidation of lipids which get enhanced during oxidative stress. In the present study, significant increase in mean MDA level was noticed in tumor affected dogs as compared to tumor free dogs (reference). This implies that the formation of free

Table 4: Assessment of oxidative stress in blood in TVT affected dogs.

Parameters	Tumor affected cases	Tumor free cases
Superoxide dismutase (SOD) (U/mg Hb)	0.12±0.01[a]	42.77±0.16[b]
Lipid peroxidation (LPO) (nmol/ml)	6.96±0.30[a]	2.75±0.14[b]
Catalase (µmol of H_2O_2 decomposed/min/mg protein)	2.64±0.16[a]	14.97±0.16[b]
GSH (U/mg Hb)	0.46±0.00[a]	2.77±0.26[b]

Data are expressed as mean ± SE. Means bearing different superscript letters differ significantly between groups at p<0.05; TVT-Transmissibble venereal tumor.

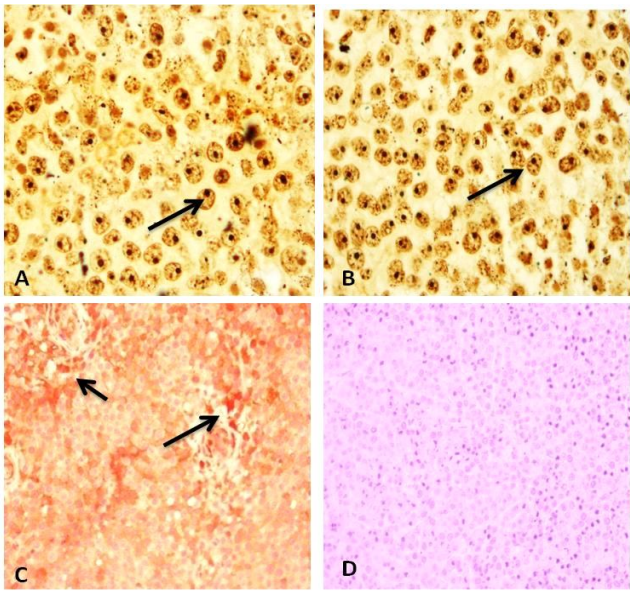

Fig 3: Demonstration of AgNoR dots and alphy antitrypsin in TVT. A: Less proliferating tumor cells of TVT showing few AgNOR dots in nucleus in tumor cells (arrow). (AgNOR staining, 1000X), B: Highly proliferating tumor cells of TVT showing more AgNOR dots in nucleus in tumor cells (arrowed). (AgNOR staining, 1000X), C: TVT showing moderate cytoplasmic immunoreactivity with alpha anti-trypsin (arrow). (IHC, 400X) D: TVT showing no immunoreactivity in negative control. (IHC, 400X).

radicals and consequent lipid peroxidation may be related to TVT formation and proliferation. Further, it has been reported that reactive oxygen species (ROS) participate extensively in T cells activation, apoptosis and hyporesponsiveness (Cornelissen *et al.* 2011). It has produced negative impact on the health of animal suppressing the immune defense system particularly T lymphocytes as observed in present study. In turn it helped in aggravating the effects of the tumor. These results correspond to previous studies on canines with mammary tumors (Hughes and Dobson, 2012). Free radicals are scavenged by antioxidant enzymes such as catalase and superoxide dismutase to balance the system. In the present study, activity of catalase, SOD and reduced glutathione was analyzed for assessment of antioxidant status and found significantly reduced in tumor affected dog (Macotpet *et al.* 2013; Birhan and Chanie, 2015). It is because of the consumption of antioxidant enzymes for the removal of excess free radicals. Further decrease in protein synthesis due to effect of tumor on liver might have caused a decrease in the activities of antioxidant.

Cytological, histopathological examination, Masson's trichrome staining (MST) and immunohistochemistry

Impression smear cytology revealed arrangement of neoplastic cells in a row or in a sheet like pattern showing pleomorphism having vacuolated cytoplasm (Fig 2 D) and hyperchromatic nuclei with prominent nucleolus (Fig 2 E). Inflammatory cells were also noticed. Characteristics punctuate basophilia of tumor cells with hyperchromatic nuclei and prominent nucleolus upon cytology are accordance with other previous report (Thangathurai *et al.* 2008). Histopathological examination revealed presence of round cells with a little fibrous septa in a sheet like manner (Fig 2 F). Cytoplasmic vacuolation was observed in tumor cells (Fig 2 G). Progressive types of tumorous lesions were found in nine cases *i.e.* number of cells were more but fibrous tissue was less. In TVT, a little proliferation of connective tissue was observed (Fig 2 H). Characteristic features such as round to polyhedral shaped tumor cells, arranged in strings and interspersed with scanty stroma observed in histopathological examination (Birhan and Chani, 2015). Studies on Immunoreactivity of alpha anti-trypsin was appeared as reddish brown/ brick red colour in cytoplasm. TVT gave moderate cytoplasmic immunoreactivity with alpha anti-trypsin which is suggestive of their histiocytic origin (Fig 3 C). Cytoplasmic immunostaining of alpha anti-trypsin was not detected in negative controls (Fig 3 D).

Immunohistochemical examination revealed that tumor cells of TVT exhibited moderate positive staining with alpha anti-trypsin antibody confirming its histiocytic origin. 51-75 per cent cells reacted positively with alpha anti-trypsin antibody in present study (Mukaratirwa and Gruys; 2003 Birhan and Chani, 2015).

Agyrophilic nucleolar organizing region (AgNOR) staining of tissue sections for assessing malignancy

AgNOR dots were counted as per previous report (Akhtar *et al.* 2005). Out of 15 cases, 12 cases have their AgNOR count below 2.5 (Fig 3 A) whereas 3 cases have their AgNOR count above 2.5 showed that they have moderate to high proliferative capacity (Fig 3 B) The AgNORs have been shown to reflect DNA transcriptional activity. Study of AgNORs has been identified as a reliable indicator of cell

proliferation and in turn, of the malignant potential of a lesion. Malignant tumor cells are characterized by extremely large AgNORs, which show a random or scattered distribution whereas benign tumors cells show single, distinct AgNORs (Akhtar *et al.* 2005). In the present study, AgNOR staining revealed that in most of the cases AgNOR dots varied from 2.02 to 3.62 which indicates moderate proliferative rate tending towards high proliferation of TVT cells (Santos *et al.* 2011; Filho *et al.* 2020).

CONCLUSION

Lipid peroxidation is found to be responsible for production of significant pathomorphological alterations, immunosuppression in dogs of 2-4 years of age suffering from transmissible venereal tumor and reduction in antioxidant enzymes. Reduction in T lymphocytes indicated hampering of cellular immune response. This finding will assist in formulating therapeutic regimen for regression of tumor along with improvement of the antioxidant status and immune system by including antioxidants and immunomodulators in therapy.

Declaration of interests

The authors declare that there is no conflict of interest.

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