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RESEARCH ARTICLE

Therapeutic Evaluation of Anti-Angiogenic and Chemotherapy with or without Cox-2 Inhibitor and Immunomodulator Drug in the Management of Canine Mammary Neoplasm

Kodakkadan Manikkan Dileepkumar, Swapan Kumar Maiti*, Naveen Kumar and Malik Mohammad Shams-uz-Zama

Division of Surgery, Indian Veterinary Research Institute, Izatnagar 243 122, (UP), India
*Corresponding author: maiti_62@rediffmail.com; swapanivri@gmail.com

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A B S T R A C T

The present study was conducted for exploring the activity of anti-angiogenic and chemotherapeutic treatment with or without cox-2 inhibitor and immunomodulator drug in the management of spontaneous canine mammary neoplasms. Anti-angiogenic drug (Tamoxifen) (n=10), chemotherapy (5-FU) (n=10), chemotherapy (5-FU) along with cox-2 inhibitor (Etoricoxib) (n=10), chemotherapy (5-FU) along with cox-2 inhibitor (Etoricoxib) and immunomodulator drug (Levamisole) (n=10) were used in 40 female dogs having spontaneous mammary neoplasms. Anticancer drug (5-FU) and anti-angiogenic drug (Tamoxifen) induced apoptosis in canine mammary neoplasms. Immunomodulator drug (Levamisole) along with cox-2 inhibitor (Etoricoxib) and chemotherapeutic drug (5–fluorouracil) induced significantly ($P<0.01$) higher apoptosis. In conclusion, chemotherapy with immunomodulator drug and cox-2 inhibitor was clinically proved better than chemotherapeutic agent (5-FU) alone in the treatment of canine mammary neoplasms.

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INTRODUCTION

In the modern industrialized world, cancer has become the most feared of all diseases. Cancer has gained considerable relevance in animals now-a-days owing to the increased awareness among people towards animal sufferings and pain. The diagnosis and management of neoplasm, therefore, represent the major challenge faced by a veterinary oncologist. With the emergence of multitude of interdisciplinary and subspecialty fields of cancer science like tumor biology, cancer chemotherapeutics, immuno-pathology etc, different management modalities like excisional surgery, chemotherapy and immunotherapy have evolved. These therapies either alone or in combination make all neoplasm patients to have an improved quality and quantity of life if not always cures. The quality of life of the animal should always be prioritized (Cassali *et al*., 2011).

Tumors of mammary gland are the second most common neoplasm of female dog representing approximately 40-50% of all neoplasms (Khimta *et al*., 2010). Chemotherapy is a kind of treatment that uses drugs to attack cancer cells. The importance of chemotherapy has been emphasized and it was reported that survival could be prolonged after chemotherapy in cancer patients (Maiti *et al*., 2011). Clinical trials of the combination of selective cox-2 inhibitors with chemotherapy in patients with a number of cancers have been initiated and preliminary results are encouraging (Liao *et al.*, 2007). Cancer in dogs depend on angiogenesis (the creation of new blood vessels) to survive and proliferate. Anti-angiogenic therapy cuts off this new blood vessels, effectively starving tumors and preventing their growth. Unlike chemotherapy, anti-angiogenic treatments are well-tolerated, have few side effects and may control disease over the course of the dog's lifetime (Ferrara and Kerbel, 2005). Considering immunosuppressive nature of mammary neoplasm, it was suggested for the use of immunotherapy to prevent cytotoxicity of cancer drugs, further metastasis and prolong survival period in canines (Todorova *et al*., 2005). 5-fluorouracil (FU) is a pyramiding analog and inhibit DNA synthesis and it is used in the chemotherapy of carcinoma of GI tract, mammary gland, liver and lungs (Hsu, 2008). The present study was therefore, designed to investigate the therapeutic efficacy of chemotherapeutic agent-5-flurouracil with or without cox-2 inhibitor (Etoricoxib) and immunomodulator drug (levamisole), anti-angiogenic

drug (Tamoxifen) in the treatment of canine mammary neoplasms.

MATERIALS AND METHODS

Necessary permission was undertaken from the Institute Animal Ethics Committee (IAEC) to conduct this research wok in clinical cases. The present study was conducted in 40 dogs of different breeds having variable sizes of spontaneous mammary neoplasms (Fig. 1A and 1B). The age of the animal varied between 2 to 15 years. They were randomly allotted to different treatment groups (I to IV) consisting 10 animals in each group. Attempts were made to allot same size of neoplasm in respective groups. We tried to maintain uniformity in different parameters including age of animal, size of tumor, histopathological types and TMN staging. Owner's consent was taken into consideration before grouping/therapy of these animals. All the CMN affected animals were intact during presentation.

In group I, patients having spontaneous mammary neoplasm were treated with anti-angiogenic drug-Tamoxifen @ 20-40mg/day orally given in two divided doses for 1 to 3 weeks depending on the response of the patient. In group II, patients were treated with chemotherapeutic drug-5- fluorouracil (5-FU) @ 150 mg/m^2 after diluting in 200-500ml of normal saline, once /week intravenously for 3-4 weeks. In group III animals, combined therapy of 5- fluorouracil (@ 150 mg/m^2 diluted in normal saline administered intravenously at weekly interval for 3-4 occasions) along with cox-2 inhibitor-Etoricoxib @ 2 to 4 mg/kg orally for 1-2 weeks were given. The patients of group IV received combined therapy as that of group III along with immunomodulator drug levamisole injection @ 2.2 mg/kg subcutaneously once in a week for two weeks.

Drug tolerance of the patient was ascertained in all the four groups by recording the various side effects, if any, reported by the pet owners. Supportive therapy was instituted to alleviate such side effects. Neoplasm biopsy samples were collected in 10% neutral buffered formalin and processed routinely by paraffin embedding technique. Sections of 4-5 micron thickness were cut and stained by Hematoxylin and eosin for histopathological examination. Radiographic examination of mammary neoplasm patients was done to detect the chances of lung metastasis. For apoptosis studies, biopsy samples of neoplasm were taken before the treatment and 1, 2 and 3 weeks after treatment. They were subjected for the flow cytometric analysis of apoptosis using FACS caliber (Becton Dickinson, San Jose, CA). The samples were processed to prepare single cell suspension and then this suspension was subjected for FACS (Fluorescence Activated Cell Sorter) analysis to study the involvement of apoptosis in neoplasm regression. The data pertaining to various parameters studied in canine cancer patients were analyzed by 'paired-t' test and analysis of variance (ANOVA) as per standard statistical methods.

RESULTS

Canine mammary neoplasm cases were presented as circumscribed nodules with variable size, consistency and mobility to the skin and muscle. They were sometimes also associated with skin ulceration and local inflammatory reactions. Multiple neoplasms were observed either in a single mammary gland or may involve multiple mammary glands, simultaneously. The age of the affected animals ranged from 2-15 yrs. More cases were recorded in the age groups of 7-9 yrs (40%) followed by 10-12 yrs (27%), 13-15 yrs (23%), 4-6 yrs (7%) and 0-3 yrs (3%). Maximum cases were recorded in Spitz (40%), followed by German shepherd (23.33%), non-descript (16.67%), Doberman (6.67%), Pomeranian (6.67%), Rottweiler (3.33%) and Labrador (3.33%). Caudal abdominal and inguinal (4^{th} and 5^{th}) mammary glands were mostly affected (65%) in the mammary chain followed by cranial abdominal and caudal thoracic (3^{rd} and 2^{nd}) with less frequency (35%).

Thoracic radiographs of CMT affected animals revealed no lung metastasis except in three cases where radio-opaque soft tissue density in the lung parenchyma was observed (Fig. 2).

Histopathologically, 16% neoplasms were benign and 84% were malignant. Spitz showed maximum malignancy (44%), whereas benign neoplasm (60%) were observed in non-descript breeds. Malignant neoplasms were observed in Pomeranian, Rottweiler and Labrador breeds whereas Spitz, German shepherd and non-descript breed showed both types. The benign neoplasms were adenoma, complex adenoma, basaloid adenoma, fibroadenoma, mixed benign tumor and ductal papilloma. The malignant neoplasms were squamous cell carcinoma, ductal carcinoma, papillary carcinoma, tubular carcinoma, fibrosarcoma, mixed tumor carcinoma, and solid carcinoma (Fig. 3A and 3B)

In group I, animals were treated with Tamoxifen @ 20-40mg/day orally in two divided doses for 1 to 3 weeks depending on the response of the patient. No observable side effects were detected in this group. There was significant increase ($P<0.05$) in apoptosis after treatment with Tamoxifen revealed the efficiency of this therapy (Fig. 4A).

In group II, animals were treated with 5- fluorouracil, diluted in 200 ml of normal saline and administered within one hour at the dose rate of 150 mg/ $m^{2,}$ once in a week intravenously for three weeks. Two animals of this group showed signs of disorientation, seizures, biting own body, pain and howling. The animals were managed with thiopentone, fluid therapy and kept under confinement. Apoptosis studies showed a significant increase ($P<0.05$) in percentage of apoptosis after 5-FU therapy (Fig. 4B).

Combinations of 5–fluorouracil and cox-2 inhibitor (Etoricoxib) were administered in the animals of group III. One animal of this group showed signs of photosensitization, pain, vomiting and wandering. These symptoms were controlled by fluid therapy and rest to the animal. A significant increase ($P<0.05$) in percentage of apoptosis was observed after therapy (Fig. 4C).

Chemotherapeutic agent with 5-fluorouracil along with cox-2 inhibitor (Etoricoxib) and immunomodulator drug (levamisole) were administered in the animals of group IV. Apoptosis studies showed a highly significant increase ($P<0.05\%$) in percentage of apoptosis (Fig.4D). Percentage of apoptosis was much more in this group as compared to other treatment groups. No untoward/side

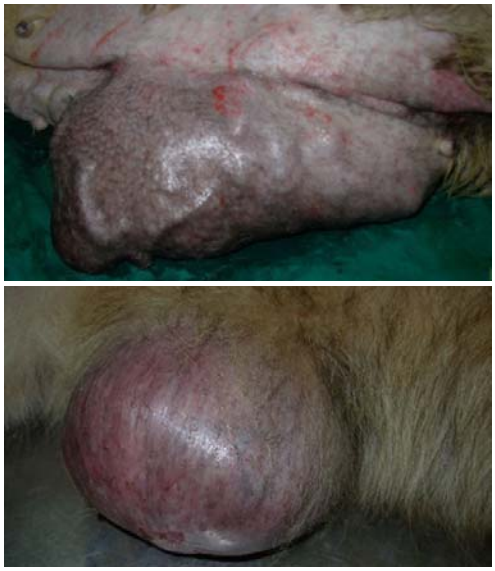

Fig. 1 (A and B): Mammary tumour in a dog

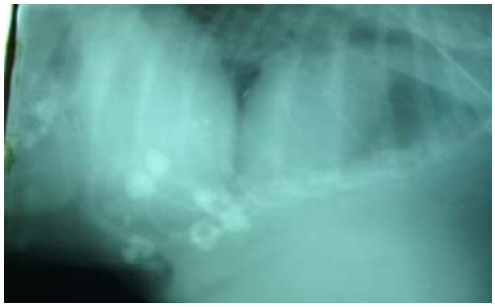

Fig. 2: Lateral thoracic radiograph showing lung metastasis in a mammary tumour affected bitch

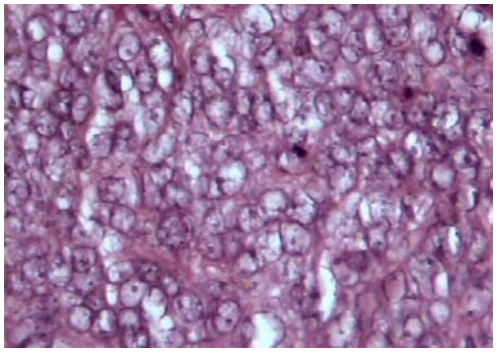

Fig. 3A: Adenocarcinoma of mammary gland (H&E stain, X100)

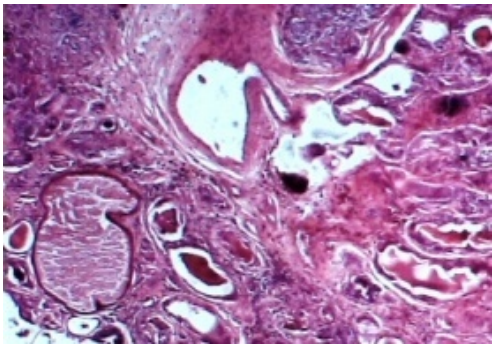

Fig. 3B: Mixed benign tumour of mammary gland (H&E stain, X100)

Table 1: Apoptosis (%) recorded in dogs with mammary tumors

Groups	Type of therapy	Before treatment	After Therapy (week)		
			1st	2nd	3rd
I	Tamoxifen	4.17±0.26	10.67±0.34*	12.09±0.32*	14.25±0.25
II	5-Fluorouracil	4.51±0.22	13.49±0.30	14.28±0.39	24.25±0.61*
III	5-Fluorouracil and Etoricoxib	4.47±0.26	17.38±0.21*	18.12±0.32*	24.53±1.13*
IV	5-Fluorouracil, Etoricoxib & Levamisole	4.47±0.71	19.63±1.60*	21.88±0.54	27.38±0.44*

*Value (mean±SE) differ significantly (P<0.05) with their base values within the group.

effects were recorded in any animal during treatment period in this group. Apoptosis studies of different groups showed increased apoptosis following therapy indicated the effectiveness of different drugs (Table 1). Percentage of apoptosis was significantly higher (P<0.01) in group IV as compared to other groups of animals (Fig. 5). There was significant difference (P<0.01) in apoptosis values between the animals in groups I and III and groups II and III.

DISCUSSION

Mammary neoplasms are the most frequent neoplasm in female dogs and constitute an important problem in veterinary medicine. Several efforts have been made towards the adoption of criteria to standardize the diagnosis, understanding tumor behavior and progression and the evaluation of prognostic factors including morphology, oncogene expression and gene alterations. The knowledge and adoption of these criteria are fundamental for the selection and success of therapies that could prevent neoplasm recurrence and increase survival (Cassali *et al*., 2011). Different surgical options for removal of canine mammary tumors has been discussed (MacPhail, 2013; Papazoglou *et al*., 2014).

Neoplasms of the mammary gland rarely occur in female dogs younger than 2 years of age. The incidence of neoplasm increases after the 5th year of age with a peak at the age of 10-12 years and subsequently decreases. Dogs were most often affected at the age of 10 years (Soremno *et al*., 2013). Benign tumors were found at the age of 8-9 years. The highest relative risks of malignant mammary tumors were reported in 13-year old dogs (Khimta *et al.*, 2010). Our observations also confirmed this report. Increased incidence of mammary neoplasm was found in many large as well as smaller breeds of dogs. The lowest occurrence was reported in Boxers and Chihuahuas. Incidences of mammary neoplasms were found higher in pure breed dogs as compared to mongrels. Even though not significant, there was also a higher relative risk of mammary neoplasm in pure breed dogs in our study (Khimta *et al*., 2010). Spayed dogs have 3 to 7 time's lower incidence of mammary neoplasm than the intact ones. The effect of spaying on the decreased incidence of mammary neoplasm, however, depends on the age of the animal at the time of surgery. Decrease incidence of mammary neoplasm was recorded in females spayed prior to the first estrous (0.5%) and between the first and second estrous (8%). Advance age at the time of spaying increases the incidence of neoplasm and no positive effect of spaying was found after the fourth estrous cycle (Misdrop, 1998). Caudal abdominal and inguinal mammary

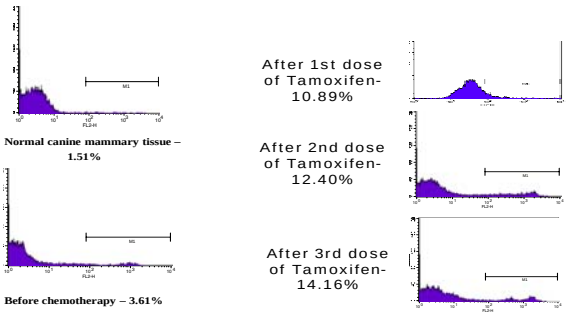

Fig. 4A: Histogram plot of flow cytometry analysis showing percent apoptotic cell (MI) in CMT cases during different course of therapy in group I, after staining with MC-540 showing percentage of apoptosis after chemotherapy with Tamoxifen.

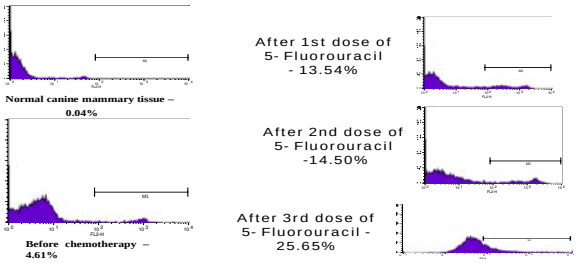

Fig. 4B: Histogram plot of flow cytometry analysis showing percent apoptotic cell (MI) in CMT cases during different course of therapy in group II, after staining with MC-540 showing percentage of apoptosis after chemotherapy with 5-Fluorouracil.

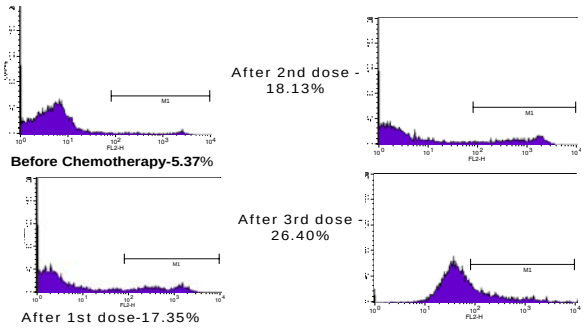

Fig. 4C: Histogram plot of flow cytometry analysis showing percent apoptotic cell (MI) in CMT cases during different course of therapy in group III, after staining with MC-540 showing percentage of apoptosis after chemotherapy with 5-FU and Etoricoxib.

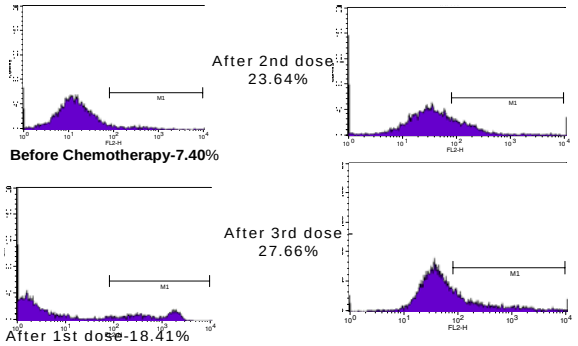

Fig. 4D: Histogram plot of flow cytometry analysis showing percent apoptotic cell (MI) in CMT cases during different course of therapy in group IV, after staining with MC-540 showing percentage of apoptosis after chemotherapy with 5-FU, Etoricoxib and Levamisole

mammary glands have higher frequency of mammary neoplasms than thoracic glands. Posterior glands have greater volume of glandular tissue to react any carcinogenic stimulus (Khimta *et al*., 2010).

Thoracic radiography provided information pertaining to the extent of organ involved and presence of metastasis in the lungs. To determine the precise clinical staging of the cancer, chest radiographs in three views (ventro dorsal, right lateral and left lateral) must be performed. The lung is the most common site for distant metastasis in dogs with malignant mammary gland tumors (Cassali *et al*., 2011). However, in this study, lung metastasis was observed only in three cases.

Clinical staging (TNM) of the mammary tumors was done as per WHO classification. Out of 40 cases recorded, 15 cases (53.85%) were in clinical stage IV followed by 10 cases (23.07%) in stage I, 8 cases (12.32%) in stage III, 5 cases (7.69%) in stage II and 2 cases (3.07%) in stage V. Clinical staging defines the extension of neoplasms and thus established prognosis and treatment to be planned (Cassali *et al*., 2011). Based on this system, size of the primary lesion (T), extent of its spread to regional lymph nodes (N) and presence or absence of distant metastases (M) was assessed.

The histological classification has become a valuable tool for predicting biological behavior of mammary neoplasms. Therefore, it is essential to conduct histopathological examination of all nodules regardless of their size, as this provides important additional information that assists the clinician to define the prognosis and best treatment plan (Cassali *et al*., 2011). In veterinary medicine, the grading systems for mammary tumors with well-defined criteria are not frequently used (Cassali *et al*., 2012).The most popular grading system is based on the cellular and nuclear characteristics (Misdrop *et al*., 1999).

The presence of hormone receptors in mammary neoplasms suggested that the hormone therapy may be an alternative treatment as in human medicine. Anti-estrogenic therapeutic benefit in veterinary medicine can be documented by carrying out studies using appropriate methodology and clinical follow up (Cassali *et al*., 2011). In addition to its effect of estrogen receptors (ER), Tamoxifen is also an anti-angiogenic drug. Neoplasm depends on neo-vascularization processes to provide oxygen and nutrients, allowing the neoplastic cells to exceed a size of 1-2 mm. Both canine and human mammary tumors are hormone dependent (Sorenmo, 2003). Most canine mammary neoplasms (both benign and malignant) expressed ER and the dogs positive for the presence of receptors have higher survival rate and are fit candidates for hormone therapy (Sorenmo, 2003). The timing of intervention appears to be important, treatment at early stage being more successful than treatment applied when the tumor burden is extensive (Ferrara and Kerbel, 2005). Angiogenesis inhibitors are attacked neoplasm by depriving cancer cells of their blood supply. Some anti-angiogenic drugs may be combined in order to hit multiple targets and to improve effectiveness (Ma and Waxman, 2008). In the present study, there was significant increase in apoptosis percentage after treatment with Tamoxifen showed the efficacy of this anti-angiogenic therapy.

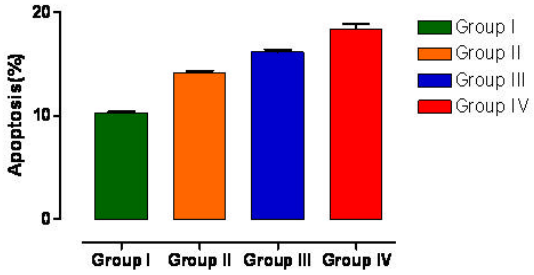

Fig. 5: Apoptosis percentage of different groups of treatment in CMT

5-Fluorouracil, a pyramiding anti-metabolite that interferes with DNA synthesis by blocking the methylation of deoxyuridylic acid; fluorouracil inhibits thymidylate synthetase (TS), or is incorporated into RNA (Hsu, 2008). Fluorouracil is indicated in the treatment of carcinoma of colon, rectum, breast, stomach, pancreas and other malignancies in human patients (Adjei *et al*., 2002). The 5-fluorouracil induced seizures in the present study were controlled with pentobarbital sodium as also reported by Albretsen *et al*. (1998). Supportive treatment included intravenous fluid administration and pain control. It was also opined that 5-fluorouracil as chemotherapeutic agent is useful in the treatment of carcinoma of GI tract, mammary gland, liver and lungs (Hsu, 2008). In this study, significant increase ($P<0.05$) in apoptosis after 5-FU therapy proved its efficacy for the treatment of canine mammary neoplasms.

Etoricoxib, selective cox-2 inhibitors are useful in the chemoprevention and therapy of human breast cancer (Half *et al*., 2002). In humans as well in canine's mammary carcinomas, there has been over expression of cox-2 (Prescott and Fitzpatrick, 2002). Cox-2 levels are higher in malignant mammary neoplasms than in benign mammary neoplasms or normal mammary gland tissue (Queiroga *et al*., 2005). It was reported that increased expression of cox-2 was associated with a graded prognosis and shorter survival time. Use of cox-2 inhibitor may be an alternative in the treatment and control of advanced neoplastic disease of the mammary glands of female dogs (Lavalle *et al*., 2009). Cox-2 inhibitor (Etoricoxib) was found useful in the prevention of animal mammary carcinogenesis (Orendas *et al*., 2007). Use of cox-2 inhibitor proved as a predictive factor for mammary cancer in dogs (Lavalle *et al.*, 2009). Two animals in group II showed signs of adverse reaction towards therapy. This may be due to 5-FU induced toxicosis. The direct cytotoxic effects of 5-FU on cancer cells, by inhibiting cell proliferation and inducing apoptosis have been reported (Sasaki *et al*., 2010). In group II, increased apoptosis percentage after treatment proved the efficacy of combination therapy in the management of canine mammary neoplasms and suggested that cox-2 inhibitor potentate the apoptotic effectiveness of 5-FU (Orendas *et al*., 2007, Lavalle *et al*., 2009, Souza *et al.*, 2009, Sasaki *et al*., 2010). Combined uses of cox-2 inhibitors and epidermal growth factor receptor (EGFR) have also been found useful for the treatment of canine mammary tumors (Guimaraes *et al.*, 2014).

Most cancers appeared susceptible to the effects of levamisole. The drug should be used as an adjunct to classical therapeutic modalities and to stabilize complete remissions. Levamisole has stimulating activity in cell mediated immune systems and has a protective effect on platelets by preventing their aggregation induced by viral neuraminidase (Pineau *et al*., 1980). Use of levamisole as an immunostimulant showed a significant increase ($P<0.05$) in mean survival time and number of survivors in mice tumor. Levamisole in dogs and human has immune-restorative effect (Pineau *et al*., 1980). Levamisole stimulate both cellular and humoral immune responses and thus prevent the immune-suppression (Shaha *et al*., 2011). In group IV, no side effects of 5-FU were observed during the treatment and highly significant increase ($P<0.01$) in percentage of apoptosis was recorded. Percentage of apoptosis was much higher in this group than any other treatment groups which proved the efficacy of combination therapy along with immunomodulator drug during chemotherapy for the treatment of canine mammary neoplasms.

Programmed cell death, also known as apoptosis plays an important role in the determination of neoplasm growth and its aggressiveness (Gonzáles-cámpora *et al*., 2000). Apoptosis plays a vital role in oncogenesis and can be used as a prognostic marker in canine mammary neoplasms (Pereira *et al*., 2003). The study of apoptosis induced by neoplastic cells was widely used in clinical medicine against lung cancer, gastric cancer and colorectal cancer and provides reliable experimental evidence (Yu-wen *et al*., 2007). In this study, apoptosis study was found very useful parameter for confirmation of effectiveness of different types of combination therapy for the management of canine mammary neoplasms.

Conclusion: The result of this study indicated that the anticancer drug (5-FU) and anti-angiogenic drug (Tamoxifen) induced apoptosis in canine mammary neoplasm. Immunomodulator drug (Levamisole) along with cox-2 inhibitor (Etoricoxib) and chemotherapeutic drug (5– fluorouracil) induced highly significant ($P<0.01$) percentage of apoptosis. The efficacy of chemotherapy with immunomodulator drug and cox-2 inhibitor was clinically proved better than chemotherapy alone.

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