THERAPEUTIC EVALUATION OF METHOTREXATE WITH OR WITHOUT COX-2 INHIBITOR IN THE MANAGEMENT OF CANINE MAMMARY TUMOURS

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Spontaneously occurred canine mammary tumours (55) were treated with chemotherapy (Methotrexate), combination chemotherapy with COX-2 inhibitor (Meloxicam) and by surgical therapy. In chemotherapy groups Hb and TEC values reduced significantly ($P<0.05$) from the base line value at 3rd week of therapy. Histologically, benign and malignant mixed mammary tumours were predominant. Methotrexate and COX-2 inhibitor drug were found effective in the treatment of canine mammary tumours and also they induced more percentage of apoptosis of spontaneous canine mammary tumours. Cox-2 inhibitor (Meloxicam) proved as a good adjunct in the treatment of canine mammary tumours.

Introduction

Tumours of mammary gland are the second most common tumours of female dog representing approximately 30-50% of all tumours (O'Keefe 1995; Maiti 2004, 2010; Maiti et al., 2009; Khimta et al., 2010). Chemotherapy is a kind of treatment that uses drugs to attack cancer cells. The importance of chemotherapy has been emphasized by Henderson et al. (1980), who reported that survival could be prolonged after chemotherapy in cancer patients. 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). The introduction of immunohistochemistry and newer techniques like nucleolar organizer region (NOR) staining have improved the objective of diagnosis of cancer (Hung et al., 2000). Silver stained nucleolar organizer region (AgNOR) count was considered to be valuable prognostic marker for canine malignant lymphomas and other canine tumours (Kiupel et al., 1998).

The present study was, therefore, designed to investigate the therapeutic efficacy of chemotherapeutic agent-Methotrexate with or without COX-2 inhibitors-Meloxicam and also to study the potential clinical application of nucleolar organizer regions (NORs) as prognostic indication in canine mammary tumours.

Materials and Methods

The study was carried out in 55 dogs of different breeds and ages with variable sizes of spontaneous mammary tumours presented at the Institute Referral Polyclinic. These 55 cases were allotted to different treatment groups randomly as mentioned below. Attempts were made to allot same size of tumours in respective groups. Owner's consent was also taken into consideration before grouping/therapy of these animals.

Group	Type of Therapy	Drug used	No of animals
A	Mono-chemotherapy	Methotrexate	15
B	Combination Chemotherapy (Chemotherapy + COX-2 inhibitors)	Methotrexate + Meloxicam	15
C	Surgical therapy		25

Animal affected with neoplasm were subjected to routine clinico- physiological monitoring by observing-rectal temperature (^{0}C), pulse (beats/min) and respiration (breaths/min) on the first day of treatment and then at weekly intervals. 5-ml of venous blood was collected for the estimation of hemoglobin (Hb), total erythrocyte count (TEC), total leukocyte count (TLC) and differential leukocyte

count (DLC) as per standard methods. Serum samples were also analyzed for alanine amino transferase (ALT), aspirate amino transferase (AST) and alkaline phosphatase using standard commercial diagnostic kits (M/S Span Diagnostics, Surat, India).

Tumour samples (biopsy/surgically excised) were collected and fixed immediately in 10% neutral buffered formalin, processed routinely by paraffin embedding technique, sections of 4-5 micron thickness were cut and stained by haematoxylin and eosin for histopathological examination.

Plain thoracic radiography for the detection of pulmonary metastases before treatment of any tumour was performed in suspected cases.

In group A, patients received anticancer drug Methotrexate at the dose rate of 20mg/m^2 body surface area (BSA) (0.65 mg/kg body weight) intravenously at weekly interval for at least 2 weeks to a maximum of 4 weeks. In group B, in addition to Methotrexate (as in group A) COX-2 inhibitors-Meloxicam was given at the dose rate of 0.3 mg/kg body weight orally daily during the entire course of treatment. Drug tolerance of the patient was ascertained in all the groups by recording the various side effects, if any, reported by the pet owners. Supportive therapy was instituted to alleviate such side effects.

In group C, dogs having very large tumour and also as per the owner's request surgical therapy was performed. All the animals were pre-medicated with atropine sulphate at the dose rate of 0.04 mg/kg body weight subcutaneously. After 10 min, general anesthesia was achieved with combination of Xylazine hydrochloride @ 1mg/kg and Ketamine hydrochloride @ 5 mg / kg body weight given intramuscularly. The anaesthetic stage was prolonged by intravenous administration of combination of ketamine hydrochloride @5 mg/kg and Diazepam @ 1 mg/kg body weight, if required.

If a single gland was affected, then only that gland was removed; if multiple glands on one side were affected, then the entire 5 glands on that side were removed; if multiple tumours on both side were affected, then both mammary chains were removed; if the lymph node (axillary/inguinal) was within the resection zone , then they also were removed; if a growth was detected in the number 2 gland on the left side-glands 1, 2, and 3 and the axillary lymph node on that side was removed; if it was found in the number 4 gland on the right side, then glands 3, 4, 5 and the inguinal lymph node on that side was removed; if the groin region was difficult to suture closed, a skin flap from the flank was needed to reconstruct the area.

Broad spectrum antibiotics and analgesics with their standard dose rate were administered for five postoperative days. Tumour recurrence was observed from a minimum of one month to a maximum of one year of surgery.

Four micrometer duplicate sections were stained for interphase nucleolar organizer regions by freshly prepared 50% aqueous silver nitrate solution using the technique described by Crocker (1992) with suitable modifications. All the AgNOR dots scattered in nucleus were counted without trying to resolve the intranucleolar dots. AgNORs in 100 consecutive nuclei were counted and mean number of AgNOR dots per nucleus was calculated for each specimen.

Tumour biopsies of the canine patients were taken before the treatment and during the course of treatment at weekly intervals in the groups A and B for the flow cytometric analysis of apoptosis using FACS caliber. The samples were processed to prepare single cell suspension as per the method described by Laakko et al. (2002), with slight modification. Then this suspension was subjected for FACS (Fluorescence Activated Cell Sorter) analysis to study the involvement of apoptosis in tumour regression during chemotherapy.

The data were analyzed by using pair't' test and analysis of variance (ANOVA) as per standard statistical method (Snedecor and Cochran, 1994).

Results and Discussion

Out of 55 cases of mammary tumour recorded, 38 cases had solitary growth and remaining 17 cases had multiple growth. Pedunculated growths were 26 and remaining 29 growths were sessile. Twenty –eight mammary growths were ulcerated and inflamed while the remaining 27 were intact and subcutaneous. Caudal abdominal and inguinal (4^{th} and 5^{th}) mammary glands were the most commonly affected (62%) in the mammary chain followed

by cranial abdominal and caudal thoracic (3rd and 2nd) with less frequency. The reason for this is that the posterior glands are having greater volume of glandular tissue to react any carcinogenic stimulus (Page, 2001; Maiti et al., 2009).

Clinico-physiological observations:

In mono-chemotherapy of mammary tumour with methotrexate (Group A), gradual increase in rectal temperature (^{0}C) was noticed up to 3rd week and thereafter decreased at 4th week. However, it remained within the normal range. In combination therapy of mammary tumour with methotrexate and Meloxicam (Group B), the mean rectal temperature (RT) remained within the normal limit without any change from the base line value throughout the period of treatment. In surgically treated group (Group C), there was no change in the mean RT before and after surgery and remained within the normal range.

Pulse rate (beats/min) recorded in all the animals of groups A and B remained within the normal limit throughout the treatment period. In group C, the pulse rate did not vary much before and after surgery and remained within the normal range (70-120 beats/min).

The respiratory rate (breaths/min) in groups A and B were increased slightly throughout the course of therapy. However, in group C, the respiratory rate remained within the normal range (18-30 breaths/min).

Hematological observations:

Hemoglobin (Hb): In group A, the Hb value significantly (P<0.05) reduced till the 3rd week and thereafter increased from the 4th week onwards. In group B, there was also a gradual decrease during the course of therapy and the 3rd week value varied significantly (P<0.05) from the first week value. In group C, the Hb values apparently increased after the surgery compared to the values before surgery. However in all groups, the Hb values remained within the normal limits (8-16g/dl).

Total Erythrocyte Count (TEC): The TEC reduced significantly (P<0.05) upto 3rd week in groups A and B when compared with the 1st week value. In group B, the 2nd and 3rd week values differed significantly (P<0.05) from the base line value. In group C, the TEC did not vary much before and after surgery and remained within the normal range (5-8 million/cubic mm).

Total Leukocyte Count (TLC): Total leukocyte count in the animals of group A, B and C did not show much change during the course of therapy and the values remained within the normal range (9-13X10^3/cubic mm)**.**

Differential Leukocyte Count (DLC): The neutrophil count in groups A and B reduced gradually during the course of therapy when compared with the 1st week value. In group A, the neutrophil count on 3rd, 4th and 5th weeks varied significantly (P<0.05) from the base line value and in group B, the 3rd week value differed significantly from the 1st week value. In group C, there was no much change in the neutrophil count before and after surgery and remained within the normal range (60-75%). In contrast, lymphocyte count in groups A and B showed gradual increase at every week of therapy especially in group A. In this group (A) the higher values during 3rd, 4th and 5th weeks of chemotherapy were statistically significant (P<0.05) from the base line value. The lymphocyte count at 3rd week of group B varied significantly from their respective first week values. In group C, there was no much variation in the lymphocyte count before and after surgery and remained within the normal range (15-30%). However, the values of monocyte, eosinophil and basophil were within the normal range (Monocyte 1-8%, Eosinophil 2-8 %, Basophil 0-1%) in all the three groups.

Biochemical parameters:

Alanine transaminase (ALT) levels in the animals of both groups A and B showed gradual increase during every week of therapy. The mean ALT value in group A animals at 5th week of chemotherapy statistically (P<0.05) differs from the base line value. In group C also, the ALT levels increased after the surgery but remained within the normal range (8-60 U/L).

Aspartate transaminase (AST) level in group A showed gradual increase during every week of therapy. The mean AST value in group A animals showed significantly higher values (P<0.05) during 4th and 5th week of chemotherapy. In group B animals, the AST values increased during the 2nd week and reduced in the 3rd week but still the variations were within the normal range (9-50 U/L). In group C, the AST values increased after the surgery compared

to the values before surgery. However, the variations were within the normal limits.

Alkaline phosphatase (ALP) levels in the animals of all the three groups remained almost unchanged during the period of treatment and remained within the normal range (10.6-101 U/L). Similar haemato-biochemical changes were also observed by Maiti et al., (2009) during treatment of canine mammary tumours with Doxorubicin chemotherapy.

Radiological examination:

Radiographs of the thorax helped to provide information pertaining to the extent of organ involved and presence of metastasis in the lungs. Lateral radiograph of thorax in the mammary tumour affected dogs revealed no radiopaque soft tissue scattered in the lungs thereby ruled out the involvement of pulmonary metastasis in all the cases subjected to surgical therapy. Radiographs of thorax taken in suspected animals of both the chemotherapy groups also revealed no lung metastasis. However, in one mammary tumour affected dog showed radiopaque soft tissue density in the lung parenchyma.

Histopathological examination:

Histopathological examination of the tumour samples that were collected following surgical excision and biopsy samples in chemotherapy cases revealed different types of tumours like cystic mucinous adenocarcinoma (5), malignant mixed mammary tumour (10), papillary adenocarcinoma (7), solid carcinoma (8), mixed benign mammary tumour (15), serous cystic adenoma (3) and squamous cell carcinoma (7).

Therapeutic evaluation:

Studies with the use of Methotrexate in CMT cases were very sparse. Hence, in the present study, use of Methotrexate for mono-chemotherapy has been studied and found 8 cases (53%) cases responded very well requiring four doses of Methotrexate and the tumour was completely regressed without any prominent side effects. However , 7 cases could not be assessed further to 2nd dose as 3 cases did not turn up and other 4 owners preferred surgery, though there was partial regression indicating much higher response to drug than that recorded by us. Side effects like vomiting, fatigue and in appetence was observed with four cases in this study. These adverse effects may be due to Methotrexate induced hematological, gastrointestinal and cardiac toxicity. Chemotherapy has become a major treatment modality in veterinary oncology (Rosenthal, 1989). Mammary tumour cases have been treated with methotrexate, an antimetabolites that is a S-phase specific chemotherapeutic agent. No observable side effects were reported in eleven cases. This may be due to the fact that low dose regimen is far less toxic as reported by Citron (2004).

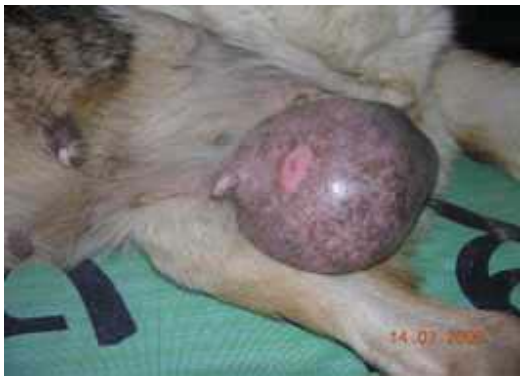
In group B, 12 cases (80%) responded very well and required three dose of methotrexate and the tumour was completely regressed. No observable side effects were reported in these cases. One case could not be followed up because the case did not report to the polyclinic while in the other two cases, the owners sought for surgical excision of the tumours growth because of the few side effects like vomiting, alopecia and anorexia observed in these cases during chemotherapy in spite of partial regression of the mammary tumour. Combination chemotherapy is based on the concept of total cell kill (Calabresi and Parks, 1980). In this study, methotrexate was combined with a COX-2 inhibitor, meloxicam to find out whether meloxicam attenuates the cell death induced by methotrexate. Similar studies were reported by Mohammed *et al.* (2003) in naturally occurring canine invasive urinary bladder cancer wherein the COX inhibitor, piroxicam has induced remission in 18% of dogs and resulted in stable tumour size in 50% of dogs when combined with cisplatin chemotherapy. In the present study, tumour regression was observed in all the 15 animals administered with this combination therapy. Marked reduction in the size of the tumour mass was noticed in twelve cases with only 2 to 3 doses. This may be due to the enhanced attenuation of methotrexate by meloxicam, as observed by Naruse *et al.* (2007) in osteosarcoma patients. The results from our study provide support for a potential role of COX-2 inhibitors as an adjunct with conventional antineoplastic agents for the prevention and the treatment of canine mammary carcinomas.

The entire tumour cases operated surgically (group C) showed uneventful recovery with no postoperative complications observed up to 14th day except in three cases, where swelling

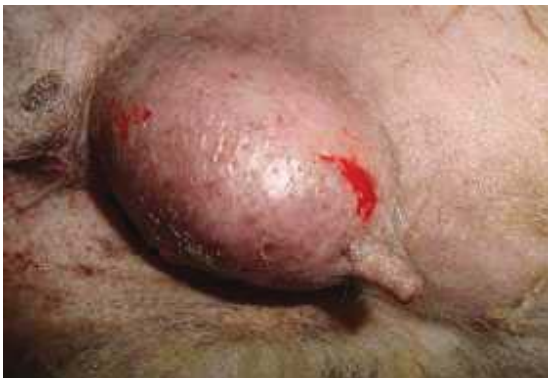
and purulent exudation at the surgical site was observed on 6th post operative day. Complete wound healing was observed in these animals on 15th postoperative day. Tumour recurrence was not observed in any of the surgically treated animals. Surgical therapy is usually considered the routine therapy for mammary tumours (Maiti et al., 2007, 2009).

AgNOR staining and counting:

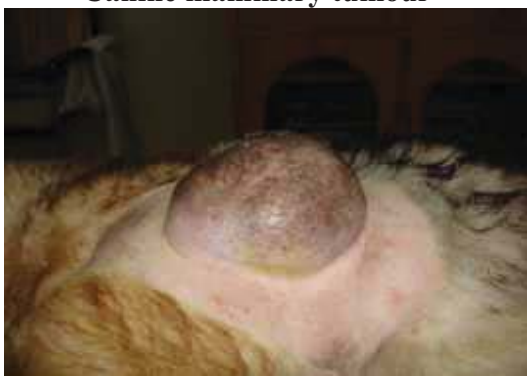
All the collected tumour samples were stained with AgNOR stain and the black AgNOR dots were counted. The AgNOR dots were in general large and round in benign tumours as compared to small and irregularly scattered black dots in malignant ones. The mean AgNOR dots per nucleus counted and calculated for each tumour type is presented in the Table 1. Based on the AgNOR counts, the tumours were classified into benign and malignant. On statistical analysis, it was revealed that the AgNOR counts were significantly higher (P>0.05) for malignant tumours than the benign one. Among the different mammary tumours, Squamous cell carcinoma revealed the highest AgNOR count (4.60) followed by cystic mucinous adenocarcinoma (3.11) and papillary adenocarcinoma (2.96).



Canine mammary tumour

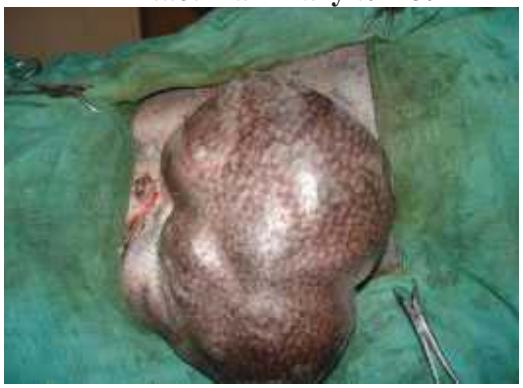


Canine mammary tumour

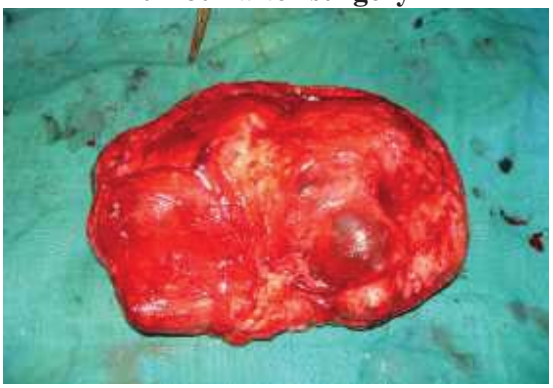


Intact mammary tumour

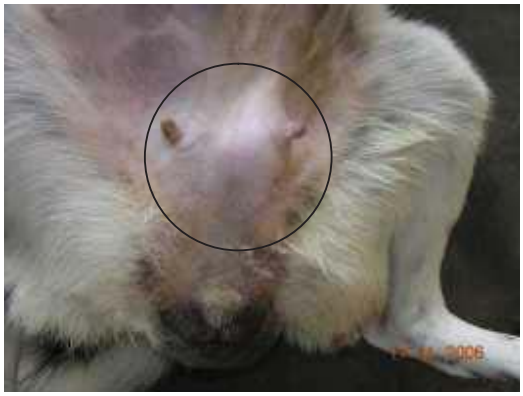
→ Tumour after surgery



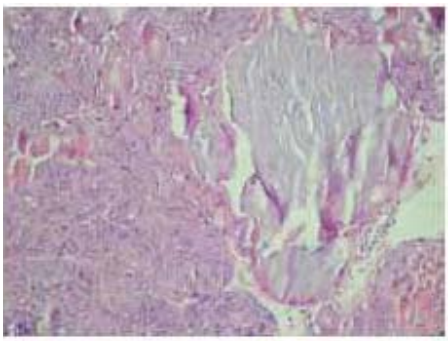
Intact mammary tumour



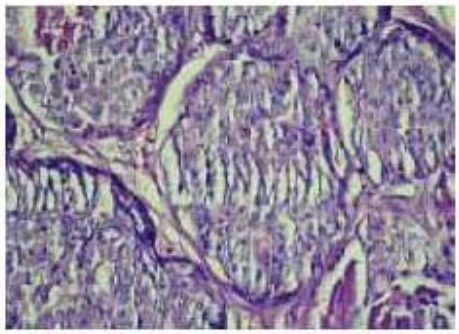
→ Tumour after surgery



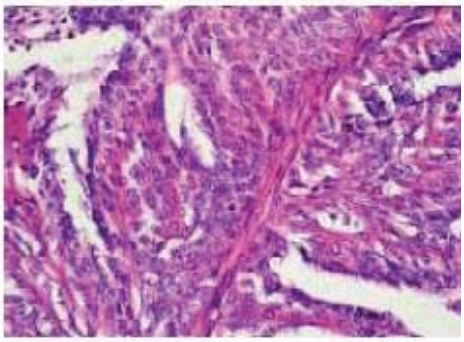
CMT before chemotherapy → **CMT after chemotherapy**



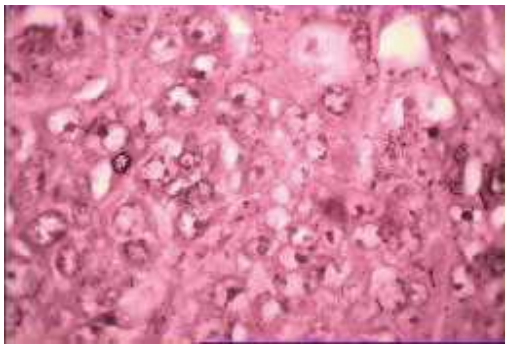
Cystic mucinous mammary adenocarcinoma (H&E X 200)



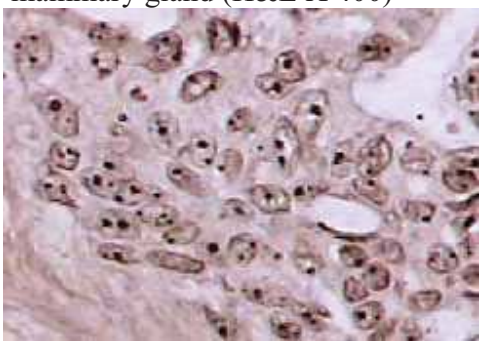
Intra acinar mammary solid carcinoma (H&E X 400)



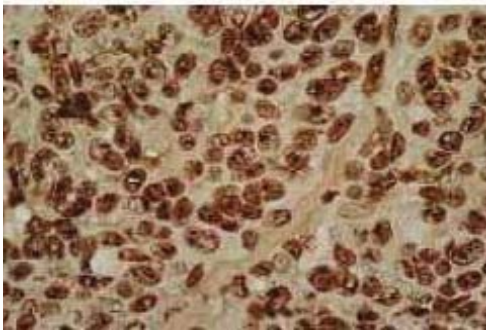
Papillary adenocarcinoma of mammary gland (H&E X 400)



Squamouc cell carcinoma of mammary gland (H&E X 200)



Adenoma (few but large & round AgNOR black dots per nucleus)



Cystic mucinous adenocarcinoma (numerous, small & irregular AgNOR dots)

Table1. AgNOR count in different types of canine mammary tumours

Tumour type	AgNOR count
Benign	
Serous cystic adenoma	2.62
Mixed benign mammary tumour	2.38
Papillary adenoma	1.80
Mammary Fibroadenoma	1.90
Benign mixed mammary tumour	1.28
Malignant	
Cystic mucinous adenocarcinoma	3.11
Papillary adenocarcinoma	2.96
Solid mammary carcinoma	2.80
Malignant mixed mammary tumour	2.66
Squamous cell carcinoma	4.60

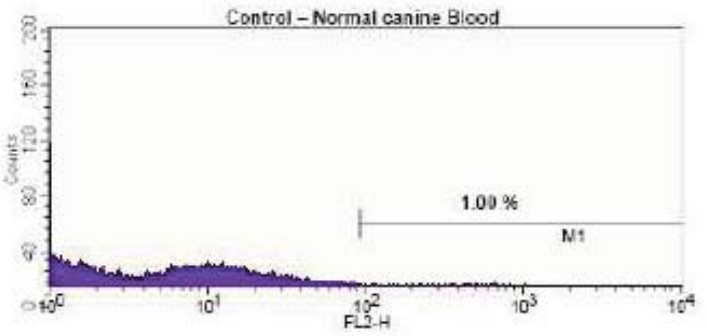
The AgNOR method of differentiation between benign and malignancy in canine mammary tumours was proved to be an inexpensive and easy to perform test for assessing the cell proliferation rate when compared to the various immuno-histochemical studies employed presently in oncological diagnosis. AgNOR counting has a great potential in diagnostic oncology in canine tumours (Kiupel *et al.,* 1998) and can also be used to arrive at the prognosis of clinical cases.

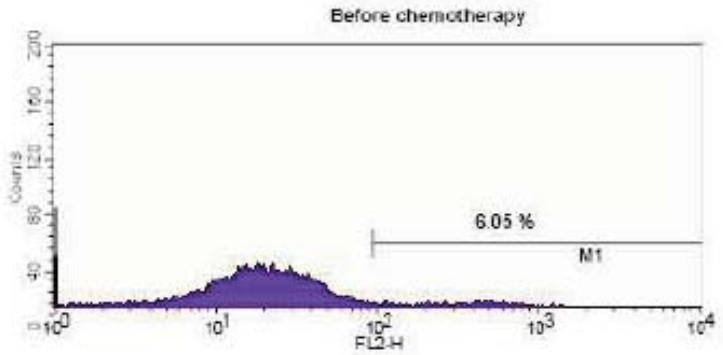
Flow cytometry studies on tumour apoptosis:

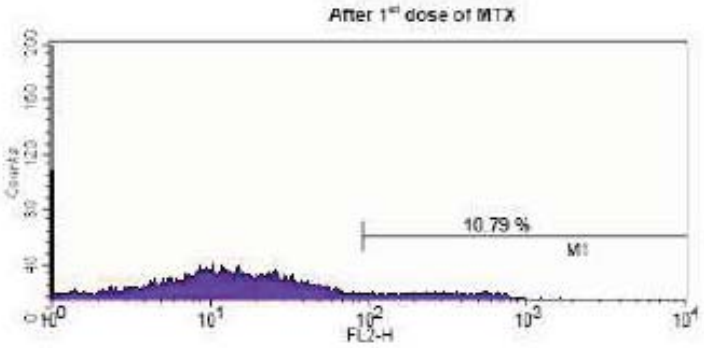
Flow cytometric analysis of apoptosis in tumour regression was performed using FACS caliber. Tumour biopsies of canine patients were collected before and during the course of chemotherapy at weekly intervals. FACS analysis was done to assess the involvement of apoptosis in tumour regression during chemotherapy by analyzing the DNA content of tumour cell population based on merocyanine (MC 540) fluorescence. Merocyanine fluorescence was measured in FL_2 filter in FACS caliber at 375 volts. Histogram plot for MC 540 fluorescence indicates that the apoptotic cell population is increasing after chemotherapy than the control. Apoptotic cell percentage was calculated from the statistical analysis of the histogram plot. Percentage of apoptotic cells in tumour biopsy samples obtained from animals during different weeks of chemotherapy was higher in the animals of group B than group A. The percentage of apoptotic cells increased at succeeding weeks of chemotherapy when compared to the 1st week values i.e. before the start of chemotherapy indicating the increase in drug induced apoptosis during tumour regression.

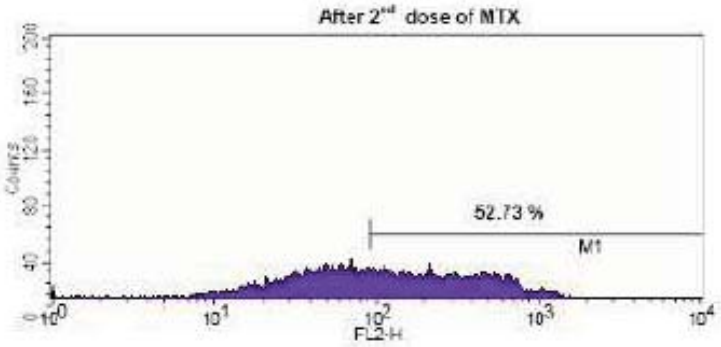
Histogram plot of flow cytometry analysis showing percent apoptotic cells (M1) in canine mammary tumour biopsy samples before and during chemotherapy with methotrexate (MTX) therapy, after staining with MC 540

Apoptosis plays a key role in many facets of biology and medicine (Hengartner, 2000) like immune function, oncology, AIDS etc. Apoptosis is a commonly described cellular outcome of treatment with many anticancer drugs (Viktorsson *et al.,* 2005). The tumour cells die by apoptosis during chemotherapy or radiotherapy and monitoring the level of apoptosis may prove useful in modulating treatment or in predicting the outcome of therapy (Gorczyca *et al.,* 1993). From the results of our study, it has been found that MC 540 readily quantifies early apoptotic cells. The drug induced apoptosis in spontaneous canine mammary tumours increases at every week of chemotherapy. The importance of flow cytometric measurement of apoptosis to monitor a patient's response to treatment during follow up period in the treatment of cancer is suggested (John, 2001). The present study also supports that flow cytometric analysis of apoptosis in the cell suspension derived from solid tumour biopsy specimen is a useful predictor of patient's


Control – Normal canine Blood
1.00 %
M1
Counts
FL2-H


Before chemotherapy
6.05 %
M1
Counts
FL2-H


After 1st dose of MTX
10.79 %
M1
Counts
FL2-H


After 2nd dose of MTX
52.73 %
M1
Counts
FL2-H

response to chemotherapy and prognostic outcome in clinical settings.

The results from this study provide support for a potential role of COX-2 inhibitors-meloxicam as an adjunct with conventional anti-neoplastic agents-methotrexate for the treatment of canine mammary tumours. Methotrexate along with Meloxicam is suggested to be used in the treatment of canine mammary tumours.

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