

Canine Malignant Mammary Gland Neoplasms with Advanced Clinical Staging Treated with Carboplatin and Cyclooxygenase Inhibitors

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Abstract. *Surgery remains the treatment of choice for female dogs with mammary gland tumors. Chemotherapy is not commonly used as an adjuvant therapy. Cyclooxygenase 2 (COX-2) has been related to angiogenesis development in tumors, disease progression and worse prognosis. The aim of this prospective study was to compare overall survival periods of female dogs diagnosed with advanced mammary tumors submitted to different treatment protocols, including surgery, chemotherapy and cyclooxygenase inhibitors. Twenty-nine female dogs were evaluated and treated with four different protocols. The overall survival of patients with low COX-2 scores was longer when compared to patients with high COX-2 scores. Different proposed adjuvant treatments associated with surgery led to a statistically significant longer overall survival when compared to surgical treatment alone. Canine patients presenting malignant mammary gland neoplasms with advanced clinical staging should be submitted to complementary therapeutic medication based on clinical staging and immunophenotypical characteristics of the disease.*

Surgery remains the treatment of choice for female dogs with mammary gland tumors, except for those with inflammatory carcinomas. The extent of the surgery depends on the size and location of the tumor and lymphatic drainage of the affected mammary gland (1).

Chemotherapy is not commonly used as an adjuvant therapy. Protocols available in the literature consist of doxorubicin in combination with cyclophosphamide or

cisplatin as a single agent, although additional studies are clearly necessary to determine a more efficient protocol for canine mammary tumors (1-3).

Carboplatin is a second-generation platinum chemotherapeutic agent which reacts within and between DNA strands, by forming DNA adducts. This chemotherapeutic agent was developed in human medicine in order to reduce side-effects seen in use of cisplatin while maintaining an equivalent efficacy. Carboplatin has a nonspecific effect on the cell cycle phase and frequent side-effects are myelosuppression, alopecia and gastrointestinal toxicity, although neuropathies, nephropathies and rare emetic episodes may occur (2, 4, 5).

Two events enhance the growth potential of neoplastic tissues: increased number of cell divisions and angiogenesis. Cyclooxygenase 2 (COX-2) is a protein that has been related to angiogenesis development in tumors, disease progression and worse prognosis. COX-2 inhibition is an important and promising target of non-specific chemotherapeutic agents for the prevention and treatment of human rectal colon cancer (6). COX-2 expression in human breast cancer might be a late event in tumor progression, in contrast to colorectal and gastric cancer, in which COX-2 plays a role in tumorigenesis and is considered to be an early event (7).

Immunostaining for COX-2 in 50% of analyzed breast tumors was observed in one study, with stronger staining in anaplastic carcinomas when compared to adenocarcinomas (8). Regarding canine mammary tumors, a different study found that all cases presented immunostaining and a positive correlation was found between COX-2 expression and worse prognosis (9). Absence of COX-2 expression was demonstrated in normal canine mammary gland tissue and variable staining degrees in different tumor types. A shorter overall survival in patients that presented tumors with higher COX-2 expression and vascular density was also recorded, with a statistically significant correlation between these two variables (10).

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The aim of this study was to compare overall survival periods of female dogs diagnosed with advanced mammary tumors submitted to different treatment protocols, including surgery, chemotherapy and cyclooxygenase inhibitors.

Materials and Methods

Twenty-nine female dogs presenting mammary tumors with advanced clinical staging ($T_3N_{1-2}M_{0-1}$) admitted to the Veterinary Teaching Hospital of the Federal University of Minas Gerais, Brazil, were evaluated in a prospective manner and randomly divided into four different treatment groups: group 1: seven animals submitted to surgical treatment alone. This group was composed of animals whose owners refused the offered adjuvant therapy for several reasons; group 2: eight animals submitted to conventional surgical excision and medication with three cycles of carboplatin at a dose of 300 mg/m², at 21-day intervals; group 3: five animals submitted to conventional surgical excision and medication with three cycles of carboplatin at a dose of 300 mg/m², at 21-day intervals. After the chemotherapy sessions, patients were submitted to medication with oral Piroxicam at a dosage of 0.3 mg/kg, every 24 hours for six months; group 4: nine animals submitted to conventional surgical excision and medication with three cycles of carboplatin at a dose of 300 mg/m², at 21-day intervals. After the chemotherapy sessions, patients were submitted to medication with oral Previcox (firocoxib) at a dosage of 5 mg/kg, every 24 hours during six months.

After surgical extirpation, the neoplasm and the lymph nodes were collected, fixed in 10% neutral formalin, processed by histological technique, embedded in paraffin, sectioned and hematoxylin-eosin stained in order to establish a histopathological diagnosis (11).

Immunohistochemical analysis. Sections of 4 µm were cut from one representative block of each case and placed onto gelatin-coated slides. Slides were deparaffinized and rehydrated in a progressive diluted series of alcohol. Endogenous peroxidase was blocked by immersion in a solution of 3% hydrogen peroxide.

Deparaffinized tissue sections were submitted to heat-induced antigen retrieval (water bath at 98°C) with antigen retrieval solution (pH 6.0) (Dako, Carpinteria, California, United States of America). Slides were then incubated at 4°C for 16 hours with the primary rabbit monoclonal anti-human COX-2 antibody (SP21, 1:10; Lab Vision, Kalamazoo, Michigan, United States of America), followed by the EnVision polymer horseradish peroxidase (Dako) for 1 hour at 37°C. Sections were then stained with 3,3'-diaminobenzidine tetrahydrochloride chromogen (DAB Substrate System; Lab Vision), incubated for 10 minutes and counterstained with Mayer's hematoxylin.

Sections from human colon carcinoma known to express COX-2 were used as positive controls for COX-2 and adjacent normal mammary tissues were used as internal negative controls. The antibody was previously tested in normal canine kidney tissue to demonstrate specificity for canine tissues. Negative controls were obtained by substituting primary antibody with normal serum.

COX-2 expression analysis in neoplastic cells. Positivity for COX-2 was indicated by the presence of cytoplasmatic staining. The number of positive COX-2 cells was evaluated semi quantitatively with the distribution score defined by the estimated percentage of

positive cells in five microscope fields (×400): 0=absence, 1=fewer than 10% of stained cells, 2=between 10% and 30%, 3=31% and 60%, 4=more than 61% of stained cells. For staining intensity, values from 0 to 3 were attributed: 0=absence, 1=weak marking, 2=moderate marking, and 3=strong marking. Distribution scores and intensity were multiplied to obtain the total score, which ranges from 0 to 12 (8, 12), and then divided into groups of low (0-5) and high (6-12) scores (10).

Survival analysis. Clinical follow-up of patients occurred from 2005 to 2010, through periodic return visits every two months to the Veterinary Teaching Hospital of the Federal University of Minas Gerais. Clinical examinations with chest radiographs were performed in order to evaluate disease evolution with possible recurrences and metastasis. Lymph node metastasis was confirmed through histopathological analysis and pulmonary metastasis was confirmed through positive radiographic images. Side-effects of chemotherapy and COX-2 inhibitors were evaluated through laboratory examinations (complete biochemistry and hematogram).

Overall survival time was defined as the period (in days) between the date of surgical removal of the tumor and death caused by the disease. Animals that died from unknown causes or causes unrelated to the tumor were censored. Overall survival was evaluated by univariate analysis (Kaplan-Meier estimated survival curves). Values were considered statistically significant when $p < 0.10$ by the log-rank test (Cox-Mantel). Median survival was defined as the period when 50% of the patients of a determined group had died.

Results

The 29 analyzed tumors were classified as: seven carcinomas in mixed tumors (24.2%), six solid carcinomas (20.8%), five tubulopapillary carcinomas (17.2%), five carcinosarcomas (17.2%), three micropapillary carcinomas (10.4%), one squamous cell carcinoma (3.4%), one anaplastic carcinoma (3.4%) and one pleomorphic lobular carcinoma (3.4%).

Regarding clinical staging, 24 animals presented metastasis in only one lymph node (82.7%) and five presented metastasis in more than one lymph nodes (17.3%). At the time of diagnosis, four animals presented pulmonary metastasis (13.8%).

Among patients with more than one positive lymph nodes, four (80%) died during the study and one (20%) remained alive. Among patients with one positive lymph node, 15 remained alive (62.5%) and 9 (37.5%) died during the study. No statistical difference was observed when comparing the overall survival of the two groups (one or more metastatic lymph nodes) ($p=0.82$; Figure 1).

The case diagnosed as a carcinosarcoma did not present COX-2 immunohistochemical expression (score 0) and the animal was submitted to surgery as single treatment (group 1). Some degree of positivity for COX-2 was observed in all the other cases (28/29). A low COX-2 score was found in 41.4% of evaluated cases (12/29) and a high COX-2 score

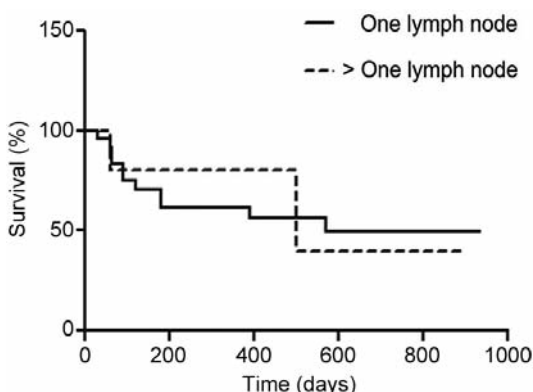


Figure 1. Overall survival curves for 29 female dogs according to lymph node positivity. Patients with one positive lymph node, 24 cases; more than one positive lymph nodes, 5 cases ($p=0.82$).

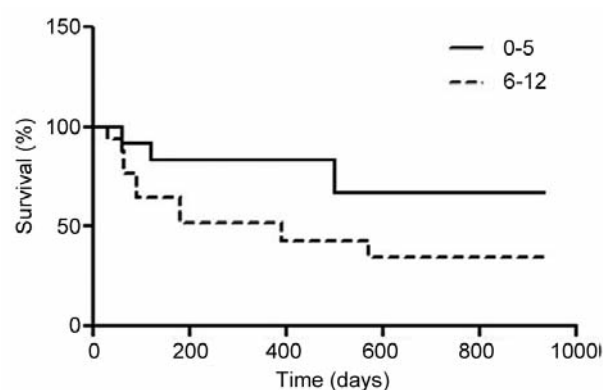


Figure 2. Overall survival curves for 29 female dogs according to cyclooxygenase staining score. Low COX-2 score, 12 cases; high COX-2 score, 17 cases ($p=0.08$).

was presented in 58.6% (17/29). A statistical difference was observed when comparing the overall survival of the two groups (0-5 and 6-12 score) ($p=0.08$). Patients with high COX-2 scores presented a median survival of 390 days while patients with low COX-2 scores did not reach the median survival (Figure 2).

The three proposed adjuvant treatments associated with surgery led to a statistically significant difference in overall survival when compared to surgical treatment alone ($p=0.07$; Figure 3). Patients of the group 1 had a median survival of 63 days; those of group 2 did not reach the median survival; those of group 3 had a median survival of 390 days and those of group 4 of 570 days. However, no statistical difference in survival according to the different adjuvant treatments was observed.

Only one animal died due to side-effects related to the use of COX-2 inhibitors. During Piroxicam therapy, a 24-hour clinical condition characterized by acute hemorrhagic gastroenteritis, not responsive to clinical treatment with hydration, anti-emetics, diet and inhibitors of gastric acid, was responsible for the death of the patient.

Serum biochemical evaluations did not reveal alterations in the patients. No severe gastric or intestinal side-effects were observed with the chemotherapeutic agent. Immunosuppression, determined by a decrease of the white blood cell count, was observed at the drug's nadir period, followed by an adequate clinical evolution; therefore no session had to be postponed.

Discussion

Immunostaining for COX-2 was observed in the majority of the canine malignant mammary gland neoplasms (96.6%) analyzed, as found in other studies (8, 9). Increased COX-2 expression is associated with disease aggressiveness and

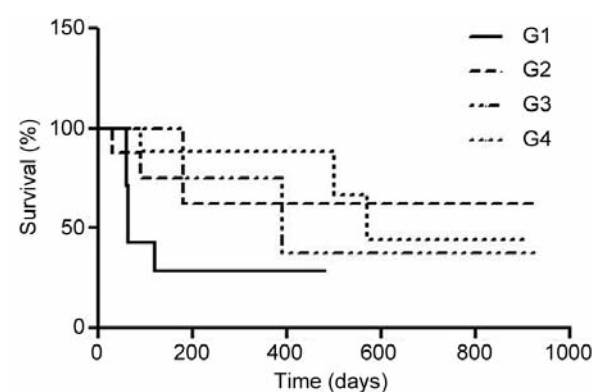


Figure 3. Overall survival curves for 29 female dogs according to therapy. Patients submitted to surgical treatment alone (G1), 7 cases; conventional surgical excision and carboplatin (G2), 8 cases; conventional surgical excision, carboplatin and Piroxicam (G3), 5 cases; and conventional surgical excision, carboplatin and Previcox (G4), 9 cases ($p=0.07$).

shorter overall survival, suggesting COX-2 inhibitors as a possible treatment for canine mammary tumors (1). In the present study, shorter overall survival was associated with high COX-2 scores (of 6-12), considered as an independent prognostic factor.

Histological types found in this study were considerably diversified. However, all patients presented advanced clinical staging with metastatic lymph nodes, which has an important prognostic value, directly impacting on with the overall survival. Canine patients with positive lymph nodes have shorter overall survival when compared to those without (13). The observed higher frequency of carcinomas in mixed tumors (24.2%) is due to its elevated frequency in the canine species (14, 15), with 18-30% of canine mammary neoplasms corresponding to this histological type.

No significant statistical difference was observed when comparing the overall survival of animals with one and more than one positive lymph nodes. The number of positive lymph nodes is considered an independent prognosis factor (16). In this study, the number of animals with more than one positive lymph nodes was limited (17.2%), possibly interfering with the statistical analysis. Moreover, longer survival periods could be a result of the complementary therapies adopted in this study.

Compared to other drugs, carboplatin is relatively affordable and well tolerated. The drug is also considered to be a reasonable option for the treatment of various carcinomas and sarcomas (1). In the present study, minimal side-effects were observed and the administration was considered easy. Animals treated with carboplatin, with or without association with COX-2 inhibitors, had a statistically significant longer overall survival when compared to animals submitted exclusively to surgical treatment, indicating this chemotherapeutic agent as being beneficial for the treatment of malignant canine mammary gland tumors.

Firocoxib inhibits COX-2 selectively and not Cox-1, is therefore considered the optimum nonsteroidal anti-inflammatory drug (NSAID) for prolonged use in canines (17). In the present study, animals treated with Firocoxib did not present side-effects, confirming the safety of the medication.

Piroxicam is a COX-1-selective NSAID that causes several side-effects, particularly in the gastrointestinal tract (18). In this study, one animal died from severe gastrointestinal alterations during Piroxicam therapy. Therefore, we suggest caution when prescribing this medication for the canine species since they have a high gastric sensitivity to the use of anti-inflammatory drugs.

Future clinical trials should concentrate on dogs with poor prognostic factors such as large, lymph node-positive, invasive, high-grade tumors, following complete surgical removal in order to establish optimal treatment options and longer overall survival (1). Based on our findings, carboplatin can be indicated for the treatment of canine mammary gland malignant tumors with advanced clinical staging. Treatment of advanced canine mammary gland tumors with COX-2 inhibitors initially suggests clinical benefits for the patient. We believe that the immunohistochemical score of COX-2 should be included as a predictive factor in the evaluation of canine mammary neoplasms with advanced clinical staging. COX-2 inhibitors will provide stronger therapeutic benefits to animals with tumors that present high COX-2 scores (of 6-12). However, this treatment remains to be further investigated in studies with a larger number of patients with more homogeneous characteristics.

Conclusion

When comparing overall survival curves of animals treated with different adjuvant therapies and those treated with surgery alone,

we verified that patients with advanced clinical staging benefit from complementary therapy. Canine patients presenting malignant mammary gland neoplasms with advanced clinical staging should be submitted to complementary therapeutic medication based on clinical staging and immunophenotypical characteristics of the disease.

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