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Canine Mammary Neoplasms - Evaluation of Tumor Microenvironment

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ABSTRACT

***Background*:** The tumor microenvironment is an important target of studies in different types of neoplasms. Understanding the role of general components such as immune, vascular and fibroblastic cells has the objective of contributing to prognosis and treatment. The aim of this study was to evaluate the relationship between mast cells and angiogenesis in benign and malignant mammary neoplasms by investigating the role of degranulation and microlocation of mast cells and neoformed vessels in canine mammary neoplasms.

***Materials, Methods & Results*:** Mammary glands (n = 122) from 50 female dogs submitted to mastectomy without chemotherapy were evaluated and categorized into 3 groups: control group (n = 46); malignant group (n = 57) and benign group (n = 19). Lymph nodes without changes (n = 59) and with metastases (n = 6) were also evaluated. To evaluate the MCD (mast cell density) and angiogenesis, Toluidine Blue (0.1%) and Gomori's Trichrome techniques were performed and adapted from previous studies. Photomicrographs of 10 hotspot areas on a 40x objective lens of the mammary glands and lymph nodes were captured to assess MCD and angiogenesis. In the absence of these areas, random fields were captured. For the mammary glands of the malignant and benign groups, 20 fields were analyzed, as the analysis considered the microlocation (peritumoral and intratumoral). Counting was performed manually using ImageJ software version 1.42q by 2 observers. The statistical analysis were performed using SPSS software version 19.0. The most frequent histological type in the malignant group was carcinoma in mixed tumor (68.42%; 39/57) and in the benign group was benign mixed tumor (57.89%; 11/19). Female dogs without breed pattern were more frequently affected represented 70% of the animals and the mean age was 9 years and 8 months ± 3 years and 1 month. The granulated density of mast cells and peritumor vessels was higher in the malignant group ($P = 0.03$; $P = 0.02$). There was also a positive correlation between intratumor and total vessel density and mast cell density. There was no significance between the malignant and benign groups in regard with fibrosis density.

***Discussion*:** In this study were observed a greater density of blood vessels in malignant group, suggesting the participation of blood vessels for neoplastic proliferation. Furthermore, these vessels were located in the peritumoral region as in previous studies. The positive correlation between MCD and blood vessels was similar to a previous study performed in canine breast carcinomas and breast cancer in women. Regarding microlocation, another study also found higher MCD in the peritumoral region than in the intratumoral region of canine carcinomas. Although there are already studies for this purpose in cases of oral squamous cell carcinoma in humans, we believe this is the first study to investigate the role of mast cell degranulation in mammary neoplasm of bitches. The MCD was not significant among the malignant and benign groups and in the mammary glands of the control group the MCD was higher, as observed by other studies. Future studies should be associated the survival time and the presence of metastases in order to confirm the findings. In view of these findings, we may conclude that a higher density of mast cells is related to a higher density of blood vessels and that these are more abundant in malignant neoplasms, which reinforces the crucial role of angiogenesis in the neoplastic development.

Keywords: tumor microenvironment, mammary tumor, mast cells, angiogenesis, bitches.

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INTRODUCTION

The impact of stromal components on the development and progression of neoplasms has been explored by studying the tumor microenvironment [6]. Most studies in breast neoplasms of women and in female dogs showed high MCD (mast cell density) associated with favorable prognoses [1,23]. However, other studies in women show the contribution of MCD in unfavorable prognoses [10]. In addition, there seems to be a difference in the microlocation of mast cells, where in prostate cancer in men, high intratumoral MCD resulted in better prognosis [9]. Conversely, there was a positive correlation between higher intratumoral MCD and vascular and lymphatic invasion in women breast carcinoma [10]. The role of mast cells remains uncertain in canine mammary neoplasms and the evaluation of microlocation was performed in only 2 studies [1,19].

Studies showed a positive correlation between mast cell density and microvessel density in canine mammary neoplasms [7,13]. Therefore, the density of microvessels may be used as a parameter for malignancy in veterinary as it is in human medicine [17]. Previous studies have not considered the microlocation of vessels in the microenvironment. Another important factor in mammary tumor microenvironment is tumor fibrosis, where a higher percentage was associated with more aggressive tumors and lower survival rates [11,21]. However, no mammary glands without changes were evaluated in these studies. The aim of this study was to evaluate the MCD, blood vessels and fibrosis of the microenvironment in benign and malignant canine mammary neoplasms, in order to find answers that may support the evaluation of these parameters in histopathological analyses.

MATERIALS AND METHODS

Sample collection and processing

The sample of the present study comprised 122 mammary glands samples and 65 regional lymph nodes of 50 female dogs of different breed and ages, submitted to mastectomy without previous chemotherapy, were selected.

The material was stored in 10% buffered formaldehyde, embedded in paraffin, cut at 3 µm, stained in hematoxylin and eosin (HE)[1] and evaluated by light microscopy. All mammary glands were evaluated, even in the absence of macroscopic alterations.

After histopathological examination, the samples were classified into groups: mammary glands without microscopic alterations (control group: n = 46), with benign neoplasms (benign group: n = 19) and with malignant neoplasms (malignant group: n = 57); lymph nodes without microscopic alterations (control lymph node group: n = 59) and with the presence of neoplastic cells (metastatic lymph node group: n= 6). Primary neoplasms were grouped according to the classification for canine mammary tumors [3]. Then, the most representative block of each sample was selected and submitted to serial cuts to be used for histochemical techniques.

Evaluation of MCD and blood vessels

The mast cells were counted in all samples, while the vessels were counted only in the neoplastic mammary glands (malignant and benign group) and the percentage of dense connective tissue in all mammary glands (control, malignant and benign groups). Toluidine Blue (1%)[2] and Gomori's Trichrome[3] techniques were performed to evaluate the MCD and angiogenesis, respectively, with methodology adapted from previous studies conducted in mouse tissue [16,18]. The 10x objective lens was used to identify 10 *hotspot* areas and the 40x objective lens was used to capture the photomicrography of the chosen fields. In the absence of these areas, random fields were selected. The images were captured in a camera coupled to an optical microscope[4] with the aid of Image-Pro Express software version 6.3[5]. This analysis was performed for mammary glands of the control group and lymph nodes. In mammary glands of the malignant and benign groups, mast cell and blood vessel densities were calculated also considering peritumoral and intratumoral microlocalization, resulting in 20 fields analyzed per sample.

In addition, for MCD, the differential count was also considered, i.e., the count of granulated and degranulated mast cells differentiated by morphology following the criteria described above [5]. As for angiogenesis, the blood vessels were considered as stained structures in longitudinal and transverse sections by morphological identification of endothelial cells, in addition to the presence of erythrocyte and/or white cell inside the lumen. Incomplete or mature vessels were not counted. The mast cell and vessels count were

performed manually with the aid of ImageJ software version 1.42q[6] by 2 observers, who counted at least twice each photomicrography (Figure 1). For each case, MCD and blood vessel density were expressed by the median of mast cells and vessels per analyzed field. For the analysis of the percentage of dense connective tissue and fibrosis, a method already recommended was performed [21]. Gomori's Trichrome showed collagen fibers in the mammary glands of the control, malignant and benign groups in green color (Figure 2).

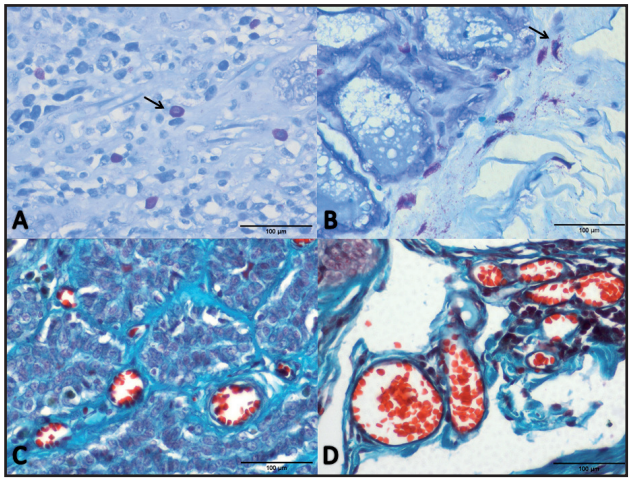

Figure 1. Photomicrographs of canine mammary glands for evaluation of MCD and blood vessel density [40x]. Toluidine blue: A- Mammary gland of the malignant group - granulated mast cell (black arrow); B- Mammary gland of the control group - mast cells in degranulation (black arrow) note the free granules in the tissue. Gomori's trichrome: C- Mammary gland of the benign group - intratumor vessels; D- Mammary gland of the malignant group - peritumoral vessels.

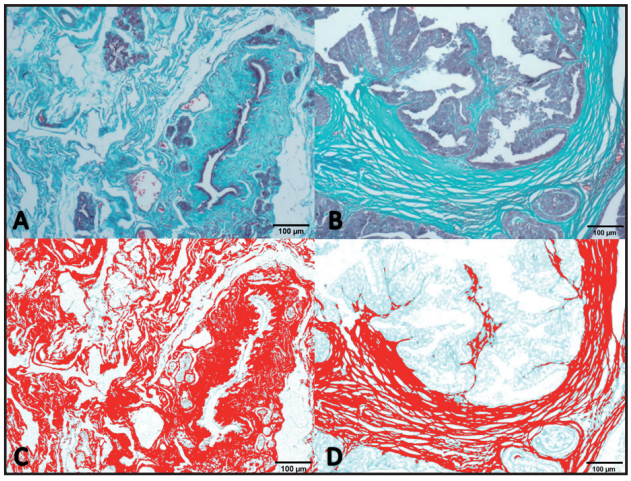

Figure 2. Photomicrographs showing areas of dense connective tissue and fibrosis by Gomori's thricrome histochemistry technique in canine mammary glands [10x]. A- Mammary gland of the control group. B- Mammary gland of the malignant group, papillary carcinoma. Areas of dense connective tissue (C) and fibrosis (D) corresponding to the previous images, evidenced in red with the aid of the "Colour deconvolution - threshold" tool of the ImageJ software.

Statistical analysis

The analyzed variables were densities of total, granulated and degranulated mast cells, microlocation of granular and degranulated mast cells, density of blood vessels and percentage of dense connective tissue and fibrosis. Mast cell density, mast cell microlocation and blood vessel density were compared between groups using Kruskal-Wallis and Mann-Whitney tests to assess the significance between pairs of groups. The percentage of dense connective tissue and fibrosis were compared between the groups by analysis of variance of 1 factor; Turkey test was used to verify the difference between the groups. The relationship between density and microlocation of mast cells and vascular density and microlocation was calculated by Spearman's correlation. In addition, the correlation between the percentage of dense and fibrous connective tissue and the greatest measurement of the tumor was evaluated by Pearson correlation. Continuous data were presented as mean ± standard error. Differences with $P \leq 0.05$ were considered statistically significant. Statistical analysis were performed using the SPSS software (version 19.0)[7], and the data distribution was evaluated by the Shapiro-Wilks test.

RESULTS

In the malignant group, the most frequent histological type was carcinoma in mixed tumor (68.42%; 39/57), followed by other subtypes of carcinomas, being 10 papillary carcinomas, 2 solid carcinomas, 2 cribriform carcinomas, 1 carcinoma *in situ* and 1 micropapillary carcinoma, in addition to 1 malignant adenomyoepithelioma and 1 malignant phylodes. Regarding the benign group, the benign mixed tumor was the most common (57.89%; 11/19), being that 5 cases of papilloma and 3 cases of adenoma were also diagnosed. Middle-aged and older bitches were more frequently affected (mean of 9 years and 8 months ± 3 years and 1 month) and those without defined breed pattern represented 70.0% of the animals. Regarding

the tumor characteristics, most tumors were located in the last 2 pairs of mammary glands, caudal abdominal (32.9%) and inguinal (35.5%). T1 tumors (< 3cm) represented 67,1%; T2 (3-5cm) - 14,5% and T3 (> 5cm) - 18.4%.

MCD was higher in the mammary glands of the control group than in the benign and malignant groups ($P < 0.001$). For the differential count, i.e., between granular and degranulated mast cells, the MCD was similar between the groups. Total and differential MCD did not differ between control and metastases lymph node groups (Table 1). Table 2 represents the MCD regarding peritumoral and intratumoral microlocation, revealing that granulated MCD was higher in the peritumoral region of the mammary glands of the malignant group ($P = 0.03$). The dense connective tissue occupied a greater area in the mammary glands of the control group when compared to the benign and malignant groups that had a smaller area of fibrous tissue ($P < 0.001$; Table 3).

The mammary neoplasms of the malignant group presented higher density of blood vessels when compared to the benign group (5.32 ± 0.26; 4.16 ± 0.41; $P = 0.042$). Regarding the microlocation of vessels, there was a higher peritumoral density in the malignant group ($P = 0.02$). There was no difference in the density of intratumoral blood vessels ($P = 0.17$; Table 4). A significant weak positive correlation was identified between total vessel density and total MCD, total and peritumoral granular MCD; also between the density of intratumoral blood vessels and total MCD, total granular MCD, peritumoral granular MCD, total degranulated MCD, intratumoral degranulated MCD and peritumoral degranulated MCD (Table 5). There was no correlation between the percentage of connective and fibrous tissue and the greatest measurement of the tumor ($P = 0.77$).

Table 1. Evaluation of total and differential MCD in mammary glands of the control, malignant and benign groups and lymph nodes of the control and metastasis groups.

	n	Mean ± Standard error	*P*
Total MCD			
Control	46	5.56 ± 0.48^{a}	
Benign	19	2.19 ± 0.59^{b}	< 0.001
Malignant	57	2.54 ± 0.27^{b}	
Control Lymph Node	59	1.01 ± 0.16	0.48
Lymph Node Metastasis	6	2.37 ± 1.28	
Granular MCD			
Control	46	2.84 ± 0.25	
Benign	19	1.99 ± 0.52	0.21
Malignant	57	2.58 ± 0.28	
Control Lymph Node	59	0.54 ± 0.09	0.76
Lymph Node Metastasis	6	0.78 ± 0.39	
Degranulated MCD			
Control	46	2.71 ± 0.27	
Benign	19	2.46 ± 0.67	0.44
Malignant	57	2.58 ± 0.29	
Control Lymph Node	59	0.49 ± 0.08	0.76
Lymph Node Metastasis	6	1.58 ± 0.90	

Different letters differ statistically.

Table 2. Evaluation of MCD in mammary glands of the malignant and benign groups regarding microlocation.

	Degranulated		*P*	Granulated		*P*
	Intratumoral	Peritumoral		Intratumoral	Peritumoral	
Benign	1.33 ± 0.44	1.12 ± 0.26	0.56	0.94 ± 0.30[b]	1.04 ± 0.27[ab]	0.03
Malignant	1.27 ± 0.21	1.31 ± 0.14		1.15 ± 0.19[b]	1.42 ± 0.15[a]	

Different letters differ statistically.

Table 3. Evaluation of the percentage of dense connective tissue and fibrosis in mammary glands of the control, malignant and benign groups.

Groups	n	Mean (%)
Control	46	46.82 ± 1.43[a]
Benign group	19	31.51 ± 2.25[b]
Malignant group	57	31.86 ± 1.31[b]

Different letters differ statistically.

Table 4. Evaluation of vessel density in mammary glands of the malignant and benign groups regarding microlocation.

	n	Mean ± Standard error	*P*
Peritumoral vascular density			
Benign	19	3.9 ± 0.49	0.02
Malignant	57	5.38 ± 0.30	
Intratumoral vascular density			
Benign	19	4.44 ± 0.53	0.17
Malignant	57	5.27 ± 0.31	

Table 5. Correlations between vessels located in the intratumoral, peritumoral and total area with mast cells [total, intratumoral (INTRA DEGR) and peritumoral degranulated, intratumoral and peritumoral granulated, total granulated and total degranulated).

Mast cell \ Density Blood vessel		Intratumoral	Peritumoral	Total
Total	r	0.342	0.124	0.225
	P	0.0001	0.289	0.05
Intratumoral degranulated	r	0.260	0.083	0.152
	P	0.024	0.478	0.192
Peritumoral degranulated	r	0.267	0.083	0.166
	P	0.021	0.478	0.154
Intratumoral granulated	r	0.226	0.064	0.121
	P	0.05	0.583	0.301
Peritumoral Granulated	r	0.322	0.186	0.256
	P	0.005	0.110	0.027
Total Degranulated	r	0.319	0.106	0.215
	P	0.005	0.365	0.06
Total Granulated	r	0.359	0.181	0.259
	P	0.002	0.121	0.025

DISCUSSION

The MCD was higher in the mammary glands of the control group, as it was found in a previous study conducted in bitches [1]. The MCD in the present study was not significant among the malignant and benign groups as observed by other authors in previous studies with mammary cancer of bitches [13]. However, it was different from other studies that observed higher MCD in mammary carcinomas, preneoplastic lesions and invasive areas [7,19]. In women, MCD increases in proportion to the increased degree of breast carcinomas malignancy [4]. These findings suggest the participation of mast cells for the development of malignant mammary neoplasms. However, due to the versatility of mast cells in different studies conducted with the most diverse types of neoplasms, we cannot conclude the real role of these cells in the tumor microenvironment considering that studies in dogs are still insufficient.

Differential MCD, i.e., granulated and degranulated between groups was also not significant. We believe that this is the first study to investigate the role of degranulation in mammary neoplasms of female dogs. In human medicine, cases of oral squamous cell carcinoma demonstrated higher degranulated MCD in the neoplastic group when compared to the control group [24]. The significance of MCD in control and metastatic lymph nodes was also not observed. However, it is difficult to find studies that investigate the MCD in lymph nodes in the literature. In women breast cancer, MCD was higher in normal axillary lymph nodes when compared to metastatic ones, suggesting prognostic significance [8].

Regarding the microlocation of mast cells, there was a higher density of granules in the peritumoral region of the malignant group when compared with the peritumoral region of the other groups. Degranulated mast cells, on the other hand, were not significant between the groups. No records that investigated the degranulation in canine mammary neoplasms were found in the literature. In women breast cancer, there was higher degranulated MCD in peritumor and non-tumor tissues [14]. Considering only the microlocation, another study also found higher MCD in the peritumoral region than in the intratumoral region in canine carcinomas [19]. In women, there was a higher MCD in intratumoral regions in more aggressive tumors with a positive correlation for prognostic parameters [2,10].

The present study observed higher density of blood vessels in malignant neoplasms, as observed in a previous study, inferring the collaboration of vessels for neoplastic proliferation [17]. In the malignant group, a higher density of blood vessels was observed in the peritumoral region, coinciding with previous studies also conducted in female dogs [20]. The density of microvessels is considered an indicator of malignancy for mammary neoplasms of female dogs and women [12]. To affirm the findings of the present study, we suggest to associate these findings with survival time and presence of regional and distant metastasis. Finally, the positive correlation between MCD and blood vessels was similar to a previous study conducted in canine mammary carcinomas and women breast cancer [7,13,15], although this did not consider degranulation. Tumor-associated mast cells seem to release granular components that potentiate angiogenesis [24].

Regarding the analysis of fibrosis, no significance was observed between malignant and benign groups, as demonstrated previously [22]. However, it disagrees with previous studies conducted in female dogs and women that found a higher percentage of fibrosis in neoplasms with worse prognosis, suggesting the possibility of using this analysis as an independent prognostic factor [11,21]. These studies did not evaluate healthy mammary glands. In addition, they had a different purpose from the present study, because it compared fibrosis between four histological types in order to correlate larger areas with worse prognoses [21]. The higher proportion of dense connective tissue in the control group may be explained by the fact that healthy mammary glands have a higher relationship between stroma and epithelial cells, unlike neoplasms that have a higher proportion of proliferated epithelial cells in relation to the stroma.

CONCLUSIONS

Our study confirmed the correlation between mast cells and blood vessels in canine mammary tumors, evidencing the contribution of angiogenesis to the development of especially malignant neoplasms. Future studies that can evaluate the microenvironment, encompassing a greater number of cases and associating with clinical characteristics such as overall survival and presence of distant metastasis may reveal other findings. Studies addressing the microenvironment of canine mammary neoplasms are still scarce in regard with the functional versatility of mast cells. The use of histochemical techniques may make the evaluation easier to perform and these can be performed in the diagnostic routine adding information on the possible behavior of these neoplasms.

MANUFACTURERS

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***Ethical approval*.** This study was approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Lavras (UFLA) under protocol number 022/19.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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