Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.277

RESEARCH ARTICLE

Antitumorogenic Effect of Mast Cells: Insights from an Experimentally-Induced Mammary Carcinoma Model in Rats and Feline and Canine Mammary Tumors

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ARTICLE HISTORY (24-471)

Received: September 8, 2024
Revised: October 16, 2024
Accepted: October 22, 2024
Published online: November 18, 2024

Key words:
7, 12-dmba
Chymase
Mammary carcinoma
Mast cells
Tryptase

ABSTRACT

Breast cancer in humans and mammary tumors in cats and dogs is one of the most important types of cancer and causes serious losses. Early diagnosis is crucial, and the treatment protocols are often complex, expensive, and inconclusive. Mast cells are considered among the important components of the immune system and have been documented to show a significant increase in cancer tissues, however their possible roles and their phenotypes in cancer are not precisely known. In this study, we examined the immunophenotypes of mast cells and their potential role in naturally occurring feline and canine mammary tumors through an experimental mammary cancer model induced by 7, 12-dimethylbenzanthracene (DMBA) in rats. The study also questioned expressions of TNF-alpha, MMP-9, and PCNA, and their possible relationship with mast cells. Mast cell count and both chymase- and tryptase-positive mast cells were increased in tumor tissues from all three species compared to the control mammary tissues. Degranulated mast cells were more common in intratumoral areas, and granulated mast cells were more common in peritumoral areas. In mammary tumors of rats, expression of PCNA correlated negatively with mast cell count; in dogs and cats, a correlation was seen, but could not be statistically substantiated. In conclusion, the increase in TNF-α, the decrease in MMP-9, and the negative correlation observed between PCNA and mast cell count indicated that an increase in mast cell count may have an anti-tumorigenic effect in mammary tumors. As a conclusion of the study, the number, localization, granulation status, and immunophenotypic characteristics of mast cells and their possible roles in mammary tumors of cats, dogs, and rats were investigated, and it is suggested that mast cells may play an important role in mammary tumors and may prove to be valuable prognostic markers.

To Cite This Article: Yavas O, Yavas SE, Cangul IT and Sonmez G, 2024. Antitumorogenic effect of mast cells: insights from an experimentally-induced mammary carcinoma model in rats and feline and canine mammary tumors. Pak Vet J, 44(4): 1131-1141. http://dx.doi.org/10.29261/pakvetj/2024.277

INTRODUCTION

Mammary tumors are among the most common tumors in cats, dogs, and humans. (Meuten, 2016). They are the most commonly encountered tumors in dogs and constitute approximately 50% of all tumors (Canadas *et al*., 2019). Feline mammary tumors are the third most common tumor observed in cats, comprising 17% of all tumors (Dagher *et al*., 2019). Breast cancer in humans is the most frequently diagnosed cancer among women (154/185), representing ¼ of all cancers diagnosed in women (Giaquinto *et al.*, 2024). Recently reported a study, the incidence of canine mammary tumors as 1340.7 per 100.000 dogs in UK (Varney *et al.*, 2023). In a recent study in cats, the incidence of feline mammary tumors was 104 per 100.000, lower than in dogs (Pickard *et al.*, 2023). The most important risk factors for mammary tumors in domestic animals are reported to be age, breed, genetic predisposition, sex hormones, diet, and COX-2 expression (Goldschmidt *et al.*, 2011; Sorenmo *et al.*, 2011). The possibility of malignant mammary tumors in domestic animals has been increasing in recent years. Especially in dogs, the incidence of malignant mammary tumors, which is known to be 50%, has increased in recent years (Salas *et al.*, 2015). Although clinical staging and histological grading help standardize treatment protocols, precise treatments are not available in dogs and cats with mammary tumors. Therefore, more than 40% of domestic animals with mammary tumors die within

one year of diagnosis (Nguyen *et al.*, 2018). This difficulty is leading to novel anticancer target cell therapies in domestic animals mammary tumors (Ke *et al.*, 2024). Therefore, cells such as mast cells are involved in biological activities that may be anti-tumorigenic and/or pro-tumorigenic. For these reasons, in current literature, tumor mast cells have been recognized as a new therapeutic target to prevent tumor progression and metastatic process (Ribatti, 2024).

The mast cell is a tissue-resident innate immune cell that plays an important role in the inflammatory response but also contributes to various immune-mediated disorders such as allergic reactions, autoimmune diseases, and cancer (Noto *et al.*, 2021; Burchett *et al.*, 2022). Mast cells in rodents are divided into 2 types according to their protease content and staining properties; these are mucosal mast cells (MMC) and connective tissue mast cells (CTMC). MMC granules predominantly include chymases (mMCP-1 and mMCP-2). Granules of CTMCs contain serglycin (Xing *et al.*, 2011). These mast cells include chymases, tryptases, and carboxypeptidase A. Mast cells can be isolated from the skin, peritoneal cavity, and intestinal submucosa (da Silva *et al*., 2014). In humans, mast cells are divided into 3 groups according to their protease content: tryptase, chymase, and both. Mast cells containing only tryptase are known as "MC-T", while those containing both tryptase and chymase are identified as "MC-TC," (Schwartz, 2006; Pejler *et al*., 2010).

Mast cells concentrate in the peritumoral or intratumoral area of different human cancer types, but there is still controversy about the increase of this cell to tumor progression (Aponte-Lopez *et al*., 2018; Ribatti, 2024). Increased quantity of mast cells has been associated with better or poor prognosis depending on the cancer type and grade for several tumors. Still, the relation between breast cancer and mast cells remains unclear (Aponte-Lopez *et al*., 2018). Some researchers have accepted mast cell infiltration in these cancers as a useful prognostic factor due to its anti-tumoral effect (della Rovere *et al*., 2007; Sang *et al*., 2016; Glajcar *et al*., 2017; Fereydouni *et al.*, 2022; Ribatti 2023), while others proposed that the existence of mast cells in breast cancers causes a pro-tumoral effect and is an adverse prognostic factor (Marech *et al*., 2014; Fakhrjou *et al*., 2016; Keser *et al*., 2017; Aponte-Lopez *et al*., 2018; Carpenco *et al*., 2019; Reddy *et al*., 2019; Segura-Villalobos *et al.*, 2022). It is known that chymase-positive and tryptase-positive mast cells increase in lung cancers (Longo *et al.*, 2022), B-cell non-Hodgkin lymphomas (Ribatti *et al*., 2010), brain (Nico *et al*., 2004), and cervical cancers (Taverna *et al*., 2013), leukemias (Ribatti *et al*., 2003), melanomas (Ribatti *et al*., 2003), hepatocellular carcinomas (Goffredo *et al*., 2013), gastrointestinal cancers (Ammendola *et al*., 2013), skin (Korhonen *et al.*, 2024) and prostate cancers (Ma *et al*., 2018).

The most widely used chemical carcinogenic in experimental mammary tumor models is 7, 12-dimethylbenzanthracene (DMBA) in the highly susceptible female Spraque-Dawley rats (Costa *et al*., 2004). DMBA is a synthetic polycyclic aromatic carbon that acts by initiating or promoting mutations in genes that are responsible for carcinogenesis (Vellaichamy *et al*., 2009). Curative effects of medroxyprogesterone acetate (MPA) have been announced in carcinogen-induced rat mammary tumors (Kiss *et al*., 1986). In DMBA-induced mammary carcinomas, subsequent progesterone administration is known to cause a more aggressive mammary tumor (Benakanakere *et al*., 2010). Therefore, in this study, we wanted to compare mast cells in mammary tumors with and without progesterone treatment by dividing the experimental model into two.

There is a paucity of knowledge on the relationship between mammary tumors and mast cells in veterinary medicine. Mast cells have been presented to raise in number in canine mammary tumors. However, their expression of tryptase or chymase has not yet been demonstrated. It is also unknown whether they increase the number of feline mammary tumors. This study was conducted to examine the presence, localization, granulation status and immunophenotype (tryptase or chymase positive) of mast cells in experimentally generated mammary tumors in rats (24 in total) and cat and dog mammary tumors (10 cats, 10 dogs) and compared them with healthy mammary glands.

MATERIALS AND METHODS

Animals: Forty-five 6-week-old female Sprague-Dawley rats weighing approximately 300g body weight were used. Animals were kept in a room set on a 12h light/12h dark cycle at an average temperature of 22°C and had *ad libitum* access to rat chow and water. Beginning by the 45th day after the application described below, a clipper was used daily to examine the experimentally-induced tumor size. When the tumor diameter reached 3 cm, the animal was euthanized, and the tumors were removed.

Group G (Gavage Group) (n=20): In this group, 20 mg of 7, 12-DMBA (Cayman Chemical, 57-97-6, 30383, Ann Arbor, USA) was dissolved in 1ml of cottonseed oil and administered orally by gavage to 20 female Sprague Dawley rats (Benakanakere *et al*., 2010). Afterward, the rats were followed daily, and no application was made until the mammary tumor reached a diameter of 3cm.

Group I (Injection Group) (n=20): In this group, 20 Sprague-Dawley female rats were administered once orally with 20mg of DMBA dissolved in 1ml of cottonseed oil and also with a single dose of 25mg medroxyprogesterone acetate (Depo-Provera, Pfizer) subcutaneously four weeks after the gavage application (Benakanakere *et al*., 2010). Afterwards, the rats were followed daily, and no application was made until the mammary tumor reached a diameter of 3cm.

Group C (Control Group) (n=5): No application was made in this group. One mammary lobe was collected from each rat and used as control mammary tissue.

Collection of Mammary Tumor Tissues from Cats and Dogs: Ten cases from cats and dogs were selected from the operated mammary carcinoma cases referred to the Department of Veterinary Obstetrics and Gynecology, Bursa Uludag University, and private veterinary clinics.

Control mammary tissues were collected from fresh cadavers of five female cats and five female dogs older than

one-year-old and not neutered and brought to the Veterinary Pathology Department, Bursa Uludag University, Turkey for necropsy.

Histopathological Examinations: Freshly taken mammary tumor tissues were fixed in 4% paraformaldehyde solution at 4°C for two days. After fixation, the samples were embedded in paraffin blocks. Consecutive 4μm sections were stained with hematoxylin-eosin and toluidine blue stains. In toluidine blue stained slides, ten areas were selected, and the number of total mast cells, granulated/degranulated mast cells, and intratumoral/peritumoral mast cells were counted under x200 magnification by two researchers. Mast cells in the center of the tumor tissue and outside the tumor margin by a maximum of one high magnification field (0.2 mm^2 field area) were considered intratumoral, and those outside one high magnification field of the tumor margin and up to two high magnification fields were regarded as peritumoral mast cells (Glajcar *et al*., 2017).

Immunohistochemical Examinations: All tissues collected and examined in this study were stained using the streptavidin-peroxidase technique with commercially available monoclonal antibodies developed against anti-chymase, anti-tryptase, anti-MMP-9, anti-TNF-α, and anti-PCNA antigens. 4μm sections were taken on glass slides from tissues fixed with 4% paraformaldehyde for 48h and later embedded in paraffin. After deparaffinization in xylene, slides were rehydrated through graded alcohols and placed in distilled water. Samples were boiled in citrate buffer (pH 6.0) in a microwave at 600 watts for 10 minutes for antigen retrieval. Later, the endogenous peroxidase activity was blocked by 3% hydrogen peroxide. After protein block, slides were incubated with antibodies for chymase (CC1, Santa Cruz Biotechnology, 1:100), tryptase (AA1, Santa Cruz Biotechnology, 1:100), MMP-9 (E11, Santa Cruz Biotechnology, 1:100), TNF-α (52B83, Santa Cruz Biotechnology, 1:100), and PCNA (PC10, Santa Cruz Biotechnology, 1:200) with streptavidin-peroxidase polymer using Thermo Fisher Scientific DAB kit protocol (Thermo Fisher Scientific, Ultra Large Volume, TL-05-HDS, United Kingdom). 3,3'-diaminobenzidine tetrahydrochloride (DAB) was added to visualize the antibody-enzyme complex. Slides were then counterstained with Mayer's hematoxylin and visualized under a light microscope. Tryptase and chymase expressions were analyzed under a light microscope in 10 fields under x200 magnification. Control mammary tissues were also examined similarly, and tumor and normal mammary tissues were compared regarding mast cell counts, tryptase/chymase positive mast cell counts, and intratumoral/peritumoral mast cell counts. Areas with intense and multiple cell staining were selected at low magnification (x40) for the cell counts.

TNF-α and MMP-9 immunohistochemical expressions were scored by two researchers by multiplying the score of positive cell number with the score of staining intensity. The score of positive cells was calculated as follows: 4 points if there is more than 80% staining of cells, 3 points if there is staining between 51% and 80%, 2 points if there is staining between 10% and 50%, 1 point if there is less than 10% staining, 0 point if there is no staining. For staining intensity, the scoring was as follows: 0: no reaction, 1: weak positive, 2: medium-intensity, and 3: intense positive. According to this evaluation method, a score between 0 and 12 was obtained after the multiplication of the two scorings. A score of 9 or higher was considered a strong positive reaction; a score between 3 and 8 was considered a moderate positive reaction; a score less than three was considered a weak positive reaction; and 0 was considered a no reaction (Weigel *et al*., 2012).

In evaluating PCNA expression, the percentage of nuclear immunopositive cells was calculated by counting 500 cells (Weigel *et al*., 2012).

Statistical Analyses: SPSS version 28 program (IBM, USA) was used for the statistical analyses. Before all groups were compared regarding the statistical differences, the Shapiro-Wilk Test assessed whether the data were normally distributed. The data would follow the normal distribution if the values obtained with the Shapiro-Wilk test were lower than 0.05. One-way ANOVA test evaluated the differences between the three normally distributed groups. Levene's test was used to analyze whether the group variances showed a homogeneous distribution. If the distribution was homogeneous, one-way ANOVA was used. In cases of heterogeneous distribution, the same groups were analyzed with the Kruskal-Wallis test. A $P<0.05$ was used for statistical significance.

RESULTS

Gross Findings and Histopathological Diagnosis: Experimentally induced mammary tumors in rats were observed between 4-13 months (mean 8.5 months) after 7,12-DMBA administration in Group G, and tumors developed between 5-9 months (mean seven months) in Group I. Experimental mammary tumors could be created in 24 of a total of 40 rats. Information about the breed, age, and sterilization status of the dogs, cats, and rats, as well as the histopathological diagnosis of tumors as shown in Fig. 1.

Demonstration of Toluidine Blue Mast Cells: The numbers of toluidine blue-stained mast cells are presented in detail in Table 1. An increase in both coarse and degranulated mast cell numbers was observed in tumors of all three animal species compared to the control mammary tissues (Fig. 2). In all tumor cases, degranulated mast cell numbers were higher than the granulated, and the intratumoral mast cell numbers were higher than peritumoral mast cell counts (Fig. 2). In canine and feline mammary tumors, there was a positive correlation between the number of toluidine blue-positive degranulated mast cells and the intratumoral mast cell numbers (canine; *P: 0.007, R:+787;* feline*; P<0.001, R:+965*) (Fig. 3).

Immunohistochemical Analysis: Tryptase-positive mast cell counts are detailed in Table 2. The number of tryptase-positive mast cells increased numerically in all four tumor groups (Group G, Group I, canine, and feline tumor groups)

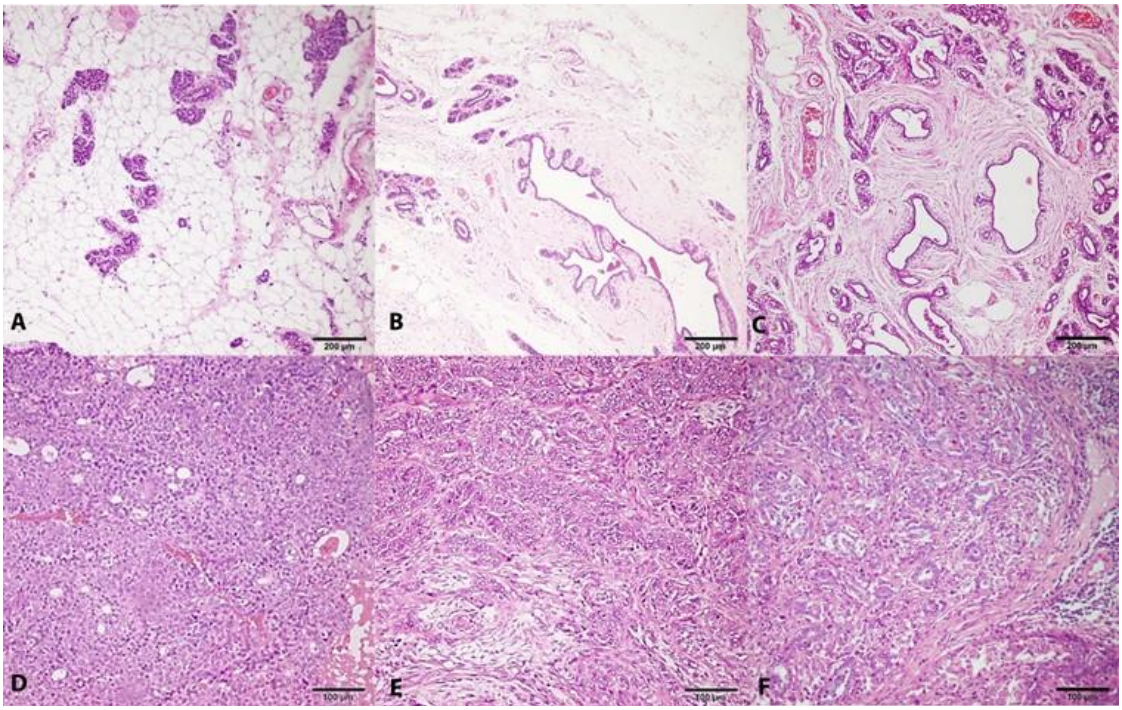

Fig. 1: Hematoxyline & Eosin staining. **A)** Control mammary tissue in rats. There is abundant adipose tissue and few mammary glands. **B)** Control mammary tissue in dogs. The tissue contains adipose tissue, connective tissue, and some glands and ducts. **C)** Control canine mammary tissue in cats. The tissue contains adipose tissue, connective tissue, and some glands and ducts. **D)** Solid carcinoma in rat mammary tissue. The neoplastic cells are arranged in solid cords and have small amounts of connective tissue stroma. **E)** Complex carcinoma in canine mammary tissue. Malignant epithelial components and benign myoepithelial components in neoplastic areas. **F)** Solid carcinoma in feline mammary tissue. The neoplastic cells are arranged in solid cords and have small amounts of connective tissue stroma.

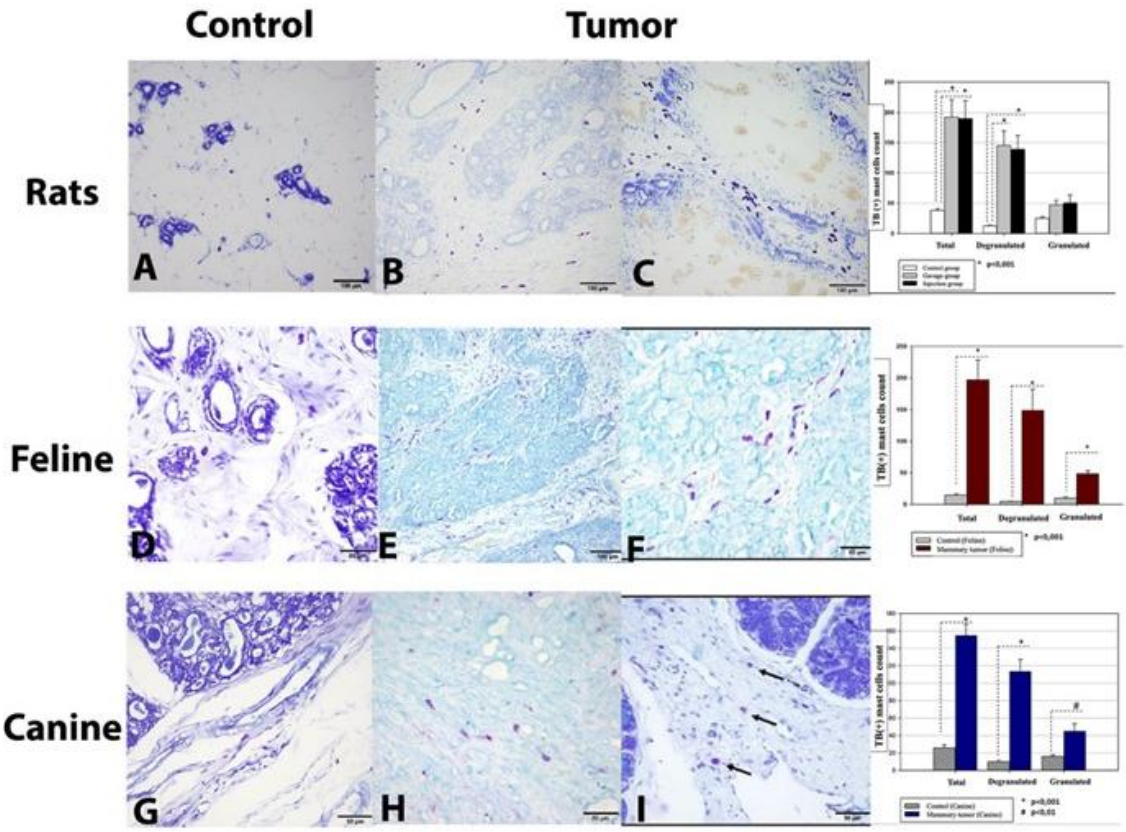

Fig. 2: Toluidine blue staining and statistical graph. **A)** Control mammary tissue in rats. Low number of mast cells are found around the mammary gland and fat tissue. **B)** High number of mast cells in rat mammary tumor tissue (Group I). Mast cells are observed both in peritumoral and intratumoral areas. **C)** High number of mast cells in rat mammary tumor tissue (Group G). Mast cells are seen among neoplastic cells and mammary glands. **D)** Control feline mammary tissue and a low number of mast cells around the mammary glands and fat tissue. **E)** High number of mast cells in the intratumoral area in feline mammary carcinoma tissue. **F)** High numbers of degranulated mast cells are observed in the intratumoral area in feline mammary carcinoma. **G)** Control canine mammary tissue and low mast cell number. **H)** High numbers of degranulated mast cells in the intratumoral area in canine mammary tissue. **I)** Granulated mast cells in the peritumoral area in canine mammary tissue (**arrows**: granulated mast cells).

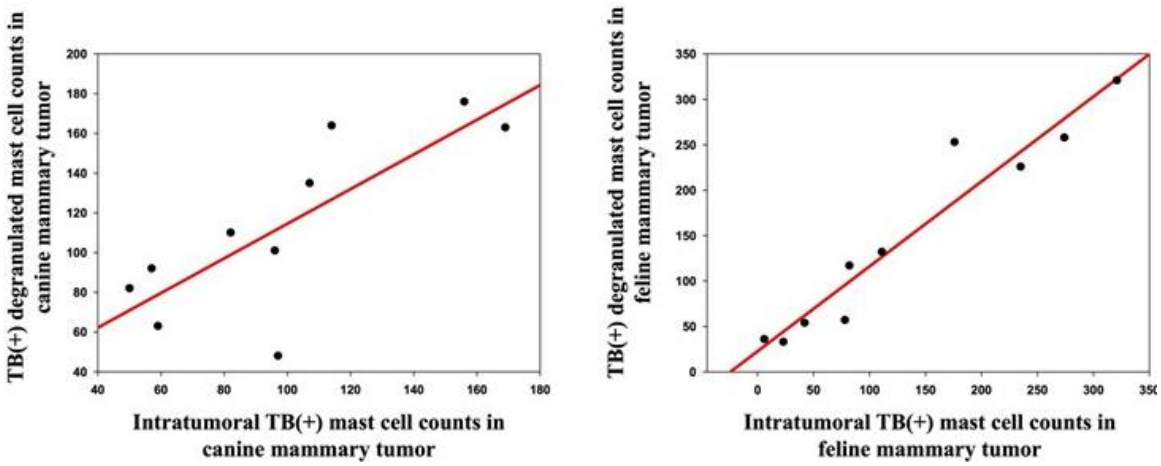

Fig. 3: Positive correlation graph between intratumoral toluidine blue-positive mast cells and degranulated mast cells in the canine mammary tumors (*P:0.007*; *R:+787*) and positive correlation graph between intratumoral toluidine blue-positive mast cells and degranulated mast cells in the feline mammary tumors (p<0.001; *R:+965*).

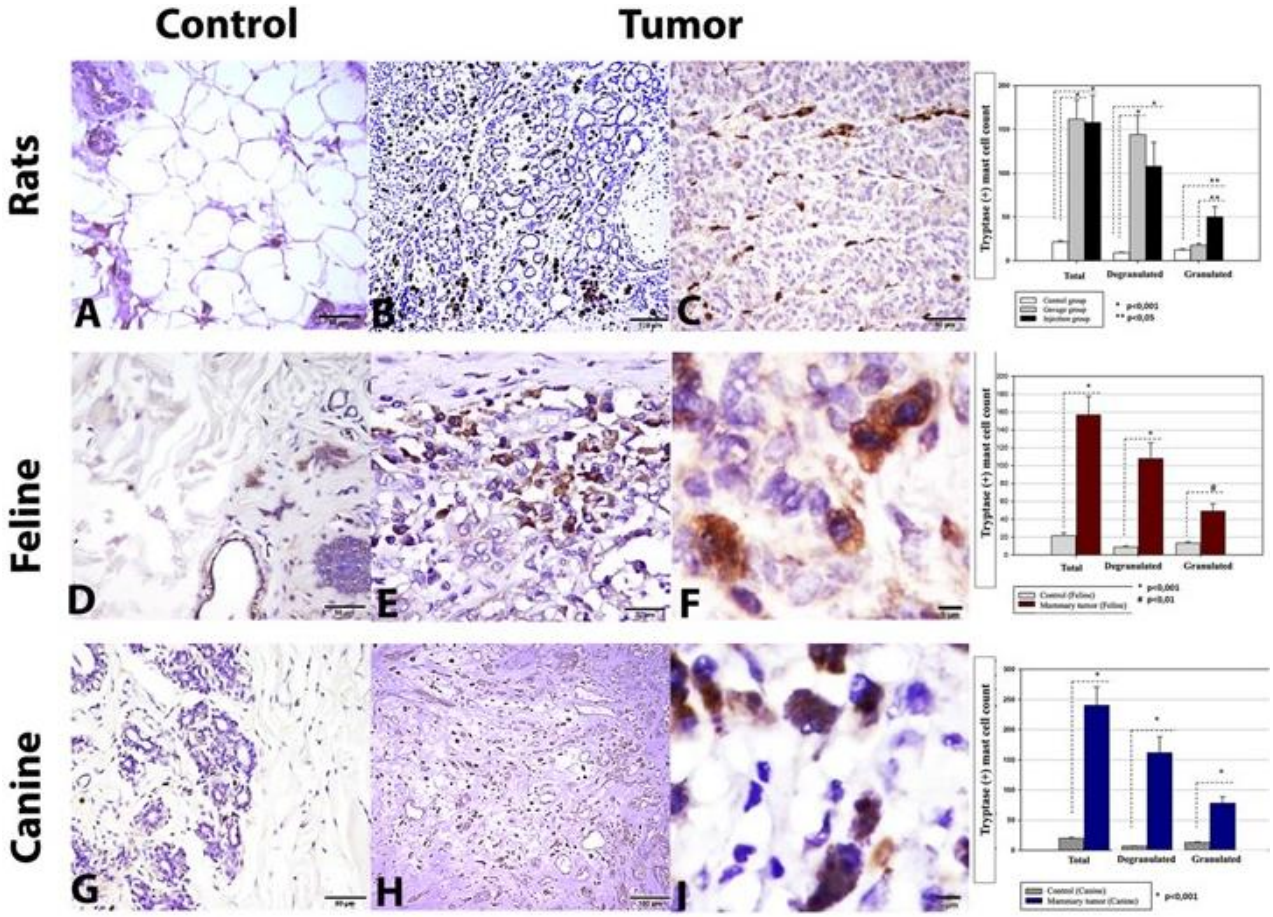

Fig. 4: Tryptase-positive mast cells, *streptavidin-peroxidase, DAB staining*. **A)** Control rat mammary tissue and tryptase-positive mast cells around the mammary gland and fat tissue. **B)** Tryptase-positive mast cells in rat mammary carcinoma in the intratumoral area (Group G). **C)** Tryptase-positive mast cells in rat mammary carcinoma in the intratumoral area (Group I). **D)** Control feline mammary tissue and low numbers of tryptase-positive mast cells around the vessels and mammary gland. **E)** High numbers of tryptase-positive mast cells among the neoplastic epithelial cells in the intratumoral area in the feline mammary tumor. **F)** Intratumoral-degranulated tryptase-positive mast cells in feline mammary tumors. These cells are among the neoplastic epithelial cells. **G)** Control canine mammary tissue and low number of tryptase-positive mast cells around the mammary glands. **H)** High number of tryptase-positive mast cells in intratumoral area in canine mammary tumor stroma. **I)** Degranulated mast cells in the intratumoral area in the canine mammary tumor.

compared to the control mammary tissues (Fig. 4), the difference being most prominent in canine mammary tumors. Intratumoral tryptase-positive mast cells were markedly more than peritumoral mast cells, with the canine tumors bearing the most pronounced difference. The highest peritumoral mast cell ratio within the total mast cell count was in feline mammary tumors. Additionally, a positive correlation was observed between the number of intratumoral mast cells and the number of degranulated mast cells in canine and feline mammary tumors (Fig. 5).

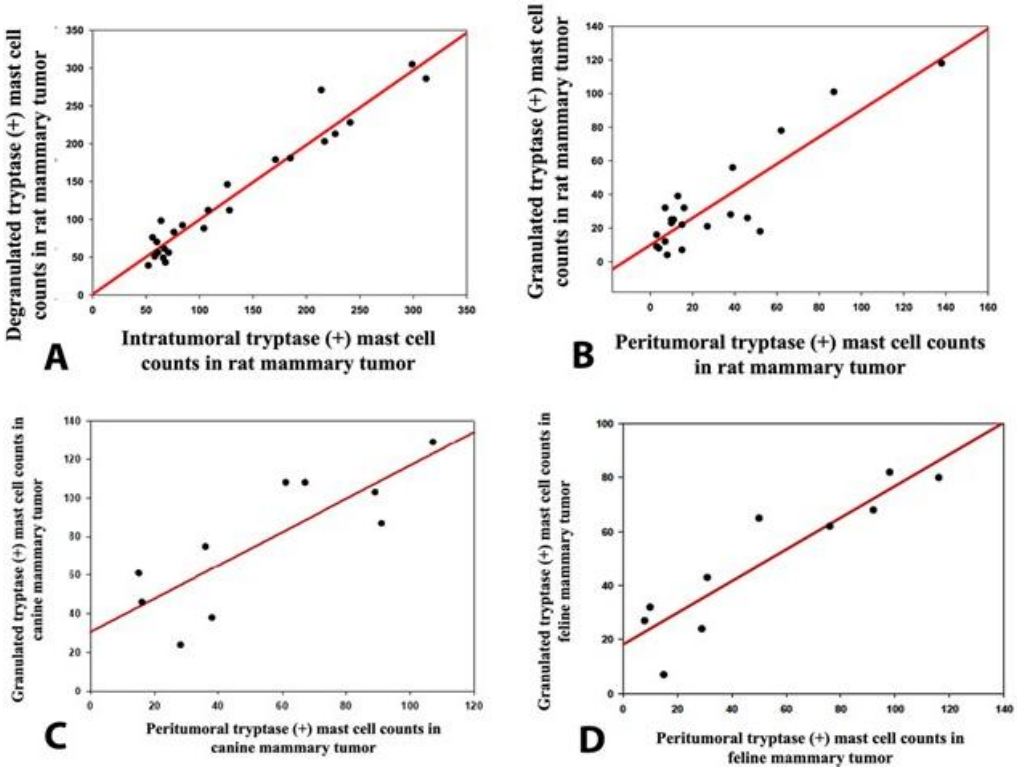

Fig. 5: A) Correlation graph showing the positive correlation between intratumoral tryptase-positive mast cells and degranulated tryptase-positive mast cells in rat mammary tumors (P<0.001; *R:+903*). **B)** Correlation graph showing the positive correlation between peritumoral tryptase-positive mast cells and granulated tryptase-positive mast cells in rat mammary tumors (*p<0.001*; *R:+805*). **C)** Correlation graph showing the positive correlation between peritumoral tryptase-positive mast cells and granulated tryptase-positive mast cells in canine mammary tumors (*P:0.004*; *R:+818*). **D)** Correlation graph showing the positive correlation between peritumoral tryptase-positive mast cells and granulated tryptase-positive mast cells in feline mammary tumors (*P<0.001*; *R:+909*).

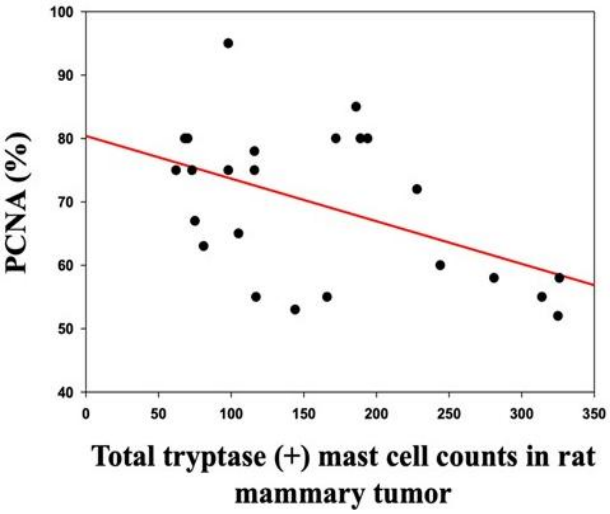

Fig. 6: Correlation graph showing the negative correlation between PCNA expression value and total tryptase-positive mast cell counts in rat mammary tumors (P:0.016; R:-487).

A positive correlation was observed between intratumoral tryptase-positive mast cells and the degranulated mast cells, and between the peritumoral tryptase-positive mast cells and granulated mast cells in the rat mammary tumor group (Fig. 5). In canine and feline mammary tumors, a positive correlation was observed between peritumoral tryptase-positive mast cells and granulated mast cells (P<0.001, R:+903; P<0.001, R:+805; P:0.004, R:+818; P<0.001, R:+909, respectively) (Fig. 5). A statistically significant negative correlation was found between the number of tryptase-positive mast cells and PCNA expression in rats (P:0.016, R:-487) (Fig. 6). In feline and canine, we observed a negative correlation between PCNA and tryptase-positive mast cells; however, this difference could not be substantiated.

The chymase-positive mast cell counts are detailed in Table 3. The numbers of chymase-positive tumoral mast cells in all four tumor groups were increased compared with the control mammary tissue (Fig. 7). Chymase-positive total mast cell count was highest in Group G and was lowest in feline mammary tumors (Table 3). The highest peritumoral mast cell ratio within the total mast cell count was observed in feline mammary tumors (Table 3, Fig. 8).

TNF-α, MMP-9, and PCNA values are given in detail in Figure 9. TNF-α values were higher than MMP-9 values in all groups. The lowest MMP-9 value was observed in rat mammary tumors without metastasis (0 in most rat mammary tumors). PCNA values in tumors were higher than the control group in all species (Fig. 9).

DISCUSSION

Besides being responsible for inflammatory and allergic reactions in humans and animals, mast cells are also known to increase in number in many tumor tissues, particularly in human breast cancers (Derakhshani *et al.*, 2019). However, the phenotypes of these cells and the reasons for their increase are still unknown (Aponte-Lopez *et al.*, 2018). In mast cell-related studies in humans, some studies have reported that increased mast cell count has a pro-tumorigenic effect, whereas others suggested an anti-tumoral action (Ribatti, 2024). Although studies on canine and feline mammary tumors are less numerous, an increase in the number of mast cells in canine mammary tumors has been documented (Sakalauskaitė *et al.*, 2022). However, these studies are both new and few. In addition, the phenotypes of increased mast cells in canine and feline mammary tumors and for what purpose they are present are not yet known.

Progesterone is an ovarian steroid hormone associated with normal mammary development during puberty and associated with preparation for breastfeeding during pregnancy. This hormone administration has been shown to

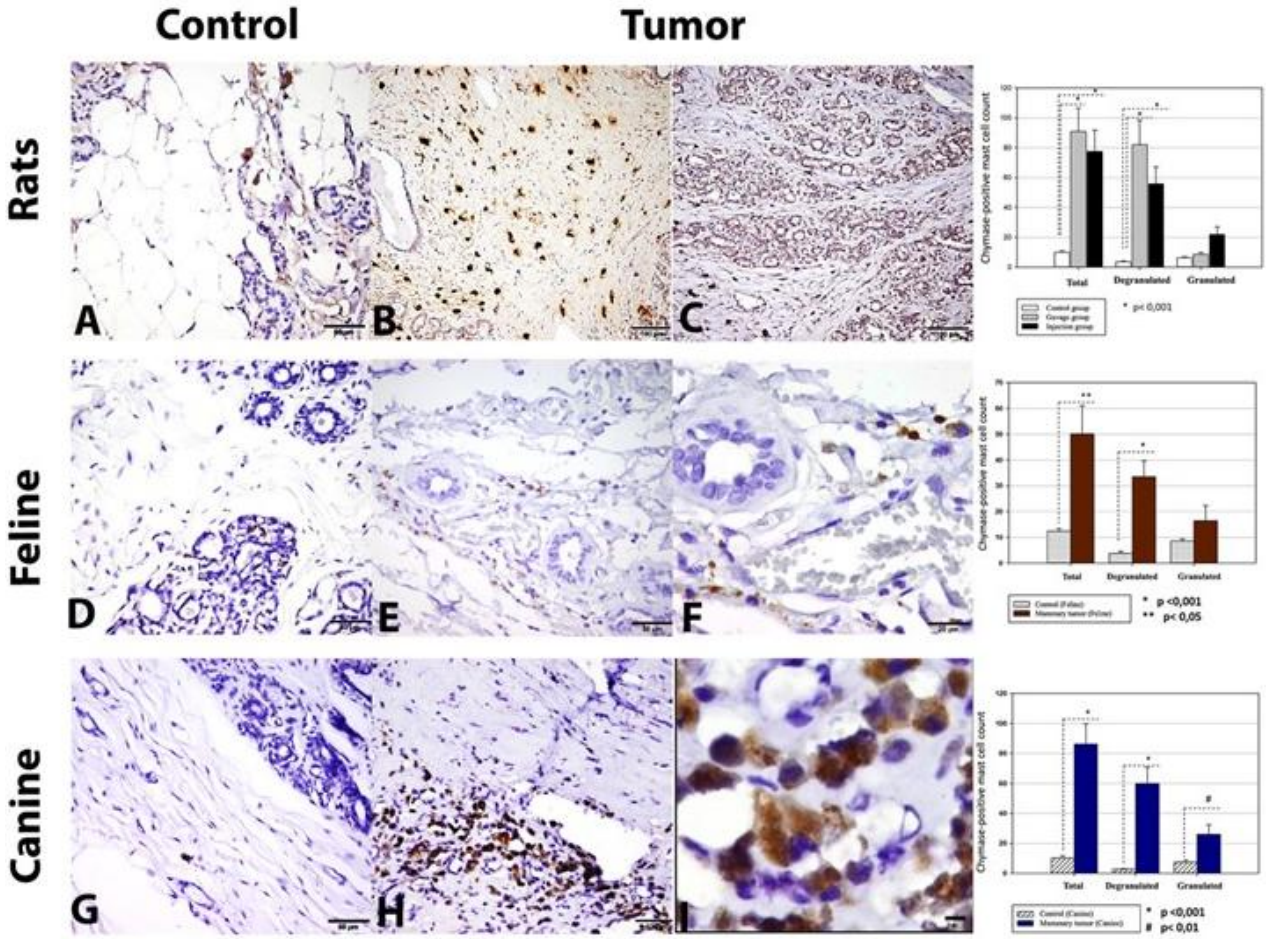

Fig. 7: Chymase-positive mast cells, *streptavidin-peroxidase, DAB staining,* and statistical graphs. **A)** Low number of chymase-positive mast cells around the fat tissue and mammary gland in control rat mammary tissue. **B)** High numbers of chymase-positive mast cells in the peritumoral and intratumoral area in rat mammary carcinoma (Group G). **C)** High numbers of chymase-positive mast cells in the intratumoral area around the neoplastic mammary glands in rat mammary carcinoma (Group I). **D)** Low number of chymase-positive mast cells around the vessels and mammary gland in control rat mammary tissue. **E)** High numbers of chymase-positive mast cells in feline mammary carcinoma in the peritumoral area around the vessels and mammary glands. **F)** High magnification of chymase-positive mast cells around the neoplastic mammary glands and blood vessels in feline mammary tissue. **G)** Low number of chymase-positive mast cells around the mammary gland in the control canine mammary tissue. **H)** High numbers of chymase-positive mast cells in the peritumoral area in the canine mammary carcinoma. **I)** High magnification of chymase-positive mast cells around the newly formed vessels in the intratumoral area in canine mammary tumors.

increase the rate of tumor formation, lead to a more malign character, and cause multiple tumors in a single animal in DMBA-induced mammary tumor models (Benakanakere *et al*., 2010). In our study, PCNA expression values were similar in the hormone-applied and not-applied groups but promoted the formation of multiple tumors. Although it is interesting that the PCNA values were almost the same, the observation of multiple tumors in the same animal in the progesterone group was in parallel with the above studies. Nevertheless, the same PCNA value is one of the handicaps of this study, and this caused us to not compare both G and I groups with each other in terms of aggressiveness.

There is still controversy regarding the increase of mast cell numbers in mammary tumors and their possible role. Although studies suggesting that mast cells have an anti-tumorogenic effect have become more critical in recent years, uncertainties on this subject continue. In the study of Glajcar *et al*. (2017), a negative correlation was observed between Ki-67 expression and tryptase-chymase positive mast cells, and the mast cells in the tumor area were proposed to have an anti-tumorogenic effect. In the study by Varricchi *et al*. (2017) on prostate cancers, the proliferation of mast cells in intratumoral areas prevented tumor growth, suggesting an anti-tumorogenic effect of these cells. della Rovera *et al*. (2007) and Shikotra *et al*. (2016) studied mast cells' anti-tumorogenic effect by establishing a correlation between TNF-α expression and mast cells. Our study revealed higher numbers of mast cells (both tryptase- and chymase-positive) in intratumoral areas of mammary tumors. These findings parallel the studies above. We also found that increased mast cell numbers in rat mammary tumors were negatively correlated with PCNA expression; a similar correlation was observed in dog and cat mammary tumors, but this correlation could not be statistically verified. The results of TNF-α and MMP-9 expressions were compatible with the above studies, proving further that the increase in mast cell numbers in these tumors has an anti-tumorogenic effect.

There are also many publications about the pro-tumorogenic effect of mast cells. These studies were generally conducted by associating the increase in mast cells with new vascular formation, increased MMP expression, presence in metastatic areas, or the presence of more mast cells in aggressive breast tumors (Kankkunen *et al*., 1997; Im *et al*., 2010; Ribatti & Crivellato, 2012; Marech *et al*., 2014; Keser *et al*., 2017). In the present study, mast cell numbers correlated with lower MMP-9 expression, contrary to the above-mentioned studies.

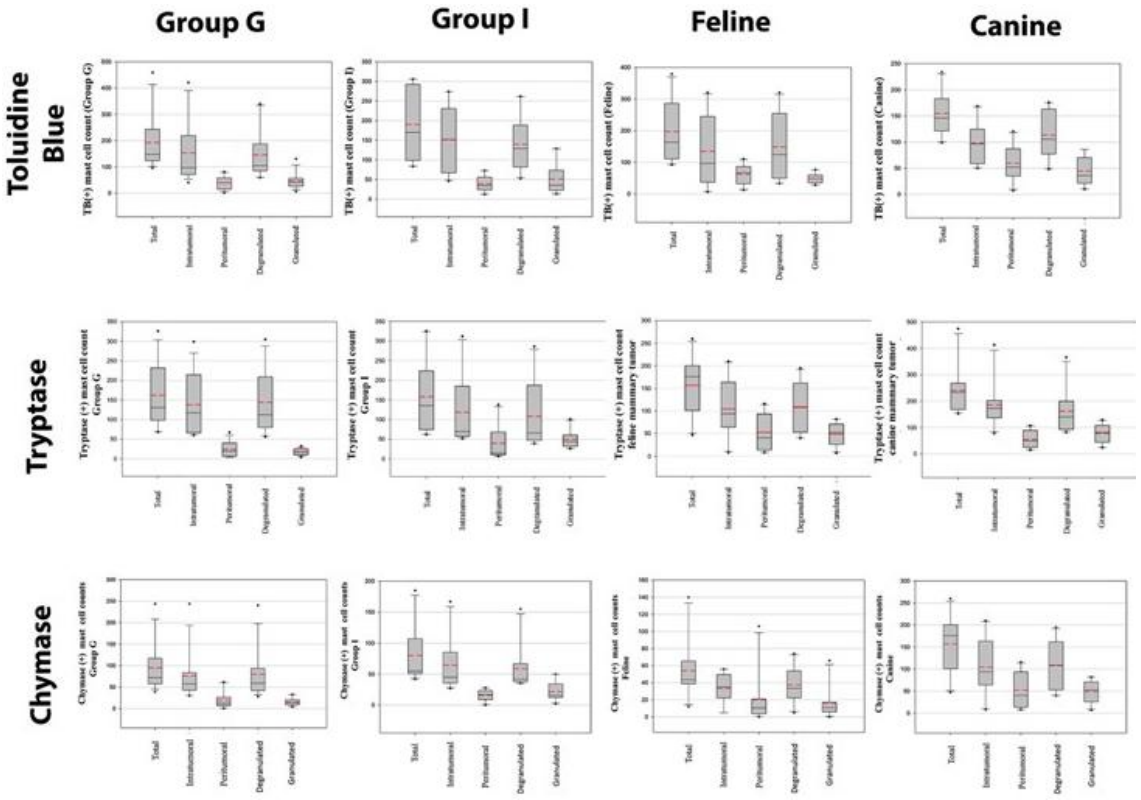

Fig. 8: Statistical scatter plot of the location and granulation status of mast cell numbers according to animal species; + values indicate the lowest and highest number, the black solid line indicates the median value and the red dashed line indicates the mean value.

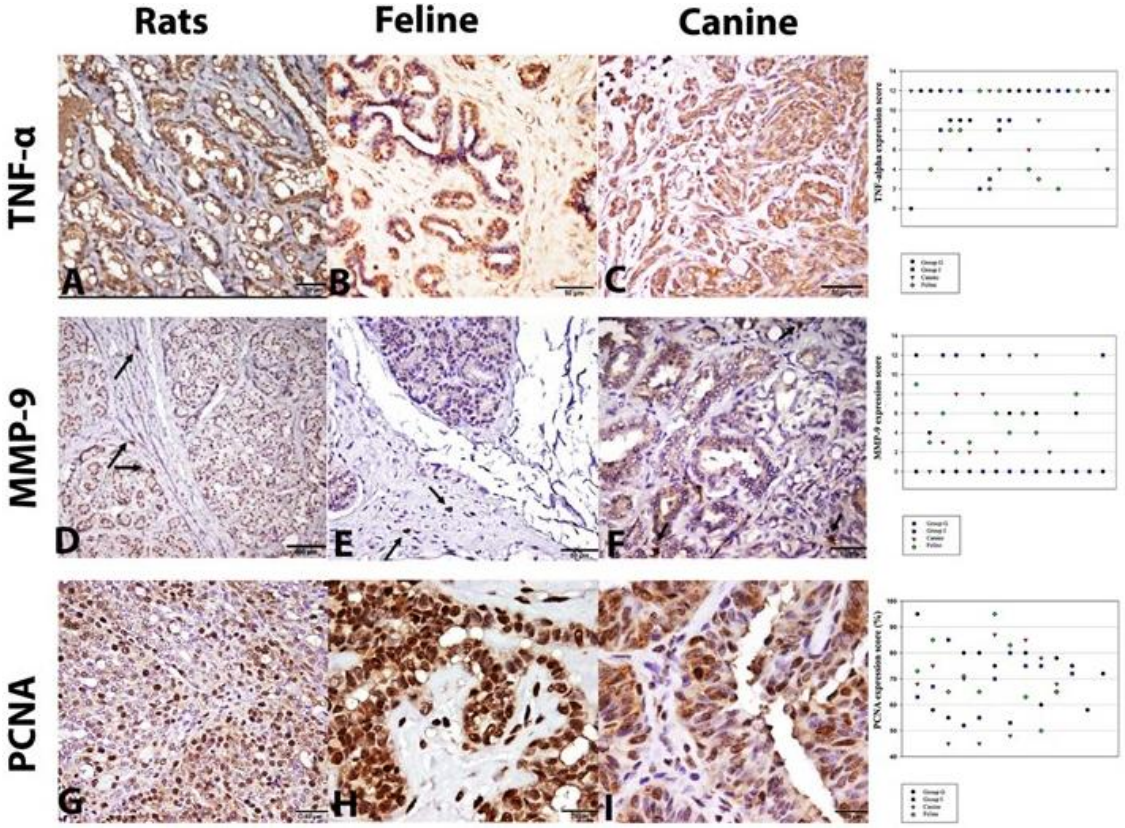

Fig. 9: Immunohistochemical staining of TNF-α, MMP-9, and PCNA, *Streptavidin peroxidase*, *DAB staining*, and distribution of measured values by groups A) High value of TNF-α expression in rat mammary carcinoma. B) High value of TNF-α expression in feline mammary carcinoma. C) High value of TNF-α expression in canine mammary carcinoma. D) Nuclear location of MMP-9 expression in rat mammary carcinoma; arrows: MMP-9 positive mast cells around the neoplastic mammary glands. E) Low value of MMP-9 expression in neoplastic feline mammary glands; arrows: MMP-9 positive mast cells in the peritumoral area in feline mammary carcinoma. F) Low value of MMP-9 expression in canine mammary carcinoma in epithelial cells; arrows: MMP-9 positive mast cells in the intratumoral area around the neoplastic mammary glands. G) High value of PCNA expression in rat mammary carcinoma, nuclear positivity in neoplastic epithelial cells. H) High value of PCNA expression in feline mammary carcinoma, nuclear positivity in neoplastic epithelial cells. I) High value of PCNA expression in feline mammary carcinoma, nuclear positivity in neoplastic epithelial cells.

Table 1: Toluidine blue-positive mast cell counts in tumors and control tissues.

Toluidine blue-positive mast cell count	Rat control tissue	Group G (DMBA gavage)	Group I (DMBA gavage + progesterone)	Canine control tissue	Canine tumor	Feline control tissue	Feline tumor
Total	3.82	19.20 (5.02*)	19.02 (4.97*)	2.58	15.72 (6.09*)	1.46	19.72 (13.50*)
Intra-tumoral	-	15.25	15.11	-	9.87	-	13.48
Peri-tumoral	-	3.97	3.91	-	5.95	-	6.24
Degranulated	1.28	14.54 (11.35*)	13.93 (10.88*)	1.6	11.34 (11.57*)	0.46	14.87 (32.32*)
Granulated	2.54	4.68 (1.84*)	5.09 (2)	0.98	4.48 (2.8*)	1.00	4.85 (4.85*)

*The number shows the fold increase between the experimental and the control group.

Table 2: Tryptase-positive mast cell count.

Tryptase-positive mast cell count	Rat control tissue	Group G (DMBA gavage)	Group I (DMBA gavage + progesterone)	Canine control tissue	Canine tumor	Feline control tissue	Feline tumor
Total	2.14	16.19 (*7.56)	15.81 (*7.38)	1.96	24.06 (*12.27)	2.16	15.69 (*7.26)
Intra-tumoral	-	13.78	11.85	-	18.58	-	10.44
Peri-tumoral	-	2.41	3.96	-	5.48	-	5.25
Degranulated	0.90	14.41 (*16.01)	10.80 (*12)	0.68	16.17 (*23.77)	0.84	10.79 (*12.84)
Granulated	1.24	1.77 (*1.42)	5.01 (*4.04)	1.28	7.79 (*6.08)	1.32	4.90 (*3.71)

*The number shows the fold increase between the experimental and the control group.

Table 3: Chymase-positive mast cells count.

Chymase-positive mast cell count	Rat control tissue	Group G (DMBA gavage)	Group I (DMBA gavage + progesterone)	Canine control tissue	Canine tumor	Feline control tissue	Feline tumor
Total	1	9.09 (9.09*)	7.77 (7.77*)	1.02	8.61 (8.44*)	1.24	5.01 (4.04*)
Intra-tumoral	-	7.97	6.49	-	6.79		3.58
Peri-tumoral	-	1.12	1.28	-	1.82		1.43
Degranulated	0.36	8.22 (22.83*)	5.58 (15.5*)	0.28	6 (21.42*)	0.38	3.36 (8.84*)
Granulated	0.64	0.88 (1.37*)	2.19 (3.42*)	0.74	2.61 (3.52*)	0.86	1.65 (1.91*)

*The number shows the fold increase between the experimental and the control group.

The granules of mast cells contain many different molecules (TNF-α, MMPs, tryptase, chymase, IL-6, VEGF, etc) (Oldford *et al*., 2010). Mast cells secrete many of these molecules according to their function in the tumor area, and there is significant heterogeneity between species, indistinct organs of the same breed, and even within the same organ (Bradding & Arthur, 2016). Heterogeneity is evident regarding substructure, mediator content, immunological and non-immunological stimulation, and pharmacological response (Bradding and Arthur, 2016). Tumor necrosis factor (TNF-α) is a critical cytokine mast cells produce (Bradding *et al*., 1994). This molecule plays an essential role in host defense. It protects against cancer development (Szlosarek and Balkwill, 2003), as revealed by the increased cancer incidence in patients receiving anti-TNF-α therapy (Fereydouni *et al*., 2022). A previous study has shown that TNF-α expression was localized in tryptase-positive mast cells and that increased TNF-α expression was independently associated with improved survival in non-small cell lung cancers (Ohri *et al*., 2010). In another study, the increase in both TNF-α expression in tumor cells and the TNF-α positive mast cells was suggested as an indicator of good prognosis, unlike MMP-9 expression, the increase of which is correlated with poor prognosis (Shikotra *et al*., 2016). In our study, TNF-α-positive mast cells were not counted, but many TNF-α-positive mast cells were noted in peritumoral and intratumoral areas. We could not substantiate a significant correlation between TNF-α expression of tumoral cells and in mast cell number. However, the increase in TNF-α expression but the low expression of MMP-9 still suggested that high mast cell numbers of in the tumor area are indicative of a good prognosis.

MMPs are primarily involved in degrading the extracellular matrix (ECM). Still, they are also involved in several biological and pathological processes, including fibrosis, inflammation, and wound healing (Vandenbroucke *et al*., 2011). MMP-9, an important family member, is closely related to mast cells. Communications between fibroblasts and mast cells activate MMP-9 release from fibroblasts (Abel and Vliagoftis, 2008). The function of the mast cell in potentiating numerous feedback in its local tissues through the discharge of dissolved mediators may depend on cooperation with extracellular matrix (ECM) molecules within connective tissues. In the present study, increased mast cell numbers in mammary tumors were associated with increased MMP-9 expression. Keser *et al*. (2017) have previously documented that the total number of mast cells increases in tumors with high MMP-9 expression and metastases, thus responsible for poor prognosis. The findings in our study contradicted this study.

One of the most critical findings in our study is the positive correlation between peritumoral mast cells and granulated mast cells and between the intratumoral mast cells and degranulated mast cells. Although a study on dogs showed that peritumoral mast cells were more abundant than intratumoral mast cells, in our research, higher numbers of intratumoral mast cells were observed in tumor stroma (Sakalauskaitė *et al.,* 2022). The positive correlation between the number of mast cells in the intratumoral region and the number of degranulated mast cells suggested that mast cells were there to perform a task in the tumor region, whereas the peritumoral mast cells had a lesser task or were bystanders in that region. Interestingly, this positive correlation was observed for both chymase- and tryptase-positive mast cells.

Mast cells have been the subject of studies on canine before. However, there is no information about the subtypes of mast cells (Sakalauskaitė, 2022). There is also no study about the role or phenotype of mast cells in feline mammary tumors. In the present study, the number of tryptase-positive mast cells was higher than the chymase-positive mast cells both in canine and feline mammary tumors. Similarly, there were more intratumoral and degranulated mast cells than their peritumoral and granulated counterparts.

Conclusions: As a result, in this study, mast cells were comparatively examined in canine and feline mammary tumors and an experimental rat mammary tumor model induced by 7, 12-DMBA. The immunophenotypes of these cells were determined for the first time, especially in canine and feline mammary tumors. One of the most exciting results of this study is that most of the mast cells in the intratumoral area were degranulated, and granulated mast cells were observed more commonly in peritumoral areas. Moreover, the negative correlation between PCNA expression and mast cell numbers and the positive correlation between the increased mast cell numbers and high TNF-α and low MMP-9 expressions suggested that the presence of these cells in tumor areas had an anti-tumorogenic effect. In light of these findings, although mast cell increases are thought to be responsible for good prognosis, future studies are needed to use these cells as a prognostic tool. Further studies are also required to answer the possible role of drugs that promote and/or inhibit mast cell degranulation (such as stem cell factor/cromolyn sodium) in cell target therapy of mammary tumors.

Ethical statement: All experiments were approved by the Bursa Uludag University Animal Experiments Local Ethics Committee (2020-04/03, BUU-HADYEK, Bursa, TURKEY).

Funding: This work was supported by grants from the Bursa Uludag University Scientific Research Projects Unit (BAP, TDK-354-2021).

Conflict of Interest: The authors have no conflict of interest.

Authors Contribution: OY and GS designed the study. OY, SEY and GS executed the experiment and analyzed the tissue samples. OY, ITC and GS analyzed data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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