BRAZILIAN CONSENSUS FOR THE DIAGNOSIS, TREATMENT AND PROGNOSIS OF CUTANEOUS MAST CELL TUMORS IN DOGS

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ABSTRACT

Mast cell tumors represent the most common malignant skin tumor in the dog. This review outlines the incidence, etiology and clinical signs of mast cell tumors. Diagnostic tests, staging and treatments are also discussed. This study was performed by the Veterinary Oncology and Pathology of UNESP, Jaboticabal and the Service of Pathology Veterinary, of UNESP-Botucatu with the support of the Brazilian Association of Veterinary Oncology and the Brazilian Association of Veterinary Pathology.

Key-words: canine, cutaneous neoplasm, diagnosis, prognosis, treatment.

Introduction

The cutaneous mast cell tumor (CMCT) is the third most common malignant cutaneous neoplasm in dogs, accounting for 11% of malignant skin tumors in dogs, being preceded only by lipoma and adenoma. Mast cell tumor (MCT) should always be considered in the list of differential diagnoses for a skin mass (London et al., 2013). Based on these facts, studies of this neoplasm, as well as diagnostic, surgical and clinic approaches are of paramount importance to establish consistent predictive and prognostic criteria for patients.

This study was performed during the Consensus for the diagnosis, treatment and prognosis of CMCT in dogs, hosted by the Department of Veterinary Oncology and Pathology of UNESP, Jaboticabal and the Department of Pathology Veterinary, of UNESP-Botucatu with the support of the Brazilian Association of Veterinary Oncology and the Brazilian Association of Veterinary Pathology.

The purpose of this consensus was to discuss the criteria to guide diagnosis, prognosis and treatment of canine CMCT in Brazil. Veterinary oncologists, surgeons and pathologists from different regions of Brazil contributed to this consensus.

Incidence and Etiology

CMCT is recognized as one of the most common cutaneous tumors in dogs, however few studies have evaluated its national incidence. Studies in CMCT in Brazil reported the incidence between 20.9 and 22.4%, ranking it as the second most common malignant neoplasm in dogs, only after mammary tumors (De Nardi, 2002; Souza et al. 2006; Meirelles et al. 2010). Some breeds are predisposed to CMCT as Boxers, Golden Retrievers, Weimaraners and Dachshunds (De Nardi, 2002; Kiupel et al. 2005; Murphy et al. 2006; Mullins et al. 2006; Costa-Casagrande et al. 2008; Furlani et al. 2008). In a survey conducted of the archives of the Department of Animal Pathology, Department of Pathology, College of Veterinary Medicine and Animal Science, USP from 1993 to 2002, 126 mast cell tumor cases were diagnosed among 1813 neoplasm in dogs, accounting for 7% of all tumors analyzed. Boxer was the breed most commonly affected (Kimura et al. 2012).

The etiology of MCT is not fully elucidated, Vail and Withrow (1996) proposed that it was associated with chronic inflammatory lesions and skin exposure to irritants, however current research has shown that mutations in the C-KIT gene contribute to the genesis of this tumor. This gene, encodes the tyrosine kinase receptor of the stem cell factor (SCF also known as kit ligand, KL or steel factor) in neoplastic mast cells of dogs, and alterations in this gene develop uncontrolled growth of these cells and origin of the malignant tumor (London et al. 1999; Reguera et al. 2000; Zemke et al. 2002; Turin et al. 2006; Webster et al. 2007).

Clinical signs

MCT is presented as masses of different sizes and according to Hanh et al. (2004), tumors larger than 3 cm are associated with worse prognosis. In addition MCT can be well delimited, elevated, firm, pruriginous, may also show erythema or ulcerated surface, and subcutaneous tissue invasion may also occur (Daleck et al. 2009). Some tumors are poorly circumscribed, elevated, soft and may not show erythema and ulceration, macroscopically very similar to cutaneous lipomas (Thamm and Vail, 2007; Daleck et al. 2009). The subcutaneous and CMCT presentations can be differentiated exclusively by histopathological analysis (Daleck et al. 2009). About 50% of the CMCT are reported in the trunk, perineal, genital and groin regions, 40% are in the limbs and 10% in the head and neck (Thamm and Vail 2007; Daleck et al. 2009).

According to Couto (2006), the biological behavior of MCT is extremely variable. In general, well differentiated MCTs (grade I) have low metastatic potential. In contrast, tumors of grades 2 and 3 commonly cause metastasis to regional lymph nodes and have high potential to spread, especially to the liver and spleen, while lung metastases are uncommon. Some researchers reported that mast cell tumors located in the oral cavity, nail bed or in the inguinal, perineal and preputial regions exhibit highly malignant behavior (Tams and Macy, 1981; Turrell et al. 1988; Fox, 1998; Couto, 2006). There is a study that states that there is no correlation between malignancy and location (Kiupel et al. 2005).

CMCTs are typically solitary lesions, but their clinical appearance can be variable and some dogs can develop more than one apparently unrelated MCT. Studies suggest that multiple MCT should be treated as individual neoplasm, while prognosis should be related to each tumor characteristics and not to the number of tumors presented in the dog (Murphy et al. 2006). However, while O'Connell e Thompson (2013) demonstrated that multiple CMCT

lesions did not affect overall survival, Kiupel et al. (2005) reported a worse prognosis for patients presented with multiple lesions.

Paraneoplastic syndromes

The clinical signs of MCT are present in up to half of dogs with mast cell tumor and include mast cell degranulation and release of histamine, heparin, chemotactic factor for eosinophils and proteolytic enzymes (Thamm and Vail 2007; Welle et al. 2008; Daleck et al. 2009). The effect of degranulation can be observed during patient physical examination by mechanical palpation of the tumor, resulting in erythema, wheal formation (Darier's sign), edema, ulceration and swelling at the primary tumor site, and possibly delayed wound healing and local coagulation abnormalities (Thamm and Vail, 2007; Welle et al. 2008; Daleck et al. 2009; Blackwood et al., 2012).

A major complication is gastrointestinal ulceration, affecting mainly the stomach and less frequently the duodenum. These lesions are usually multiple and superficial although in some situations perforations can be present. They occur due to increased histamine blood levels that stimulate the H2-receptor of parietal cells, therefore resulting in excessive production of gastric acid and increased gastric motility. Furthermore, histamine causes damage to the vascular endothelium of arterioles and venules and releases fibrolysin, leading to intravascular thrombosis and ischemic necrosis of the stomach mucosa. Heparin, in turn, tends to block the effects of histamine, but it is present in low concentrations in undifferentiated malignant mast cell tumors (Welle et al. 2008; Daleck et al. 2009; Blackwood et al., 2012). In such cases, clinical signs such as hematemesis, anorexia, hematochezia, melena, anemia, abdominal pain and in some cases intestinal perforation and peritonitis may be observed (Thamm and Vail, 2007; Welle et al. 2008; Daleck et al. 2009; Blackwood et al., 2012). According to Welle et al. (2008) gastrointestinal ulcers were observed in about 35 to 83% of dogs with mast cell tumors during necropsy examination.

The delayed wound healing and dehiscence, observed after MCT surgical resection, are possibly related to the release of vasoactive amines and proteolytic enzymes by mast cells, which once bound to H1 and H2 receptors may lead to suppression of fibroblast growth factor reducing fibroplasia (Welle et al., 2008; Daleck et al., 2009). Furthermore, local bleeding can also be observed during surgical resection, probably due to defective coagulation caused by release of heparin (Welle et al., 2008).

Circulatory collapse, although rare, can occur in the presence of a massive release of histamine by neoplastic cells. Dogs with extensive disease are particularly at risk (Welle et al., 2008; Blackwood et al., 2012).

Diagnostic approach

The diagnostic evaluation of animals with suspected CMCT usually has three goals: 1) definitive diagnosis by cytological and histopathological examination; 2) clinical staging; 3) documentation of paraneoplastic syndromes. Immunohistochemical techniques have been applied to differentiate MCT from other anaplastic round cell tumors. MCT are vimentin positive and the majority is tryptase and CD117 (KIT) positive. Other markers like chymase, MCP-1 and IL-8 can be used (London and Thamm, 2013).

Cytology

Fine needle aspiration (FNA) cytology gives a diagnosis for 92–96% of MCT. Mast cells quickly exfoliate and are easily identifiable by metachromatically staining intracytoplasmic granules but the grading cannot be established by cytology (Blackwood et al., 2012).

Furthermore, the assessment of tumor grade in cytological smears does not allow following strictly the grade system proposed by Patnaik et al. (1984), since the evaluation of specific criteria, such as level of tissue invasion, cannot be determined by cytological examination. The sample should be collected by fine needle aspiration (FNA) without aspiration. The needle chosen for the cytology should be 13x4.5mm (26G) in order to reduce blood contamination, thus increasing the accuracy of the diagnosis (De Nicola, 2009).

Among round cell neoplasms, MCT has a characteristic morphology, as the presence of fine to coarse intracytoplasmic basophilic granules evidenced by the Romanowsky stains and their derivatives (De Nicola, 2009; Strefezzi et al. 2009; MacNeill, 2011; Grandi et al. 2014). Evaluation using Diff-Quik stain, commonly used in the routine, can be inconclusive since it may not stain mast cells granules (DeNicola, 2009; MacNeill, 2011). Regardless of the stain used, cells that constitute high grade tumors, may not exhibit abundant cytoplasmic granulation because they lose them during the differentiation process. Strefezzi et al. (2009) demonstrated that nuclear morphometry could be associated with disease prognosis, showing a correlation between average nuclear area with survival time.

Camus et al. (2016) developed a cytologic grading scheme for canine MCTs, based on correlation with histologic grade, to predict

treatment planning and prognostication. The cytologic grading scheme classified a MCT as high grade if it was poorly granulated or had at least 2 of 4 findings: mitotic figures, binucleated or multinucleated cells, nuclear pleomorphism, or anisokaryosis (>50% variation in nuclear size) and the high tumor grade was also associated with increased probability of additional tumors or tumor regrowth. Other MCT markers such as CD25, interleukin-2 receptor, c-kit mutations, and proliferation markers such as AgNOR and Ki67 complement cytologic and histologic grades may add additional prognostic information.

However, Hergt et al. (2016) also proposed criteria to use a 2-tier histologic grading system on cytology specimens and concluded that cytologic grading of MCT in the dog is helpful for initial assessment, although, the reliability of cytology using the 2-tier grading system is considered inadequate at this point. Cytologic grading resulted in 36 high-grade and 105 low-grade tumors. Agreement between histologic and cytologic grading based on the 2-tier grading system was achieved in 133 cases (sensitivity 86.8%, specificity 97.1%, kappa value 0.853), but five high-grade tumors on histology were classified as low-grade on cytology.

In this consensus, we indicate that cytology should be used only for screening diagnosis, and the histopathology as definitive diagnosis and grading of canine MCT.

Histopathology

Cutaneous mast cell tumor

Histopathological grading is the primary tool to predict the biological behavior of cutaneous and subcutaneous MCT (Bostock, 1973; Patnaik et al. 1984; Preziosi, Morini and Sarli, 2004; Kiupel et al. 2011; Thompson, 2011b).

The histopathological results depend on the adequate sampling of the material for diagnosis. Therefore, few recommendations on how the surgeon should send the sample for histopathologic analysis are described as follows: (1) corticosteroids or chemotherapy should not be used for tumor cytoreduction before the histopathological diagnosis; this will underestimate the assessment of cell proliferation index (mitotic index and immunohistochemical analysis with the antibody Ki67). When cytoreduction is necessary before the surgical procedure, it is recommended to perform a previous incisional biopsy in order to not compromise the evaluation of the proliferation index, and the referral form should specify whether this procedure was performed and the therapeutic protocol used for this purpose; (2) another possible option is excisional biopsy of the tumor with macroscopic safety margins of at least 3 cm whenever possible. This would enable the assessment of microscopic margins in greater detail, as well as the distinction between cutaneous and subcutaneous MCT, since these exhibit different behavior; (3) in cases of large tumors, when it is impossible to obtain clean margins, carefully perform incisional biopsy in order to avoid mast cell degranulation and risk to the patient; (4) immediately after biopsy, include a tumor sample in a 10% formalin solution at a rate of 1:9 parts of tissue fixative for a maximum of 24 to 48 hours. Subsequently, transfer the tissue sample to a 70% alcohol solution. A prolonged formaldehyde fixation, as well as a short one, can impair the immunohistochemical staining and molecular biology analysis, prognostic and predictive values.

The large surgical pieces should be sectioned during the fixation in formaldehyde, for 24 to 48 hours, to obtain a minimum of four pieces per sample. The pieces should be identified with different Indian ink on the edges, for deep and lateral margins, or using suture stitches (Fulcher et al. 2006; Kamstock et al. 2011; Thompson, 2011a). Multiple nodules should be sent separately, and properly identified so histopathological analysis will be done individually for each node. The sentinel lymph node (SLN) should be referred and analyzed for the presence of metastasis. Samples of tumor and lymph node should be fixed with a maximum thickness of 1 cm2.

For more details on how to submit surgical specimens, to identify the surgical margins and tumor sample processing, we recommend the article *Recommended Guidelines for Submission, Trimming, Margin Evalu ation, and Reporting of Tumor Biopsy Specimens in Veterinary Surgical Pathology* (Kamstock et al. 2011)

The grading system proposed by Patnaik et al. (1984) is the most widely used system for the classification of CMCT. According to this system, CMCT grade I consist of rows or clusters of monomorphic neoplastic mast cells well differentiated with rounded nuclei, small intracytoplasmic granules with cell proliferation confined to the dermis. CMCT grade I present rare mitotic figures, show no binucleate cells and may have minimal stromal reaction or necrosis. CMCTs grade II are moderately to markedly cellular, with moderately pleomorphic neoplastic mast cells, with round and/or pleomorphic nuclei and intracytoplasmic granulation of varying sizes. They extend into the deep dermis, subcutaneous tissue and occasionally deeper. MCT grade II have zero to two mitotic figures per high power field (HPF), discrete

areas of edema, necrosis and hyalinization of collagen. The grade III of CMCT are markedly cellular and composed of neoplastic mast cells with remarkable pleomorphism with rounded pleomorphic vesicular nuclei, containing multiple prominent nucleoli. The cells are arranged in dense sheets that replace the subcutaneous tissue and deep planes. Grade III mast cell tumors contain three to six mitotic figures per HPF, areas of hemorrhage, edema, necrosis and hyalinization of collagen. According to the original study, 94% of dogs with CMCT grade I survived more than 1500 days, compared with 56% of dogs with grade II and 7% of dogs with grade III.

Although the Patnaik system is considered the "gold standard" for the prognosis of mast cell tumors, the prevalence of CMCT grade II and inter observer variability when grading the same tumor, reduce the accuracy of this classification system. Still, according to Patnaik criterion, for grade III mast cell tumors, specifically the high mitotic index (3-6 mitotic figures per HPF) excludes certain tumors with low proliferative index that could display aggressive biological behavior (Strefezzi, Xavier and Catão-Dias, 2003; Preziosi, Morini and Sarli, 2004; Northrup et al. 2005; Pinczowski et al. 2008; Strefezzi et al. 2009; Kiupel et al. 2011).

In order to decrease inter observer variability and have a more reliable histological classification, Kiupel et al. (2011) proposed another grading of CMCT. This histopathological classification is based on low and high grade of malignancy. At least one of the following characterizes CMCT of high malignancy: (1) at least seven mitotic figures counted in ten HPF, counted in areas of higher number of mitotic figures; (2) at least three multinucleated cells (three or more nuclei) in ten HPF; (3) at least three bizarre nuclei, markedly pleomorphic nuclei in ten HPF; or (4) karyomegaly (Kiupel et al. 2011; London e Thamm, 2013).

In the study of Kiupel et al. (2011), three CMCT samples that were classified as high-grade malignancy did not have the seven mitotic figures counted in ten HPF, which indicates that the number of mitotic figures should not be the only criterion to classify high grade MCT. There was 100% agreement among pathologists in the grading of low and high malignancy MCT while according to this classification average survival time was less than four months and more than two years, respectively, for dogs with high and low-grade tumors (Kiupel et al. 2011).

This consensus proposes that CMCT should be classified according to Patnaik et al. (1984) and Kiupel et al. (2011), including the histopathological diagnosis of the two grading systems (e.g., "CMCT grade III according to Patnaik and high-grade malignancy CMCT according to Kiupel").

Similarly, this item should also indicate the mitotic index (MI) in ten HPF, followed by MI reference. MI has been described as a prognostic factor independent of other histopathological features while higher mitotic rates are associated with a worse prognosis (Romansik et al. 2007; Elston et al. 2009). However, the cutoff values reported for the number of mitotic figures counted in ten HPF may vary from five to ten (Bostock, 1973; Romansik et al. 2007; Sueiro et al., 2007; Elston et al. 2009).

Until further prospective studies define cutoff values statistically calculated for canine cutaneous mast cell tumors, the authors of this consensus recommend using the methodology of Romansik et al. (2007) ≤ 5 and> 5 mitotic figures or Elston et al. (2009) 0; 1-7 and> 7 mitotic figures, quoting the reference source in the report. Thus, as higher values of mitotic figures (>5 mitotic figures or >7 mitotic figures) are related to a worse prognosis, the use of chemotherapy in the postoperative period of some patients is recommended.

Subcutaneous mast cell tumors

Many MCT arise in the dermis and extend to deeper levels, but there is a group of MCT that are confined to the subcutaneous adipose tissue and it seems that these tumors exhibit less aggressive biological behavior (Newman et al. 2007). Recently, a study of 306 dogs categorized histopathologically the subcutaneous MCT and determined some prognostic markers for these tumors (Thompson et al. 2011a).

Thompson et al. (2011a) classified the subcutaneous MCT in three patterns determined by light microscopy (4x): (1) circumscribed, (2) combined (infiltrative/circumscribed), and (3) infiltrative. In this study, the decreasing survival time was related to MI>4 ten HPF/40x and infiltrative histological pattern and the presence of multinucleated neoplastic mast cells.

Histochemistry

Histochemical techniques like Giemsa, toluidine blue, Alcian Blue-Safranin are of utmost importance to establish the differential diagnosis with other round cell neoplasms and to identify intracytoplasmic granulation, especially in cases in which the cytoplasmic granules are scarce, thus facilitating visualization. Simões and Schoning (1994) compared various histochemical methods as hematoxylin-eosin and toluidine blue, Alcian blue, PAS, Giemsa, and others, commonly used to identify neoplastic canine mast cell with techniques of immunohistochemistry and lectin-histochemistry and found that, routine histochemical stains are efficient to mark intracytoplasmic granules of mast

cells. Difficulty remains in the undifferentiated mast cells, which only were identified with higher frequency by immunohistochemistry technique in this study.

Although AgNOR count is strongly correlated with tumor grading and this method can provide greater objectivity to the classification of Patnaik et al. (1984), the consensus established that mast cell AgNOR staining should not be adopted in routine due to the lack of standardization and interpretation of the technique. Instead, only immunohistochemistry technique should be used to determine cell proliferation using Ki-67 antibody.

Immunohistochemistry analyses

There are numerous studies using immunohistochemistry to analyze immune markers that help understand the biological behavior of MCT. However, few antibodies have real predictive and prognostic value (Kamstock et al. 2011).

The antibodies that best characterize the aggressiveness and malignant potential of cutaneous and subcutaneous MCT are the Ki-67 (proliferation index) and KIT (membrane receptor for stem cell factor) (Abadie et al., 1999; Preziosi, Morini and Sarli, 2004; Webster et al., 2004; Scase et al., 2006 Webster et al., 2007; Maglennon et al., 2008; Strefezzi et al., 2010).

The methods used to calculate the proliferation index vary considerably between studies (Abadie et al. 1999; Scase et al. 2006; Webster et al. 2007; Maglennon et al. 2008; Strefezzi et al. 2010). There is inter observer variability while examining different tumor areas and total fields on a slide, as well as different techniques used in immunohistochemistry for the same antibody.

Therefore, we recommend the methodology proposed by Webster et al. (2007) for cell proliferation using the Ki-67 immunostaining for CMCT and the method of Thompson et al. (2011b) for subcutaneous MCT. For CMCT, Ki-67> 23 positive cells/5 HPF is associated with high risk of recurrence and/or metastasis (Webster et al. 2007).

Studies have determined expression patterns of KIT protein in CMCT (Preziosi, Morini and Sarli, 2004; Webster et al. 2004). Three localization patterns of the KIT protein were identified, namely membranous (KIT pattern 1), focal cytoplasmic with loss of membrane labeling (KIT pattern 2) and diffuse cytoplasmic (KIT pattern 3). These patterns were associated with tumor aggressiveness showing that focal and diffuse cytoplasmic patterns had an unfavorable prognosis (KIT patterns 2 and 3) (Webster et al. 2004). It is indicated that immunohistochemistry reports use the nomenclature adopted by Webster et al. (2004) for the c-KIT patterns in order to create a single and standard report.

Molecular biology for gene C-KIT

In 1999, two studies described the presence of mutations in the C-KIT gene in canine MCT (London et al. 1999; Ma, Longley and Wang, 1999). KIT is a membrane receptor with tyrosine kinase activity for stem cell factor (SCF), which stimulates mast cell growth. These two studies described duplication of DNA in the C-KIT gene, called internal tandem duplications (ITDs). These mutations were found in exons 11 and 12 of the gene.

When the SCF binds to the KIT receptor without mutation, the cytoplasmic portion of the receptor undergoes autophosphorylation. In the presence of ITD in exon 11, the receptor is constitutively phosphorylated, regardless of SCF presence. This KIT phosphorylation activates signaling pathways that stimulate the growth of neoplastic mast cells (London et al. 1999).

Therefore, the presence of this mutation is directly responsible for the uncontrolled proliferation of the tumor, which in this case carries a poorer prognosis (Webster et al. 2007). The discovery of an aggressive behavior of MCT by a constitutive activation and signaling of a tyrosine kinase receptor, result in research about the use of tyrosine kinase inhibitors in MCTs (London et al. 2009; Yamada et al. 2011).

After a decade of research in canine populations, the ITD mutation was found in approximately 30% of cutaneous MCT. Other mutations, deletions and insertions have also been found in this tumor, but rarely observed (Ma, Longley and Wang, 1999; London et al. 1999; Downing et al. 2002; Zemke et al. 2002; Reguera, Ferrer and Rabanal, 2002; Jones et al. 2004; Riva et al. 2005; Webster et al. 2006a; Webster et al. 2006b; Webster et al. 2007; Letard et al. 2008; Ohmori et al. 2008; London et al. 2009). Only one study examined the presence of mutation in the C-KIT gene in subcutaneous MCT; and it was absent in all cases (n = 60) (Thompson et al. 2011a).

The test to detect C-KIT mutation is routine because the result can guide therapeutic choices. Several laboratories offer C-KIT mutation analysis, most commonly for exon 11 ITD since alterations in other exons are not commonly reported (Avery, 2012).

The PCR test is performed on samples embedded in paraffin, of which the genomic DNA of tumor cells is extracted. PCR using specific primers amplify the gene segment where the mutations is most commonly inserted (e.g., exon 11) and the products are separated by gel electrophoresis (Jones et al. 2004). Jones et al.

(2004) demonstrated the efficacy of the method when compared to other types of development and highlighted that the incidence of punctual/small mutations is very rare in canine MCT, which avoids the use of sequencing as a diagnostic method. Positive control may not be used reliably as a control method due to different mutation patterns (duplicates, deletions and insertions). It is recommended to use a standard molecular weight control, so the reaction control is warranted and optimizes the identification of different types of changes that may arise in the interpretation of the PCR results.

Analyses of mutation to the gene *C-KIT* should be included in a panel of markers for mast cell tumors, since they have prognostic and predictive value (Avery, 2012). These tests must discriminate which mutation, deletion, or insertion polymorphisms should be searched, since many of them do not lead to constitutive phosphorylation of the *C-KIT* gene (Webster et al. 2007; Létard et al. 2008; Yamada et al. 2011; Avery, 2012).

Staging

Staging must be performed to define the extension of the disease since it greatly influences the therapeutic choice, and also the prognosis (Daleck et al. 2009; London e Thamm, 2013). Current staging of MCTs practiced by many veterinarians involves a minimum of lymph node assessment, abdominal ultrasound and thoracic radiography; however, Warland et al. (2012) concluded that thoracic radiography was not useful in the staging of canine MCT. The World Health Organization (WHO) has proposed a clinical staging system for canine MCT; however, several studies have questioned its use, especially

for MCT grade III (Murphy et al. 2006; Thamm and Vail, 2007; Daleck et al. 2009). Because of this, the authors of this consensus propose to adopt the staging system described at the Southern European Veterinary Conference (SEVC) in Barcelona 2008 (Table 1).

Table 1 – Clinical Staging System for Canine Mast Cell Tumor

Stage	Tumor	Regional lymph nodes	Metastasis
I	Single, <3cm, well-circumscribed	-	-
II	+1 node, <3cm, interlesional distance>10cm, well-circumscribed	-	-
III	1 or +, >3cm, interlesional distance <10cm, poorly circumscribed or ulcerated	-	-
IV	Any lesion type	+	-
V	Any lesion type	- or +	+

Suffixes a: without systemic signs, b: with systemic signs.

MCT usually metastasizes to lymph nodes (LN), spleen and liver. Lung involvement is infrequent, bone marrow and peripheral blood in systemic dissemination may be compromised (London e Thamm, 2013).

Warland et al. (2012) found in their study that no tumors spread distantly metastasis without first showing local LN metastasis. For this reason, the authors suggest that the local LN is sentinel to the evaluation of metastasis of MCT. Histological examination of these LNs provides information on the status of lymphatic metastasis for the entire lymphatic system. However, abdominal organs were not aspirated unless ultrasonographic abnormalities were found. Is important to say that not all local LNs are possible to palpate and

collect FNA, like iliac LN. In the same study, 30.9% of tumors had metastasized at presentation; similar to another study did by Krick et al. (2009).

Radiography and ultrasonography are widely used as a screening tool. However, Book et al. (2011) showed that ultrasonography sensitivity to detect spleen and liver metastasis is 43% and 0%, respectively, which suggests that FNA or ultrasound guided biopsy should be performed in all cases of CMCT regardless of ultrasonographic findings. Krick et al. (2009) demonstrated that cytological evaluation of lymph nodes of dogs with MCT provides valuable clinical information and correlates with tumor grade and prognosis, in addition of being a practical and non-invasive technique. Dogs with positive cytological evidence of infiltration by mast cells in the spleen, liver, or both, have a worse prognosis (Book et al. 2011).

Hume et al. (2011) conducted a retrospective study in dogs with MCT grade III and described higher survival time in patients without lymph node metastasis at the time of diagnosis, when compared to dogs with positive lymph node metastasis. Therefore, lymph node metastasis constitutes an important prognostic factor and should be treated accordingly. Dogs with positive lymph node metastasis treated with surgery or radiation therapy had a mean survival of 240 days, whereas untreated dogs had 42 days.

The SLNs are important in the staging of several types of human cancer, providing information as prognosis and treatment strategy in these cases. In veterinary medicine the assessment of the SLN during the staging should be incorporated, especially in breast cancer, osteosarcoma, synovial sarcomas and MCT (Tuohy et al. 2009). Therefore, we recommend FNA and cytologic evaluation of regional lymph nodes or SLN, for metastasis evaluation prior to wide surgical excision (London e Thamm, 2013).

Treatment

Surgery

The main treatment of canine MCT is surgical resection. Whenever possible, a 3 cm for lateral and one fascial layer for deep margins of the lesion should be considered. Moreover, the removal of the fascia musculature and muscle is indicated to remove the tumor as a block (Daleck et al. 2009; MacPhail, 2014).

It is necessary to avoid manipulation of the lesion during the procedure to prevent mast cell degranulation and systemic adverse effects on the patient (MacPhail, 2014). It is further recommended that SLN are removed intraoperatively. In cases of tumor removal prior to lymph node, surgeons should replace all the material and surgical fields (Hume et al. 2011).

When the lesion is present in the scrotum, it is recommended to perform scrotal ablation and orchiectomy. If the prepuce is also compromised, penectomy associated with scrotal urethrostomy should be indicated (MacPhail, 2014). In the limbs, reconstructive techiniques as pedicled or bi-pedicled, tubular or hinge flaps are the best forms of surgical correction in order to preserve the limb. However, amputation of the compromised limb is more appropriate, but this technique is not always accepted by the owners due to the cosmetic appearance of the patient (MacPhail, 2014; Daleck et al. 2009).

Healing by second intentions is recommended when it is not possible to use reconstructive techniques or appropriate sutures that promote tissue closing. It is preferable to have a postoperative open wound, which heals secondarily than the occurrence of compromised margins (Daleck et al. 2009; MacPhail, 2014).

Antineoplastic chemotherapy

Antineoplastic chemotherapy is an important technique to treat unresectable tumors, in the postoperative of high grade and grade III mast cell tumors and, when surgical margins are compromised by neoplastic cells (Welle et al. 2008). In addition, chemotherapy is indicated in cases of advanced clinical staging to downstage the tumor for subsequent surgery (Daleck et al. 2009).

Neoadjuvant chemotherapy main objective is to promote cytoreduction in cases of difficult surgical resection. The protocols more commonly used are lomustine as a single agent at a dose of 60 to 90mg/m2, every 21 days, for a total of 2 sessions or, vinblastine associated with prednisone, according the protocols in Table 2. It is important to say that cytoreduction using neoadjuvant chemotherapy usually has a temporary response lasting an average 40-70 days, so surgical procedure is indicated during this period (Rodasky and De Nardi 2008; Daleck et al. 2009).

In most studies, vinblastine is used to treat MCTs at a dose of 2.0mg/m2 every 1 or 2 weeks, associated or not with prednisone (Table 2) (Davies et al. 2004; Trumel et al. 2005; Thamm, Turek and Vail, 2006; Hayes et al. 2007). In dogs, dose limiting is characterized by neutropenia (Golden and Langstin, 1988; Davies et al. 2004; Trumel et al. 2005; Thamm, Turek and Vail, 2006). Vinblastine nadir is observed approximately 7 days after administration (Rosenthal, 1981). In order to improve treatment effectiveness, some studies suggest gradual increase of dosage to 2.33, 2.67 and 3mg/m2 weekly (Vickery et al. 2008; Rassnick et al. 2008). Vinblastine can be safely administered in dose of 3.00mg/m2, above this level some hematological and gastrointestinal effects may be observed (Vickery et al. 2008; Rassnick et al. 2008). The highest tolerated dose is 3.5mg/m2 every two weeks (Bailey et al. 2008). The authors of this consensus recommend the use of vinblastine associated with prednisone as the first option of treatment, in the postoperative of high grade and MCT grade III, and when surgical margins are compromised. When the response is not positive with the use of these drugs, we suggested the use of lomustine 70 to 90mg / m2.

Table 2– Chemotherapy protocol of vinblastine and prednisone to treat MCT in dogs.

Week	Vinblastine (2mg/m2)	Prednisone
1st	X	X (1 mg/ kg)
2nd	X	X (1 mg/ kg)
3rd	X	
4th	X	
5th		
6th	X	
7th		X (0.5 mg/ kg)
8th	X	
9th		
10th	X	
11th		
12th	X	

Multidrug protocols have promoted higher response when compared to single agent chemotherapy (partial response) (Govier, 2003). Thus, numerous chemotherapy protocols have been described in the literature (Table 3); however, there is little data regarding disease-free interval and other important criteria that

allow compare different protocols (Taylor et al. 2009). It is known that MCT have different response rates to different chemotherapy protocols and the occurrence of adverse effects may be common among patients (Webster et al. 2008).

Table3 -Published chemotherapy protocols to treat advanced MCT in dogs and remission rates (RR) (Adapted from Taylor et al. 2009).

Chemotherapy Protocol	n	n (DGI)	RR (C+P)
Prednisolone	25	25	20%
Prednisolone and vinblastine	41	28	47%
Prednisolone, vinblastine and cyclophosphamide	35	11	63%
Lomustine	19	19	40%
Chlorambucil and prednisolone	21	21	38%

n= total number of animals

n(DGI)=total number of dogs gravely ill (Patients severely compromised by disease progression)

RR= remission rate (C= complete) and (P= partial)

Taylor et al. (2009) treated 21 dogs with unresectable MCT grade II and III with a combination of prednisolone 40mg/m2 orally SID for 14 days and 20mg/m2 EOD thereafter, and chlorambucil 5mg/m2 orally SID. In cases when complete remission was achieved, treatment was discontinued after six months, if not, treatment was continued. Three dogs achieved complete remission; nine in stable disease, five in partial remission and four in disease progression (overall response rate 38%). According to the authors, the study showed poorer response when compared to other protocols.

Hosoya et al. (2009) used lomustine (60 mg/m2 every three weeks) and prednisone (40mg/m2 SID, for seven days, followed by 20mg/m2 EOD) in partially resected MCT grade II. The animals received treatment for four to six months. In this study, no animals developed local recurrence and regional or distant metastasis. Furthermore, 100% of animals were disease free at one year interval and 77% at two years interval.

Hayes et al. (2007) evaluated prednisolone (1mg/kg) and vinblastine (2mg/m2, about eight doses for each patient) in dogs with CMCT grade III after surgical resection. In this study, the animals without lymph node metastasis had a survival time of 800 days. In contrast, dogs with lymph node metastasis lived an average of 481 days after diagnosis. Furthermore, Wesbter et al. (2008) found that treatment with vinblastine (2mg/m2 once per week for four weeks) and prednisone (2mg/kg orally daily) after surgery was beneficial to dogs with MCT grade III when compared to those only treated with surgery. Thus, according to the authors, the results of this study validate the use of adjuvant vinblastine and prednisolone. In addition, dogs with c-KIT mutation treated with this protocol had longer disease-free interval and survival. In contrast, Rungsipipat et al. (2009) observed a partial response rate of 78.2% in dogs with CMCT grade III that were treated with adjuvant vinblastine (2mg/m2 at weeks one, two, three, four, six, eight, ten and twelve) and prednisolone (1mg/kg, for four weeks, after 0.5 mg/kg for eight weeks).

Cooper, Tsai and Bennet (2009) evaluated the effect in dogs with grade II and III CMCT of chemotherapy with lomustine (60mg/m2) and vinblastine (2mg/m2), divided into two groups - macroscopic and microscopic disease. The authors showed that this protocol was well-tolerated and suitable for disease control since the response lasted longer compared to other agents.

Rassnick et al. (2010) evaluated the protocol with lomustine (70mg/m2), vinblastine (3.5mg/m2) and prednisone (1-2mg/kg). A total of 17 dogs had unresectable MCT. The response rate in animals that did not undergo surgery was 65% (five with complete remission and six with partial remission).

To date, few studies have been published involving the mechanisms of multi-drug resistance (MDR) in canine MCT. Despite the expression by different methods of detection of key genes and proteins involved in MDR in MCT (MDR1, P-glycoprotein, Glutathione-S-transferase pi, protein associated with multidrug resistance 1, p53, etc.) no studies have confirmed the exact mechanism involved in chemotherapy resistance. Apparently, there is no histological association with the expression of MDR mechanisms that could justify the reasons why undifferentiated tumors respond unsatisfactorily to chemotherapy (Ginn et al. 2000; Jaffe et al. 2000; Miyoshi et al. 2002; Nakaichi et al. 2007).

The use of glucocorticoids as the sole treatment of canine MCT remains controversial regarding the induction of MDR. Recently, Teng et al. (2012) evaluated prednisone as the sole treatment of CMCT. These authors found that prednisolone caused a significant reduction in tumor size although it promotes gradually resistance since response rate was short (81.5% of patients showed maximal response for three weeks). The majority of MCTs overexpressed both P-glycoprotein and signal transducer and activator of transcription 3 (STAT3) before and after prednisolone treatment.

Previously, Matsuda et al. (2011) showed that the sensitivity in vivo and in vitro of canine MCT to glucocorticoids (GC) is associated

with the expression of the glucocorticoid receptor (GR) in neoplastic mast cells; however, there was a great difference in sensitivity to GC among the various MCTs and MCT cell lines. The degree of GC resistance was inversely correlated with GR expression. Interestingly, this study did not find a relationship between GC sensitivity and pathological grade of the MCT and neither between breed of dog and GR expression. Overexpression of MDR1 has been known to be involved in drug resistance in many tumors, however, this study also showed that the high expression of MDR1 was not evident in any of the canine MCT cell lines used. The authors suggested that the detection of the expression of GR can be useful for predicting mast cell tumor sensitivity to glucocorticoids.

The metronomic chemotherapy regimen has been studied with primary results so far (Colleoni et al. 2002; Pasquier et al. 2010). For example, chlorambucil metronomic regimen (4mg/m2/day/35 weeks) resulted in complete regression of MCT in a dog (Leach et al. 2012).

Electrochemotherapy

The electrochemotherapy (ECT) has emerged as a feasible technique to treat superficial and mucosa tumors since the 1990s (Mir et al. 1991). It associates electroporation with chemotherapy to boost cytotoxic effect of the drug (Mir et al. 1998).

 Kodre et al. (2009) reported ECT as single therapy to treat CMCT and showed recovery rates similar to those obtained after surgical excision without associated chemotherapy and lesion regression for 31.5 months average. Spugnini et al (2006) treated dogs with incomplete surgical margins and found that in 85% of cases the average recurrence rate was 52.7 months.

Adverse effects such as degranulation were reported in two cases out of 28 (Spugnini et al. 2006). Kodre et al. (2009) stated that due to the vasoconstriction caused by ECT no mast cell degranulation occurs and they did not observe this adverse effect in 12 treated patients. The median size of the tumors treated by ECT in this study was 2.9 cm3. These authors also concluded that ECT is an easy, effective and safe local treatment of MCT and can be an alternative treatment to surgery, specifically for smaller nodules in which a complete response with long duration can be obtained after only one treatment session, or when the nodule is unresectable because of the location.

Tyrosine kinase receptor inhibitors

Tyrosine kinase inhibitors (TKI) may be used in cases of unresectable or recurrent MCT where conventional therapy is not appropriate or available (Blackwood et al. 2012). Besides being involved in the normal cell cycle, studies suggest that tyrosine kinase receptors have a key role in the neoplastic process. Overexpression of these receptors and mutations in proto-oncogenes that encode these receptors cause uncontrolled cell proliferation that leads to tumor development (London, 2009). In addition, these receptors are involved in angiogenesis, which is important for the nutrition of malignant cells and a route for dissemination of metastasis (Thurson et al. 2004).

The participation of the receptor tyrosine kinase KIT in mast cell represents one of the greatest examples of the action of these receptors in the development of malignancies in dogs (London, 2009). After its discovery in carcinogenesis, a large effort has been directed at the development of strategies to inhibit its function in cancer cells and angiogenesis (Argyle, 2007).

The major strategy developed in veterinary medicine in order to inhibit these receptors was the use of so-called "small molecule kinase inhibitors tyrosine". These act by blocking ATP from bind to the receptor by a competitive inhibition mechanism (reversible or not) preventing the phosphorylation and signals of cell proliferation and angiogenesis (London, 2009).

The tyrosine kinase inhibitors currently available for the treatment of MCT in dogs are Toceranib (Palladia®), Masitinib (Kinavet®/Masivet®) and Imatinib (Gleevec®). The option of using this type of therapy in this tumor must be assessed following the clinical indications of drugs, such as the presence of C-KIT mutation, evaluated by molecular analysis as previously discussed, along with immunohistochemical findings of expression patterns of the KIT protein. According to the authors the treatment with the tyrosine kinase inhibitor should be instituted for patients with C-KIT mutation and patients with KIT pattern 2 and KIT pattern 3 in the immunohistochemistry analysis as discussed previously. However, there is no evidence that MCTs with standard KIT pattern 2 or 3 respond to treatment in the same way as mastocytes with C-KIT gene mutation.

Dogs with MCT that do not have C-KIT mutation can also benefit from TKI due to the antiangiogenic effect achieved by inhibition of vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF), but in these cases the response to therapy is generally lower. Currently, TKI are indicated in many other circumstances like multiple drug resistant, unresectable tumors, grade II and III, presence of metastasis and as rescue therapy in recurrent tumors (London et al. 2009; Soria et al. 2009; Ogilvie and

Ahn, 2010; Hahn et al. 2010, Phillips et al. 2010; Macedo et al. 2012).

Recommended doses for TKI are: Imatinib 5 to 10 mg/kg/ VO/SID, Masitinib 12.5 mg/kg/VO/ SID and Toceranib 3.25 mg/kg/VO/ EOD or Monday-Wednesday-Friday scheme. The literature indicates that these drugs should be administered continuously lifelong (London, 2009). The authors of this consensus recommend the use of Masitinib 12.5 mg/kg, VO, SID as a treatment for MCTs with a mutation in the C-KIT gene and for chemoresistance over six months. After this period the dose may be reduced to 6.25 mg/kg, VO, SID, throughout life.

Similar to what happens with chemotherapeutic agents; TKI also have side effects (London et al., 2009). These effects result from the chronic inhibition of the tyrosine kinase receptor in normal cells, which depend on these for survival and proliferation in normal conditions. The main side effects are gastrointestinal issues and neutropenia, but signs of hepatotoxicity, nephrotoxicity, hypoalbuminemia and hypertension have also been reported (London, 2009).

Radiotherapy

Radiation therapy in dogs with MCT can be performed prior to surgery, post-operative or palliative (only to pain control) (Dobson and Scase, 2007). In cases where the diffuse and/or extensive nature of tumor mass prevents surgery, radiotherapy should be taken into consideration, although studies on the use of radiation as a single therapy to treat canine MCT are scarce (Dobson, Cohen and Gould, 2004).

Adjuvant radiotherapy to treat MCT grade II when complete surgical resection was not possible, showed 12 months of disease-free survival in 94-97% of cases (Al-Sarraf et al. 1996; Frimberger et al. 1997; Ladue et al. 1998). The use of this therapy in incompletely excised MCT grade III was also indicated for tumor management (Hahn, King and Carreras, 2004). MCT located in head and distal region of limbs are important examples of difficult complete removal of tumors while preserving local anatomy; therefore, radiotherapy is recommended (Dobson, Cohen and Gould, 2004).

The tumor response to radiation is dose-dependent. An increased dose can lead to a significant increase in tumor response. Furthermore, a study performed to determine the optimal dose (maximum effect on the tumor and minimal toxicity to healthy tissues), used small radiation fractions (4x 800 cGy) in 7 day intervals to treat dogs with different grades of unresectable MCT, reported 85% partial or complete response rate (Dobson, Cohen and Gould, 2004). In another study, radiotherapy protocol was three sessions (54 Gy) per week and showed remission response of 94% in one year and 88% in five years (Al-Sarraf et al. 1996). For better results regarding disease-free periods, irradiation is recommended directly on the tumor and regional lymph nodes (Dobson, Cohen and Gould, 2004).

In a weekly radiotherapy study, many animals developed mild erythema in the treated area without sever signs related to degranulation and histamine release. Alopecia, hyperpigmentation and thickening of the skin in the affected area were also observed as late effect (Dobson, Cohen and Gould, 2004).

La Due et al. (1998) evaluated the effectiveness of megavoltage radiation therapy to treat 56 dogs. Radiation treatment length ranged from 14 to 28 days. The average disease-free interval was 32.7 months, 15 months for dogs older than 7.5 years and 62 months for dogs younger than 7.5 years. The data presented in the paper show that megavoltage beam radiation is effective to treat mast cell tumors.

Carlsten et al. (2012) performed a multicenter prospective trial with a combination of hypofractionated radiation treatment, Toceranib, and prednisone for measurable canine MCT and they concluded that this protocols is a viable treatment option for unresectable MCTs, well tolerated and efficacious in the majority of dogs and that the response rates and durations were higher than those reported for Toceranib as a single-agent treatment for MCT.

Prognosis

The biological behavior of canine MCT varies widely from low metastatic potential to extremely aggressive lesions, leading to metastasis and death. Several prognostic factors of MCT in dogs such as clinical staging, tumor growth rate, breed predisposition, systemic signs, recurrence, patient age, tumor size, histopathological grade, mitotic index, proliferation index Ki-67 and Kit immunostaining pattern were discussed in the text and are shown in Table 4. These factors can be used in an attempt to predict tumor biological behavior, as well as to direct the treatment (London and Seguin, 2003).

Table 4 - Prognostic factors for cutaneous mast cell tumors in dogs

PROGNOSTIC FACTOR	NEGATIVE	POSITIVE	AUTHOR
Tumor size	>3 cm	< 3 cm	Hanh et al. 2004
Tumor site*	Perineum, prepuce, inguinal region, paws, head, limb, foot	Other sites	Gieger et al. 2003; Couto et al. 2006; Hanh et al. 2008; Warland et al. 2012
Number of tumors*	Multiple tumors	Single tumor	Kiupel et al. 2005
Metastasis	Present	Absent	Hume et al. 2011; Book et al. 2011; Warland et al. 2012
Surgical margin*	Compromised margin	Free margins	Ozaki et al. 2007
Mitotic Index**	>5	< 5	Romamsik et al. 2007;
Nuclear Morphometry in Cytology (Papanicolaou)	nuclear area$\geq$62,39μm2	Nuclear area <62,39μm2	Strefezzi, Xavier and Catão-Dias, 2003
KIT pattern immunostain	KIT 2 e 3	KIT 1	Kiupel et al. 2004
Mutation of the gene c-KIT	Present	Absent	Webster et al. 2006
Ki – 67 Index	>23	<23	Webster et al. 2007
Tumor intravascular density	>14.1 mm-2	<14.1 mm-2	Preziosi, Morini and Sarli,2004

*There is no consensus among all authors that tumor site, presence of multiple tumors or compromised margins are associated with a worse prognosis.

** Some authors suggest as cutoff value for the mitotic index > or < than 7 (Elston et al. 2009).

Future Perspectives

Researchers have tried to find new strategies for the treatment of MCT in dogs. A recent genetic study evaluates the effectiveness of intramuscular or intra tumoral therapy using eletrogene

with interleukin-12 encoding plasmid (IL-12). These treatments resulted in complete remission in two out of three dogs with MCT and a reduction in volume in up to 83% of the tumors (Pavlin et al. 2011).

Advances in epigenetics show the existence of hypoacetylation of histones in cancer. With this, the experimental treatments with an inhibitor of histone deacetylation, AR-42, induced reduction in growth and apoptosis "in vitro" in a strain of canine MCT, proving to be a promising therapy for this tumor (Lin et al. 2010). A Brazilian study evaluated the Trichostatin A (TSA), an inhibitor of histone deacetylase that has antiproliferative effects and induces apoptosis in cancer cells. The action of the drug "in vitro" in MCT grade III cells showed deleterious effects on the growth and proliferation of tumor cells, suggesting a good chemotherapeutic potential (Nagamine et al. 2011).

Final Comments

According to the available literature, we suggest:

• Cytological analysis only as a diagnostic screening;

• Histopathological examination to confirm the cytologic diagnosis, tumor grade (using the classifications proposed by Patnaik et al. 1984 and Kiupel et al. 2011) and assessment of surgical margins;

• Use of the mitotic index as an aid in determining prognosis;

• Staging must be performed to define the extent of disease since it greatly influences the therapeutic choice and prognosis;

• Treatment based not only on grade system but also on clinical signs, histopathologic and immunohistochemical findings.

• Chemotherapy as neoadjuvant and adjuvant therapy

• Tyrosine kinase inhibitor should be instituted for patients with C-KIT mutation and patients with KIT pattern 2 and KIT pattern 3 in the immunohistochemistry analysis

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