UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

TERAPIA CITORREDUTORA PRÉ-OPERATÓRIA, ASSOCIADA OU NÃO À QUIMIOTERAPIA METRONÔMICA ADJUVANTE COM CICLOFOSFAMIDA E PIROXICAM, EM CÃES COM CARCINOMA DE CÉLULAS ESCAMOSAS CUTÂNEO

VINICIUS GONZALEZ PERES ALBERNAZ

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SUMÁRIO

Albernaz, V.G.P., **Terapia citorredutora pré-operatória associada ou não à quimioterapia metronômica adjuvante com ciclofosfamida e piroxicam em cães com carcinoma de células escamosas cutâneo**, Botucatu, 2019, 77p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho".

RESUMO

O carcinoma de células escamosas (CCE) é uma neoplasia epitelial originada dos queratinócitos da pele de cães. Sua etiologia está relacionada à exposição a raios solares ultravioletas, o que o coloca como uma das neoplasias mais frequentes em países tropicais de clima quente. O CCE cutâneo tem comportamento invasivo local, baixa capacidade de metástase e frequentemente encontra-se associado à ceratose actinica. Neste estudo, objetivou-se avaliar o efeito do tratamento pré-operatório com piroxicam (Px) na expressão de COX-2 e Ki67, indicadores de inflamação e proliferação celular, respectivamente, em cães acometidos por CCE cutâneo. Além disso, o intervalo livre de doença (ILD) por um período de pelo menos 180 dias após excisão cirúrgica associada a regime de baixa dose diária com Px (0.3mg/kg) e ciclofosfamida (CYC; 15mg/m^2) foi determinado nesta população. Não houve diferença estatística significativa na expressão de COX-2 após o tratamento pré-cirúrgico com Px; no entanto, houve diminuição no índice proliferativo marcado com Ki67 ($P<0.05$). Não foi encontrada alteração significativa no ILD no grupo tratado com Px e CYC (160 dias), quando comparado com o grupo controle retrospectivo (145 dias), tratado somente com ressecção cirúrgica. Não houve diferença estatística no ILD quando analisados o grau histológico, índice mitótico, tempo de evolução, metástase de linfonodos e o comprometimento das margens cirúrgicas. Adicionalmente, cães com estadiamento T4, independente do tratamento, estavam 3.2x e 4.8x mais propensos a recidiva precoce quando comparados aos estádios T3 e T2, respectivamente. Os resultados deste estudo demonstram o efeito anti-proliferativo do Px, independente da inibição de COX-2. O tratamento com baixa dose de Px e CYC associado a cirurgia não foi acompanhado por um aumento do ILD, em comparação com animais submetidos apenas ao tratamento cirúrgico.

Palavras-chave: prognóstico, AINEs, intervalo livre de doença, carcinoma espinocelular.

Albernaz, V.G.P., **Pre-operative cytoreductive therapy associated or not to adjuvant metronomic chemotherapy with cyclophosphamide and piroxicam in dogs with cutaneous squamous cell carcinoma.** Botucatu, 2019, 77p., Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho".

ABSTRACT

Squamous cell carcinoma (SCC) is an epithelial neoplasm that arises from skin keratinocytes of the dogs. Its etiology is related to ultraviolet sunlight, which puts it as one of the most frequent neoplasm in tropical hot weather countries. Cutaneous SCC have a locally invasive, low metastatic behavior, and is often associated with actinic keratosis. This study aimed to evaluate the effect of pre-operative treatment with piroxicam (Px) on COX-2 and Ki67 expression, indicators of inflammation and cell proliferation, respectively. Besides that, the evaluation of disease-free interval (DFI) of these animals for at least 180 days after surgical excision associated with a daily low-dose treatment with Px (0.3mg/kg) and cyclophosphamide (CYC; 15mg/m^2). There was no statically significant difference between COX-2 expression before and after treatment with Px; However, there was a significant decrease on Ki67 proliferative index ($P<0.05$). No significance was found in DFI when comparing the group treated with Px and CYC (160 days) and the control retrospective group (145 days), treated only with surgical resection. There was no statically difference on DFI when accessing histological grade, mitotic index, evolution time, lymph node metastasis, and incomplete surgical margins ($P>0.05$). Additionally, dogs with T4 stage, independent of the treatment, were 3.2 and 4.8-fold more likely to develop an early recurrence when comparing with T3 and T2, respectively. The results of this study demonstrate the antiproliferative effect of the Px independent of the COX-2 inhibition. The treatment with low-dose Px and CYC in association with surgery was not followed by an improvement on DFI in comparison with surgically treated animals.

Keywords: Prognostic, NSAIDs, disease-free interval, spinocellular carcinoma.

CAPITULO 1

1. Introdução

O carcinoma de células escamosas (CCE) cutâneo, também conhecido como carcinoma epidermóide é uma neoplasia maligna proveniente do epitélio escamoso da pele e da membrana mucosa (WEBB et al., 2009; FERNANDES et al., 2015). Sabe-se da influência da radiação ultravioleta (UV) como um fator desencadeante da neoformação, que frequentemente ocorre em pele não pigmentada com baixa cobertura pilosa e exposta continuamente a luz solar (NIKULA et al., 1992; MELNIKOVA & ANANTHASWAMY, 2005; WEBB et al., 2009).

Levantamentos epidemiológicos realizados no Brasil colocam o CCE como a primeira (FERNANDES et al., 2015) ou segunda (SOUZA et al., 2006) neoplasia cutânea mais comum, podendo atingir até 15% do total de tumores cutâneos, enquanto a média de ocorrência relatada em outros países não ultrapassa 6% (BOSTOCK, 1986; KALDRYMIDOU et al., 2002; PAKHRIN et al., 2007; VILLAMIL et al., 2011; HAUCK, 2013). A maior incidência e gravidade destes casos em países tropicais colocam o CCE como uma das principais neoplasias a serem estudadas no Brasil.

A confirmação do diagnóstico deve ser feita por exame histopatológico. A avaliação imuno-histoquímica (IHQ) tem sido frequentemente utilizada como auxílio na classificação específica e prognóstica de diversas neoplasias em medicina veterinária, incluindo o CCE (PESTILLI DE ALMEIDA et al., 2001; MILLANTA et al., 2016), embora haja poucos estudos apresentando dados envolvendo CCE cutâneo em cães (AL-DISSI et al., 2007, BONGIOVANNI et al., 2011, BADAGI et al., 2012, POGGIANI et al., 2012).

A excisão cirúrgica é a principal forma de tratamento na maioria dos casos; entretanto, a possibilidade de se ressecar completamente o tumor é dependente de seu tamanho, distribuição e localização anatômica (WEBB et al., 2009, HAUCK, 2013). Nos casos induzidos por radiação UV, novas lesões podem ocorrer mesmo após o controle local da lesão primária (WEBB et al., 2009). Ainda, em animais acometidos por múltiplas lesões, frequentemente associadas a luz solar, a exérese cirúrgica pode não ser curativa (NORTHRUP & GIEGER, 2010), o que impulsiona o uso de terapias adicionais no período pós-cirúrgico.

Os inibidores da cicloxigenase-2 (COX-2) têm sido utilizados na tentativa de controlar a longo prazo as proliferações neoplásicas (NORTHRUP & GIEGER, 2010).

Associados aos inibidores da COX-2, os quimioterápicos orais administrados de forma metronômica visam proporcionar controles mais efetivos das neoplasias, com baixos índices de toxicidade (KERBEL & KARMEN 2004; ELMSLIE et al., 2008; BILLER, 2016).

2. Revisão de Literatura

Os carcinomas de células escamosas, tumores malignos da epiderme envolvendo diferenciação dos queratinócitos apresentam comportamento biológico localmente agressivo, podendo exibir infiltração óssea e osteólise (HAUCK, 2013). O desenvolvimento da neoplasia inicia-se com lesões pré-neoplásicas denominadas CCE *in situ*; no entanto, sabe-se que este compartilha características biológicas com o CCE, sendo duas fases diferentes na progressão da mesma doença (POGGIANI et al., 2012).

Dados baseados na análise de 9000 tumores de pele, oriundos de diferentes centros de patologia do mundo mostram que a casuística de CCE representa cerca de 6% dos tumores cutâneos em cães (HAUCK, 2013). Estudos internacionais desenvolvidos na Coréia do Sul (PAKHRIN et al., 2007), EUA (VILLAMIL et al., 2011), Grécia (KALDRYMIDOU et al., 2002) e Reino Unido (BOSTOCK, 1986) demonstram porcentagens menores na incidência do CCE cutâneo (0,2%, 1,25%, 2,3%, e 5,4%, respectivamente).

Por outro lado, levantamentos epidemiológicos realizados no Brasil colocam o CCE como a primeira ou segunda neoplasia cutânea mais comum, representando de 7% (SOUZA et al., 2006) a 15% (FERNANDES et al., 2015) do total de tumores cutâneos, atrás somente do mastocitoma (Souza et al. 2006). Em países considerados tropicais e de clima quente como Tailândia (RUNGSIPIPAT et al., 2003), Austrália (ROTHWELL et al., 1987), Índia (GUPTA; TIWARI, 2009) e Zimbábue (MUKARATIRWA et al., 2005), porcentagens semelhantes às brasileiras são relatadas, sendo estas 6,7%, 6,9%, 11,5% e 15,4%, respectivamente.

A predisposição para a maior incidência de CCE cutâneo em países de clima quente reforça o pressuposto de que a radiação UV solar interfere no seu surgimento (NIKULA et al., 1992). Nos CCE induzidos por luz UV, o dano celular já pode estar presente em outros sítios anatômicos, mesmo após controle local da neoplasia, levando ao surgimento de novas lesões e alta taxa de recidiva da doença (WEBB et al., 2009).

A infecção por papilomavirus é uma conhecida causa desta neoplasia em humanos (Valls-Ontañón et al., 2019), equinos (Zhu et al., 2015) e felinos domésticos (Hoggard et al., 2018), mas a relação da presença do vírus com o surgimento de CCE cutâneo canino tem sido questionada (SABATTINI et al., 2016). Outros fatores desencadeantes como radiação ionizante, agentes químicos, imunossupressores e inflamação crônica também são relatados (HAWROT et al., 2003).

A maior parte dos CCE progride lentamente, sendo que metástases para linfonodos e à distância são raras e não costumam ocorrer até fases tardias da doença (HAUCK, 2013). Acomete normalmente cães entre oito e dez anos de idade e não há predisposição racial (PESTILLI DE ALMEIDA et al., 2001; WEBB et al., 2009; MILLANTA et al., 2016). Lesões profundas, ulceradas, proliferativas, elevadas em placas hiperêmicas e com crescimento em forma de couve-flor podem ser observadas na pele dos animais acometidos (WEBB et al., 2009; HAUCK, 2013). A doença geralmente se desenvolve em áreas glabras ou de hipotricose e de pouca pigmentação, na maior parte dos casos no abdome (POGGIANI et al., 2012). O CCE também pode ocorrer na cavidade oral (MESTRINHO et al., 2015), plano nasal (LASCELLES et al., 2000), tonsila (MAS et al. 2011), e leito ungueal (BELLUCO et al., 2013) com características biológicas diferenciadas do CCE cutâneo.

A cirurgia é o tratamento de eleição para a maioria dos pacientes, considerando que geralmente o tumor não é responsivo à quimioterapia (WEBB et al., 2009). Os relatos do uso de quimioterapia adjuvante de máxima dose tolerada no tratamento do CCE cutâneo em cães são esporádicos e não demonstram bons resultados. A cisplatina como agente único foi descrita em dois cães com CCE metastático, tendo como resultados remissão completa em um e remissão parcial com posterior recidiva no outro animal (HIMSEL et al., 1986). Resultados semelhantes foram descritos com o uso de bleomicina, (BUHLES & THEILEN, 1973), actinomicina-D (HAMMER et al., 1994) e mitoxantrona (OGILVIE et al., 1991). Não há relatos do uso de radioterapia no controle de CCE cutâneo em cães; porém, resultados insatisfatórios são descritos em estudo avaliando cães acometidos por CCE nasal (Lascelles et al., 2000).

A confirmação do diagnóstico deve ser feita por exame histopatológico (HAUCK, 2013). A classificação histopatológica de Anneroth et al. (1984) foi desenvolvida para CCE humano, sendo considerada um valioso fator prognóstico e preditivo para a ocorrência de metástase nodal (AKHTER et al., 2011). Ainda, esta classificação apresenta padronização que pode ser estendida para medicina veterinária.

A avaliação IHQ tem sido frequentemente utilizada como auxílio na caracterização específica e prognóstica de diferentes tumores, incluindo o CCE (POGGIANI et al., 2012; MILLANTA et al., 2016). Além da expressão da proteína p53, outros biomarcadores utilizados em cães incluem Ki67 (POGGIANI et al., 2012), E-caderina, ciclina A, ciclina D1, metaloproteinase 2 e 9 (ONO et al., 2011), β-catenina (BONGIOVANNI et al., 2011), cicloxigenase-2 (PESTILLI DE ALMEIDA et al., 2001; POGGIANI et al., 2012 MILLANTA et al., 2016) e CD31 (MAIOLINO et al., 2001). A seguir, destacam-se particularidades de alguns destes anticorpos.

Cicloxigenase-2

As ciclooxigenases (COX) 1 e 2 catalisam as etapas iniciais da síntese de prostaglandina G_2 e prostaglandinas H_2 por meio da conversão do ácido araquidônico (DUBOIS et al., 1998). A isoforma COX-2 é indetectável na maioria dos tecidos de mamíferos; no entanto, é rapidamente induzida em locais inflamados por meio de fatores de crescimento, promotores tumorais, hormônios, endotoxinas bacterianas e citocinas envolvidas na produção de prostaglandinas (ROUZER & MARNETT, 2009).

A superexpressão de COX-2 tem impacto em todos os estágios de carcinogênese, como a iniciação (PANG et al., 2014), promoção e progressão tumoral para estágios metastáticos (GREENHOUGH et al., 2009). A superexpressão de COX-2 ocorre precocemente durante a carcinogênese induzida por raios UV e diante do aumento local de prostaglandina-E_2 (RUNDHAUG et al., 2007).

Em uma variedade de neoplasias que acomete humanos e animais, a expressão de COX-2 está relacionada a piores prognósticos (QUEIROGA et al., 2011; PRADA et al., 2012). A marcação de COX-2 parece estar associada a características de malignidade, como a presença de ulceração, pleomorfismo nuclear, angiogênese, infiltração de macrófagos e linfócitos (GREGÓRIO et al., 2017), ocorrência de metástase, recidiva tumoral, aumento da proliferação celular mensurada pelo Ki67 e o índice mitótico em tumores melanocíticos (MARTÍNEZ et al., 2011). Em neoplasias mamárias caninas, a associação de alta expressão de COX-2 e outros marcadores, incluindo Ki67 e CD31, foram relacionados a fenótipos malignos, maior grau histológico, presença de êmbolo neoplásico intravascular, metástase em linfonodos, e menor tempo de sobrevida global (CARVALHO et al., 2016a; CARVALHO et al., 2016b).

Aproximadamente 53% dos CCE em cães e gatos expressam COX-2 (MILLANTA et al., 2016). A superexpressão de COX-2 é significativamente mais alta nos CCE cutâneos que não-cutâneos e está frequentemente associada a presença de granulócitos (BARDAGÍ et al., 2012; MILLANTA et al., 2016). Após exposição solar, a expressão de COX-2 encontra-se aumentada em CCE cutâneos como mecanismo de proteção dos queratinócitos, permitindo sua proliferação e sobrevivência (POGGIANI et al., 2012). Queratinócitos irradiados com radiação UV *in vitro* expressaram COX-2, relacionando o aparecimento de tumores de pele após exposição solar com a ação da COX-2 (PESTILLI DE ALMEIDA et al., 2001).

A expressão de COX-2 não parece ter relação com a presença de invasão linfática e a graduação tumoral; porém, está correlacionada com a progressão do CCE (PESTILLI DE ALMEIDA et al., 2001; MILLANTA et al., 2016). Além disso, pode ser considerada um alvo farmacológico promissor para inibidores COX sistêmicos e tópicos, como estratégia preventiva e/ou terapêutica (BARDAGÍ et al., 2012; MILLANTA et al., 2016). O CCE pode também ser utilizado como um modelo pré-clínico de terapia antineoplásica com alvo na COX-2 (MILLANTA et al., 2016). Estudos em humanos mostram que anti-inflamatórios não esteroidais (AINEs) com especificidade para COX-2 podem reduzir a incidência de diversas neoplasias epidérmicas e lesões pré-neoplasicas, como a dermatite actínica (BARDAGÍ et al., 2012).

Ki67

O Ki67 é uma proteína nuclear essencial na manutenção do ciclo celular, que se expressa em todas as suas fases ativas (G1 a M) (BERGIN et al., 2011). A expressão desta proteína é eficaz em distinguir células proliferativas, visto que não está presente em células em descanso, representando um importante marcador de proliferação tumoral (BERGIN et al., 2011; PEREIRA et al., 2013). Sua ligação exclusiva com o ciclo celular não leva à expressão em células normais, em células sob estresse ou apoptose (COATER et al., 1996).

A análise IHQ de Ki67 é utilizada para determinar o índice de proliferação (ou o número relativo de células ativas envolvidas no ciclo celular), sendo considerado ainda como fator prognóstico em diversos tipos de tumores em cães e humanos (BROWN & GATTER, 2002; BERGIN et al., 2011; VASCELLARI et al., 2012). A expressão de Ki67 é feita por meio da marcação nuclear de células imunorreativas e representada em

porcentagem. Esta porcentagem é o critério mais comum para se obter o índice de proliferação (TANEJA et al., 2010).

Desde que o Ki67 surgiu como fator prognóstico, este é considerado independente da graduação histológica (WEBSTER et al., 2007). A expressão de Ki67 é útil no prognóstico de diversos carcinomas, visto que o índice proliferativo está aumentado em neoplasias mais agressivas, representando a elevada taxa de crescimento e de recidiva (AL-DISSI et al., 2007; JENSEN et al., 2010; GIOACCHINI et al., 2015). A baixa expressão de Ki67, por outro lado, foi relacionada com maior tempo de sobrevida em cães com CCE (POGGIANI et al., 2012).

A possibilidade de diferenciação do CCE de outras lesões pré-neoplásicas, como a dermatite actínica, pelo Ki67 foi avaliada; porém, não foram encontradas diferenças, inviabilizando a expressão do Ki67 como ferramenta diagnóstica auxiliar neste caso (POGGIANI et al., 2012). Já em mastocitomas, observou-se índice proliferativo significativamente maior em tumores com alto grau e intensidade de imunorreatividade para COX-2 (GREGÓRIO et al., 2017).

CD31

A molécula de adesão celular endotelial plaquetária 1 (PECAM-1), conhecida como CD31, é um marcador sensível e específico para células endoteliais e outras células hematopoiéticas (NEWMAN et al., 1990; MCKEANNEY et al., 2001). A marcação imunohistoquimica desta proteína permite obter a densidade microvascular intratumoral (DMVi). A DMVi é calculada com base no número de micro vasos presentes em 10 campos de alta magnificação (400x) e avaliadas em software de análise de imagens (MAIOLINO et al., 2001).

CD31 é um marcador de células endoteliais mais sensível quando comparado a outros marcadores como Fator VIII e CD34, visto que não há reação conjunta com o endotélio linfático (CHARPIN et al., 1997). No entanto, sua marcação não permite diferenciar os vasos marcados como vasos dormentes ou vasos tumorais neoformados ativos (HOLLEMANN et al., 2011). A maior DMVi nos CCEs grau IV em relação aos graus I e II sugere que a angiogênese pode ser fator determinante para o fenótipo invasivo e agressivo da neoplasia (MAIOLINO et al., 2001). Em humanos, a formação de novos vasos também acompanha a progressão do CCE cutâneo (FLORENCE et al., 2011). Acredita-se que o aumento da angiogênese leva a maior capacidade de invasão de tecidos profundos e metástase (TSE et al., 2007).

A angiogênese é fator necessário para que o tumor obtenha acesso a oxigênio e nutrientes utilizados para seu crescimento (KOCH & DISTLER, 2007). Este processo ocorre pelo desenvolvimento de novos capilares a partir de preexistentes (BIKFALVI, 2004) ou pela incorporação de vasos do hospedeiro, e nesse caso não há necessidade de angiogênese (HU & CHENG, 2009). A inibição da angiogênese tumoral, como terapia única pode interromper a progressão tumoral, mas não erradicar a neoplasia, pois vários são os mecanismos envolvidos no processo de vascularização da neoplasia (RAJABI et al., 2017).

2.1. Modalidades de tratamento clínico

Anti-inflamatórios Não Esteroidais

Os anti-inflamatórios não esteroidais (AINE) são fármacos analgésicos e anti-inflamatórios que agem por meio da inibição das enzimas COX-1 e COX-2 (CHAN, 2002). Dentre estes, destaca-se o piroxicam (Px), AINE derivado do ácido enólico, pertencente ao subgrupo dos oxicans e inibidor COX não seletivo, que apresenta fácil absorção após administração oral; porém, com início de ação lento (VERBEECK et al., 1986; GROSSER et al., 2018). Sua metabolização se dá pela hidroxilação do anel piridil por meio do citocromo P450 CYP2C9 e a excreção dos metabólitos pela urina e fezes (GROSSER et al., 2018). Em humanos, concentrações plasmáticas estáveis são obtidas após 7 a 12 dias de tratamento contínuo (GROSSER et al., 2018).

Além de sua excelente ação anti-inflamatória, analgésica e antipirética, o Px possui ainda efeito quimiopreventivo (MOHAMMED et al., 2003; PALMERINI et al., 2007; ALKAN et al., 2012). Adicionalmente, o Px é uma opção prática e de baixo custo, o que o torna uma excelente escolha para a prática clínica (CHOISUNIRACHON et al., 2013)

Os inibidores COX-2, possuem efeito anti-metastático, anti-angiogênico, anti-proliferativo e melhoram a resposta imune local por meio do bloqueio da inflamação crônica, além de otimizar a atividade de diversos quimioterápicos (MCMILLIAN et al., 2011; KNAPP et al., 2016). Isoladamente, inibidores COX-2 tem atividade preventiva e terapêutica em diversos tumores, melhorando a qualidade de vida e com resultados semelhantes ao uso de agentes citotóxicos (DE NARDI et al., 2011; KNAPP et al., 2016). O uso de Px demonstrou resposta semelhante às relatadas por outros tratamentos quimioterápicos citotóxicos em CCE oral de cães (SCHMIDT et al., 2001). Em modelo

de melanoma canino, o Px promoveu bloqueio da angiogênese por meio de atividade anti-VEGF e efeito anti-proliferativo, demonstrado pela diminuição na expressão de Ki67 em relação ao controle (CHOISUNIRACHON et al., 2013), Além dos benefícios no tratamento de neoplasias, os AINEs são a base do tratamento da dor crônica e perioperatória (EPSTEIN et al., 2015).

Entretanto, apesar dos benefícios claramente demonstrados em pacientes com neoplasias, alguns efeitos adversos, principalmente devido a inibição de COX-1 podem limitar o uso de AINEs (CHA; DUBOIS, 2007; EICHSTADT et al., 2017). O principal fator de risco para o desenvolvimento de efeitos adversos com o uso de Px está relacionado à idade do paciente (EICHSTADT et al., 2017).

A taxa de eventos adversos gastrointestinais com o uso de Px como agente único varia entre 6% e 23% em cães com tumores (KNAPP et al., 1992; KNAPP et al., 1994; ALLSTADT et al., 2015). A escala do Veterinary Comparative Oncology Group – Common Terminology Criteria for Adverse Events (VCOG-CTCAE, 2011) determina cinco graus de gravidade, com descrições únicas para cada evento adverso (i.e., condições clínicas, achados clinicopatológicos, ou doenças) associado ao tratamento antineoplásico. Dos pacientes que apresentam eventos adversos gastrointestinais após administração do Px, 40,9% e 48,7% correspondem a graus I e II na escala VCOG, respectivamente (EICHSTADT et al., 2017), e podem ser tratados com a interrupção temporária do medicamento e uso de protetores gástricos (KNAPP et al., 1994).

Em relação a possível toxicidade renal dos AINEs, causada pela inibição da síntese de prostaglandinas, esta parece ser baixa em cães recebendo unicamente Px (KNAPP et al., 1992; KNAPP et al., 1994; KNAPP et al., 2000; SCHMIDT et al., 2001). Azotemia renal associada a diminuição da densidade urinária foi relatada em 9% dos 137 pacientes tratados com Px (EICHSTADT et al., 2017). Em um estudo envolvendo a administração de ciclofosfamida (CYC), furosemida e em 85% dos casos Px, nenhum animal desenvolveu doença renal (SETYO et al., 2017).

Quimioterapia Metronômica

A quimioterapia metronômica (QM), ao contrário da quimioterapia de máxima dose tolerada (MDT), é a administração contínua e a longo prazo de baixas doses de drogas citotóxicas, em intervalos frequentes e regulares (MAITI, 2014; BILLER et al., 2016). Prolongar o tempo de sobrevida sem o objetivo curativo é o alvo inicial do tratamento destes pacientes (BILLER et al. 2016). Nestes casos, o objetivo da QM é

estabilizar a doença, ao invés de diminuir o volume tumoral (MAITI, 2014; BILLER et al., 2016). Ainda, os protocolos metronômicos se mostram opções atraentes em decorrência de seu baixo custo, facilidade de administração e baixos índices de toxicidade (MUTSAERS, 2009; BILLER et al., 2016).

Diferentemente da quimioterapia de MDT, que visa atingir células tumorais de rápida replicação, o alvo do regime metronômico são as células endoteliais e a angiogênese tumoral (BILLER et al., 2016). Outros mecanismos propostos são a promoção da imunidade antitumoral do animal, efeito sobre células neoplásicas e células tronco tumorais e a indução da dormência tumoral (BILLER, 2014; GASPAR et al., 2017). Embora muitas formas de atuação sejam abordadas para justificar o efeito antitumoral da QM, as mais importantes estão envolvidas com a inibição da neovascularização por meio da citotoxicidade endotelial (MUTSAERS, 2009) e normalização dos vasos tumorais por meio do aumento da proteína anti-angiogênica endógena tromboespondina-1 (HAMANO et al., 2004). Os vasos sanguíneos tumorais são considerados patogênicos devido à baixa cobertura de células murais, causada pelos altos níveis de proteínas pró-angiogênicas no tecido tumoral (JAIN, 2005). Esses vasos induzem hipertensão intersticial e baixa oxigenação do tumor, tornando-o resistente à quimioterapia e radioterapia (CARMILIET; JAIN, 2011; VIALLARD; LARRIVÉE et al., 2017). O balanceamento de proteínas anti-angiogênicas, por meio do aumento dos níveis de trombospondina-1 (TSP-1) e diminuição das pró-angiogênicas como o fator de crescimento vascular endotelial (VEGF) aumenta a porcentagem de estruturas vasculares normais (JAIN, 2005; BILLER 2014). A quimioterapia em baixas doses com CYC como agente único promove o desbalanceamento das proteínas angiogênicas, por meio da redução dos níveis de TSP-1 endógeno (PATTEN et al., 2010). Para equilibrar as proteínas angiogênicas, é frequente a associação à AINEs, pois estres inibem proteínas pró-angiogênicas, como o VEGF (CHA; DUBOIS, 2007; CHOISUNIRACHON et al., 2013).

Em regimes metronômicos de administração da CYC, observa-se atividade anti-angiogênica persistente durante todo o tratamento, ao contrário do que ocorre na MDT, mesmo em células resistentes ao fármaco (BROWDER et al., 2000). Frequentemente, os quimioterápicos metronômicos são combinados com a administração de um inibidor COX-2 (KERBEL & KARMEN 2004; ELMSLIE et al., 2008; SETYO et al., 2017; GASPAR et al., 2017). A maioria dos estudos com QM envolvem a administração de CYC por via oral (ELMSLIE et al., 2008; BURTON et al., 2011; GASPAR et al., 2017).

Independente do efeito anti-angiogênico proposto pela QM, observou-se diminuição do índice proliferativo em modelo experimental de melanoma canino, evidenciando efeito citotóxico do Px e da CYC (como agentes únicos e combinados) para células tumorais (CHOISUNIRACHON et al., 2013).

Apesar da característica promissora dos protocolos de QM, os tipos de neoplasia responsivos à terapia e como avaliar estas respostas são fatores ainda pouco conhecidos (BILLER et al., 2016). Algumas neoplasias caninas avaliadas em estudos metronômicos são o hemangiossarcoma (LANA et al., 2007), sarcoma de tecidos moles (ELMSLIE et al., 2008; BURTON et al., 2011), adenocarcinoma mamário e pulmonar (MARCHETTI et al., 2012), osteossarcoma (BRACHA et al., 2014) e carcinoma de células transicionais (SCHREMPP et al., 2013). Ressalta-se que em diversos casos foi utilizado protocolo de CYC associado ao Px (LANA et al., 2007; ELMSLIE et al., 2008) ou celecoxibe (MARCHETTI et al., 2012). Nestes estudos, diferentes respostas foram observadas, incluindo retardo no desenvolvimento de novos tumores (ELMSLIE et al., 2008) e taxas de sobrevida semelhantes às de quimioterapia de MDT (LANA et al., 2007). Em estudo de MARCHETTI et al. (2012), avaliando resposta à QM em 15 cães, sendo a maioria destes acometidos por adenocarcinoma mamário, observou-se 33% de doença estável, um caso de remissão completa e ganho de qualidade de vida.

De modo geral, o tratamento com baixas doses de CYC é relativamente atóxico e de baixo risco comparado com os efeitos colaterais observados com a quimioterapia intravenosa de MDT (LANA et al., 2007; MARCHETTI et al., 2012). No entanto, independente da tendência de poucos efeitos adversos, a possibilidade de cistite hemorrágica estéril (CHE) deve ser monitorada (BILLER, 2014). A CHE se caracteriza pelos sinais de hematúria, estrangúria e polaquiuria na ausência de infecção do trato urinário (SETYO et al., 2017; HARPER; BLACKWOOD, 2017). Na urinálise observa-se hematúria na ausência de aumento de leucócitos e mínima presença bacteriana (SETYO et al., 2017). O contato da acroleína, um metabólito inflamatório resultante da metabolização da CYC pelo fígado, com a parede da bexiga parece ser a causa da CHE (HARPER; BLACKWOOD, 2017).

A incidência deste efeito colateral varia entre 0-30% (LANA et al. 2007; ELMSLIE et al., 2008; BURTON et al., 2011; SETYO et al., 2017; HARPER; BLACKWOOD, 2017). Os fatores de risco para o desenvolvimento de CHE, ainda são pouco claros nos tratamentos metronômicos de CYC (SETYO et al., 2017); entretanto, a idade e doses cumulativas parecem influenciar em regimes MDT (GAETA et al., 2012).

Estratégias como administrar a CYC pela manhã, acesso fácil a água e prover liberdade total para o animal urinar reduzem o contato da urina contendo acroleína com a parede da bexiga (BEST; FRY, 2013; SETYO et al., 2017). A administração concomitante de furosemida, na dose diária de 0,5-1 mg/kg, parece diminuir significativamente a incidência de CHE em cães recebendo baixas doses de CYC (SETYO et al., 2017). O tratamento para a CHE geralmente envolve a interrupção da quimioterapia, que pode ser reintroduzida após a resolução clínica (HARPER; BLACKWOOD et al., 2017; SETYO et al., 2017). A eliminação de CYC pela urina ocorre após a administração intravenosa e oral, mas decai nos dias seguintes e é indetectável no segundo dia em diante (KNOBLOCH et al., 2010). Estes dados não estão disponíveis para a administração diária de baixas doses de ciclofosfamida. A maioria dos animais toleram bem o tratamento, e não é necessário interromper a medicação devido a efeitos adversos (e.g. sinais gastrointestinais VCOG grau I e II), com exceção da CHE (SETYO et al., 2017; HARPER; BLACKWOOD, 2017). Mielotoxicidade não foi observada em nenhum animal em tratamento com QM (HARPER; BLACKWOOD, 2017).

Estudos iniciais com o uso de QM tem demonstrado respostas positivas em pacientes veterinários; no entanto, em virtude do aumento de popularidade desta modalidade terapêutica, maiores investigações dos riscos e benefícios são necessárias para estabelecer diretrizes de seu uso desta (ELMSLIE et al., 2008; BILLER et al., 2016; GASPAR et al., 2017). Até o presente momento, não há estudos com o uso de QM em pacientes com CCE cutâneo em cães.

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CAPÍTULO 2

1. Trabalho Científico 1

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Piroxicam decreases the Ki67 proliferative index independent of COX-2 inhibition in naturally occurring canine cutaneous squamous cell carcinoma.

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Abstract

COX-2 is highly expressed in canine cutaneous squamous cell carcinoma (SCC) and has an important role in tumor promotion. The non-selective COX inhibitor Piroxicam (Px) is known for its anti-proliferative effect over several tumors. In order to evaluate the role of piroxicam in canine cutaneous SCC, this research investigated COX-2 and Ki67

expression before and under treatment with Px. Fifteen client-owned dogs with SCC were biopsied to confirm the diagnosis (non-treated sample), and then treated with Px (0.3mg/kg daily) for 21 days prior surgical resection of the tumor (treated sample). The non-treated and treated samples were referred for immunohistochemistry. There was a statistically significant difference in Ki67 expression between non-treated and treated samples ($P<0.05$), with lower proliferative index in treated samples, but not in COX-2 ($P>0.05$). No correlation between both markers was found ($P>0.05$). These results suggest that piroxicam may improve the clinical management of canine cutaneous SCC prior surgery.

Keywords: Dogs, skin cancer, oncology, immunohistochemistry, keratinocyte

Highlights

- Piroxicam down-regulated proliferative index (Ki67) *in vivo*.
- Canine cutaneous squamous cell carcinoma is a highly proliferative tumor.
- There was no correlation between Ki67 and COX-2 expression.
- There was no association of Ki67 and COX-2 with T-stage, lymph node metastasis, and Histological grade

Introduction

Cutaneous squamous cell carcinoma (SCC) is a malignant tumor of the epidermal keratinocytes (Hauck et al., 2013). It is one of the most common skin cancers in dogs, comprising 15% of all cutaneous tumors in Brazil (Fernandes et al., 2015). The carcinogenesis of the SCC has an intimate relationship with the exposure to sun UV radiation (Melnikova et al., 2005), which makes it common on glabrous and low pigmented sites (Poggiani et al., 2012). SCC can arise from other locations, such as the

oral cavity, tonsils, nail bed, and nasal cavity, but these exhibits a distinct biological behavior (Lascelles et al., 2000; Schmidt et al., 2001; Mas et al., 2011; Belluco et al., 2013).

Cyclooxygenase-2 (COX-2) enzyme is fundamental for the synthesis of prostaglandin G_2 and H_2, the mediator of several inflammatory processes (Greenhough et al., 2009). COX-2 overexpression has been associated to the early solar induced carcinogenic process (Rundhaug et al., 2007), as well as acting at tumor initiation, promotion and progression to metastatic stages (Greenhough et al., 2009; Pang et al., 2014). The COX-2 inhibition by a non-steroidal anti-inflammatory drug (NSAID) is considered an ancillary antineoplastic therapy to treat or prevent SCC (Bardagí et al., 2012; Millanta et al., 2016).

Piroxicam (Px) is a non-selective COX-2 inhibitor commonly used as an analgesic and antipyretic, is also indicated for long-term cancer management (Knapp et al., 2016; Grosser et al., 2018). Px has demonstrated an anti-angiogenic and anti-proliferative effect in different tumor cell lines and even on oral SCC (Schmidt et al., 2001; Choisunirachon et al., 2013). Currently, Px is often used alone or combined with cytotoxic or metronomic drugs in the treatment of bladder transitional cell carcinoma (Knapp et al., 2016), intranasal cancer (Woodruff et al., 2018), mammary carcinoma (Rossi et al., 2018), soft tissue sarcoma (Elmslie et al., 2008), and primary lung carcinoma (Polton et al., 2018). Finally, Px is considered a low-cost and practical drug for clinical routine (Choisunirachon et al., 2013).

Ki67 is a nuclear protein essential to cell cycle, and its expression effectively detects cells in active phases of mitosis (Pereira et al., 2013). The percentage of cells expressing Ki67 is called proliferative index (Vascellari et al., 2012), and is useful as a prognostic factor (Gioacchini et al., 2015; Smith et al., 2017; Gregório et al., 2017). In

canine cutaneous SCC, Ki67 immunolabelling was previously associated with survival time (Poggiani et al., 2012).

In the present study, Px effect prior surgery over cutaneous SCC was investigated *in vivo* by its anti-inflammatory and antiproliferative properties.

Material and methods

Case Selection and Samples

This prospective study was approved by the Ethics Committee on Animal Use (CEUA/FMVZ-UNESP) under protocol 5/2017. All owners were informed and consent before dogs were included in the experiment. We randomly selected 15 client-owned canine patients with naturally-occurring cutaneous SCC. Animals were staged with physical examination, triple thoracic radiographs views, abdominal ultrasound examination, urinalysis, complete blood count, and serum biochemistry (i.e. creatinine, urea, alanine aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, total serum protein, albumin, and globulin).

The epidemiological information regarding age, sex, breed, body weight, skin and fur color, and history were recorded. Macroscopic aspects of all tumors were noted, including two-dimension size, distribution (i.e. focal, multifocal, and disseminated), morphological aspects (i.e. nodular, plaque, and ulcer), local invasiveness, presence of ulceration, secondary infection, and actinic dermatosis, as well as lymph node enlargement. Owners informed evolution time since first notice of the lesions, the occurrence of high solar exposure, and comorbidities. Clinical staging was established according to World Health Organization proposed TNM system for skin tumors (Owen 1980) for each tumor (in patients with multiple lesions), but the higher stage was considered for statistical purpose.

The cases were included based on the following inclusion criteria: (1) Absence of distant metastasis; (2) No previous treatment (i.e. primary tumor); (3) T2 or higher TNM; (4) no diagnosis of kidney or liver disease; (5) Tumors amenable to surgical resection; (6) Adequate samples for immunohistochemistry labeling.

All dogs were submitted to incisional biopsy under sedation and local anesthesia to confirm the diagnosis of SCC and to provide a control histopathological sample for further immunohistochemical evaluation (non-treated sample). In patients with multiple lesions, only the largest tumor was used for evaluation. After that, patients were treated with full-dose Px (0.3mg/kg, PO q24h) throughout approximately 21 days (median of 27; range, 21 – 33) until the definitive resection surgery procedure. Surgery was performed with at least 1 cm of healthy tissues margins. At this moment, a sample (treated sample) was obtained from the same lesions and adjacent to the pre-operative biopsy site.

Histopathological Analysis

Both non-treated and treated tumor samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of 3 µm of each block were sectioned and histopathological diagnosis was made in hematoxylin & eosin stained slides. Mitotic index (MI) was defined as the number of cells in mitosis process in 10 high-power fields (HPF, x400). Histological grading was according to Anneroth et al. (1984). Surgical margins were examined histologically for neoplastic cells.

Immunohistochemistry

3µm thick sections were placed over charged slides (Starfrost®, Knittel Germany). dewaxed and rehydrated in ethanol. Antigenic retrieval was performed in a pressure cooker (Pascal®; Dako, Carpinteria, CA, USA) in citrate solution (pH 6.0). Endogenous

peroxidase activity was blocked by 3% hydrogen peroxide (Dinâmica®, SP, Brazil) in methanol (Dinâmica®, SP, Brazil). The slides were washed in phosphate buffered saline (PBS) throughout the whole process. The primary antibodies for COX-2 (Dako, Carpinteria, CA, USA; Clone CX294, 1:100) and Ki67 (Dako, Carpinteria, CA, USA; Clone MIB-1, 1:500) were incubated overnight at 4ºC. The immunoreactivity of these primary antibodies for canine squamous cell carcinoma has been demonstrated previously (Poggiani et al., 2012). A polymer system (EnVision®, Dako, Carpinteria, CA, USA) was used as secondary antibody for 30 minutes and the reaction revealed with 3,3'-Diaminobenzidine tetrahydrochloride (DAB, Dako, Carpinteria, CA, USA). After final washing in distilled water, the sections were counterstained with Harris hematoxylin, mounted, and assessed in an optical microscope (0400S Opticam®, SP, Brazil). The positive controls used was canine kidney for COX-2 and the epidermis as internal control for Ki67.

Quantification of Immunoreactivity

The percentage of COX-2 positive immunolabelled cells was assessed as semiquantitative evaluation (Score 0 = no labelling, 1 = 1 – 19%, 2 = 20 – 49% and 3 = ≥50% of marked cells) and according to staining intensity (Score 0 = No labelling, 1 = weak, 2 = moderate, 3 = strong) in 400x high power throughout the tumor (Prada et al., 2012). The product of percentage score and intensity score was the immunoreactivity score (IS) (Prada et al., 2012).

We selected the highest labeling fields for Ki67 analysis ("hot spots"). Immunoreactivity to Ki67 was evaluated by manually counting the positive cells in 1000 tumoral cells in 5 high-power fields (x400) and expressed as percentage of positive cells, the proliferative index (PI) (Dowsett et al., 2011).

Statistical Analysis

All statistical analysis was carried out using GraphPad Prism 5.0 software (San Diego, CA). A value of $P<0.05$ was considered statistically significant. Data are shown as mean ± standard deviation, or median, as indicated. Normality was assessed using the Shapiro-Wilk test. The immunolabelling scores and MI were compared using the Wilcoxon test for paired samples and the dependence between them evaluated with Spearman's correlation test. ANOVA and Mann-Whitney tests were used to compare the scores according to clinicopathological variables. In order to associate the difference in immunomarkers and clinicopathological variables Chi-Square or Fisher's exact test were used.

Results

In this study, there was no gender prevalence (7 male and 8 female dogs). The mean age at surgery was 9.7 ± 3.0 years (range, 4 – 15). Most dogs were from medium to large breed, with a mean body weight of 21.8 ± 10.6 kg (range, 6 – 47). The following breeds were represented- Mixed breed (8), Pitt Bull (4), Cocker Spaniel (1), Boxer (1), and Dalmatian (1). Owners reported all dogs had solar exposure. About 86% of the dogs had pink skin while 73% had white fur. The mean time from tumor first notice by the owner and surgery was 4.0 ± 2.8 months (range, 1 – 12).

The median number of tumors per patient was 1 (range, 1 – 4). Considering the largest tumor, the mean size of the longest diameter was 5.5 ± 3.0 cm (range, 2 – 12) while the shortest axis had a mean of 4.7 ± 27.8 cm (range, 1.7 – 11). The sum of both axis diameters had a mean of 10.3 ± 5.6 cm (range, 3.7 – 23.2 cm). Patients had a predominance of focal (46%, 7/15), followed by multifocal (33%, 5/15), and

disseminated (20%, 3/15) distribution. Most patients with multifocal and disseminated distribution exhibit signs of solar dermatosis and countless neoplastic lesions. The abdomen was the most common location for skin SCC, accounting for 80% (12/15), followed by limbs with 13% (2/15) and head with 6% (1/15).

All tumors had an ulcerated surface, while 53% (8/15) had signs of tumor infection and 26% (4/15) exhibit infiltration to adjacent structures (i.e. muscle, fascia, and penile body). Considering the TNM system, 26.6% (4/15) were T4, 40% (6/15) T3 and 33.3% (5/15) T2. On physical examination, 40% (6/15) of the dogs had local lymph node enlargement, but fine needle aspirate biopsy and further histology confirmed SCC metastasis in 26% (4/15), they were staged as N1b while the non-metastatic were N1a. None of the dogs had distant metastasis on 3-view thoracic radiographs and abdominal ultrasound. They were classified as M0 on TNM staging system.

After at least 21 days of piroxicam treatment, most of the dogs had stable disease; only one patient showed a partial response (i.e. at least 30% decrease in tumor size). None of the dogs, while receiving piroxicam, experienced treatment-related toxicity, been well tolerated by all patients. Curative-intent surgical resection was performed with 1-cm of free health tissues in 73% of the patients (11/15) and 2-cm in 20% (3/15) and only one case with >3-cm (amputation). Primary closure of the wound bed was possible in 80% of the cases (12/15), while the rest required a skin flap. One dog underwent forelimb amputation and another one a penectomy.

Histological Grade and Surgical Margins

According to Anneroth et al. (1984) grading system, 9 SCC were grade I (60%) and 6 grade II (40%). No patient had a grade III or beyond. The MI had an overall median

of 15 mitosis/10 hpf (range, 3 – 54). Grade I patients had a median MI of 22 mitosis/10 hpf (range, 3 – 46) and grade II had 11 mitosis/10 hpf (range, 3 – 54).

Surgical margins were free of neoplastic cells in 80% (12/15) of the patients; among them 75% (9/12), 16.6% (2/12) and 8.3% (1/12) were resected with 1-cm, 2-cm and >3-cm margin, respectively. Three dogs had incompletely resected cutaneous SCC; being two by incomplete lateral margin (both with 1-cm margin) and one with deep margin contamination (2-cm margin). A 1-cm surgical margin allowed complete resection on 81.8% (9/11) of the cutaneous SCC.

Immunohistochemistry

All patients (15/15) expressed immunoreactivity for COX-2 in both non-treated and treated samples. COX-2 immunostaining was focal and predominantly in the cytoplasm of neoplastic keratinocytes. In regard of labeling intensity on non-treated samples, there was weak, moderate and strong positivity in 2 (13.3%), 3 (20%) and 10 (66.6%) cases, respectively. Regarding the distribution, 66.6% (10/15) and 33.3% (5/15) had a percentage score 1 and 2, respectively; there was no score 3 stained tumors (Table 1). A non-significative (P=0.063) low positive correlation (Spearman's r=0.49; 95% CI, -0.046 to 0.807) between intensity and distribution could be found. The non-treated IS had a median of score 3 (range, 1 to 6), with 33.3% (5/15) exhibiting an immunoreactivity >4.

In treated samples, there was the prevalence of strong positivity in 7 (46.6%), followed by four of each for weak (26.6%) and moderate (26.6%) intensity. Concerning the percentage of stained cells, 80% (12/15) were score 1, and 20% (3/15) 2; with no score 3 (Table 1). No correlation was found between intensity and percentage of stained cells (Spearman's r=0.26; 95 CI, -0.296 to 0.695) on treated samples (p = 0.269). The

treated animals IS had a median of 3 (range 1 to 6), with 13.3% (2/15) of the cases exhibiting a IS higher than 4.

There was no significant difference ($P>0.05$) and correlation (Spearman's $r<0.21$; $P>0.05$,) between non-treated and treated COX-2 staining when comparing intensity, distribution and IS. However, 53.3% (8/15) of the patients had a decrease in IS after treatment (Figure 2), while 26.6% (4/15) had an increase. Three patients had a stable IS.

Table 1. Summary of the scores for non-treated and treated COX-2 immunolabelling according to cases. The IS is the product of the percentage score and intensity score.

Cases	Non-treated COX-2			Treated COX-2		
	% Cells	Intensity	IS	% Cells	Intensity	IS
1	2	3	6	1	3	3
2	1	2	2	1	3	3
3	1	1	1	2	2	4
4	1	1	1	1	1	1
5	2	3	6	2	3	6
6	1	3	3	1	1	1
7	1	2	2	1	3	3
8	2	3	6	1	3	3
9	1	3	3	1	3	3
10	1	3	3	1	2	2
11	1	2	2	1	1	1
12	2	3	6	1	2	2
13	2	3	6	1	2	2
14	1	3	3	1	1	1
15	1	3	3	2	3	6

Legend: % Cells = percentage score; Intensity = Intensity score; IS = immunoreactivity score; Grey lines = patients that exhibit a decrease in COX-2 expression after treatment.

Ki67 was expressed in all tumor samples. Immunolabeling was present in the nuclei with a granular pattern. The positive cells were present all over the tumor tissue but focus of a high density of positive cells ("hot spots") could be found. The mean Ki67 score on the non-treated sample was 60 ± 17.2 (range, 18 to 77) with 13.3% having less than 50% of positive cells. The treated sample had a mean of 52.2 ± 17.9 (range, 19 to 83) with 40% exhibiting less than half of the cells labeled. There was no statistical significance between non-treated and treated PI ($P>0.05$); nonetheless, when excluding

outliers (Figure 1A and 1B), there was a significative difference with the use of piroxicam (P=0.03). Outliers were detected based on the interquartile range. In fact, 66.6% of the patients exhibited a decrease in the Ki67 PI after treatment (Figure 2). No correlation was found between non-treated and treated PI (Spearman's r=0.08, P>0.05).

There was no dependence between all immunohistochemical markers and MI, tumor size, or evolution time in Spearman's test. All comparison resulted in a P>0.05 and a r<0.4.

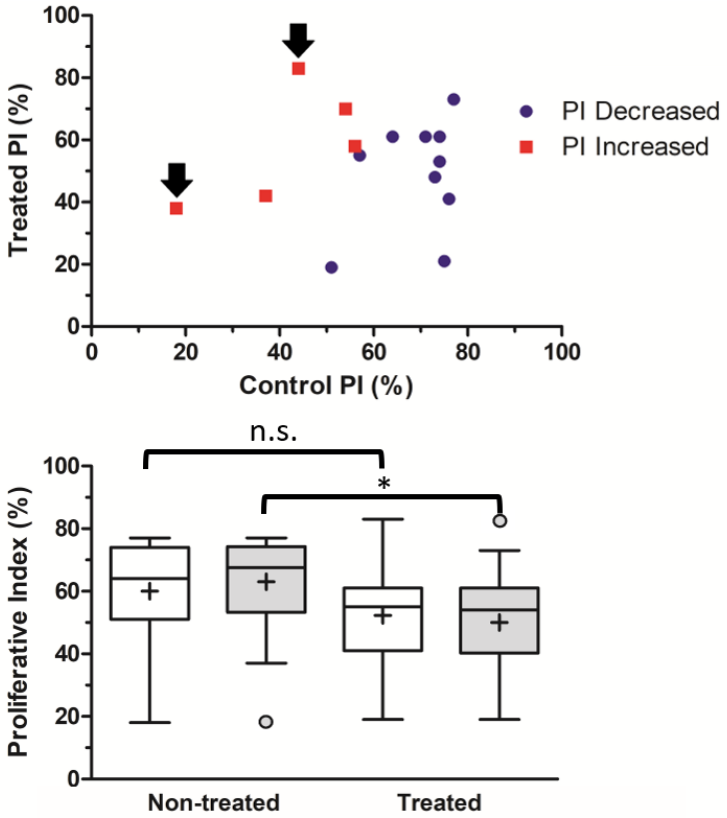

Figure 1. (A) Distribution graph of the proliferative index (PI) in non-treated and treated samples of canine squamous cell carcinoma. Black arrows indicating the outliers excluded (the lowest and highest PI). Blue points represent the patients in which the PI decreased after treatment, while the red squares are the cases that had an increase in PI. (B) Boxplot of non-treated and treated with (white) and without (grey) outliers. * shows statistical significance. n.s. show non-significance. Grey circles are the outliers.

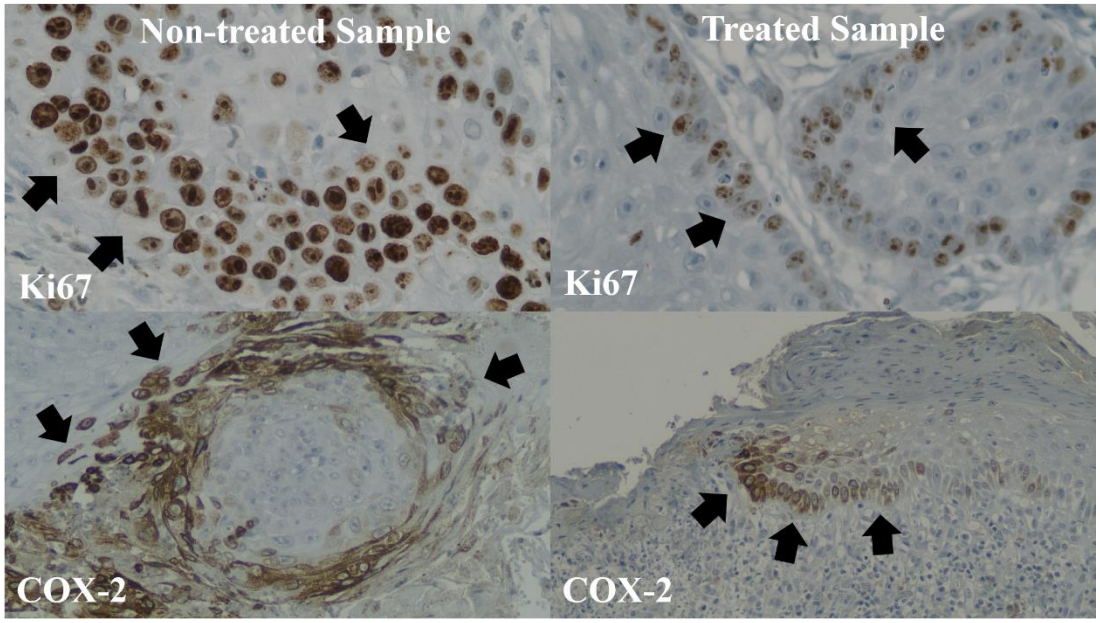

Figure 2. Photomicrography of canine cutaneous SCC immunolabelled for Ki67 and COX-2. Black arrows show stained cells. The non-treated sample "hot spot" had an 82% of the cells expressing Ki67, while after treatment with Px, the PI dropped to 42% of positive cells for Ki67 (400x). The COX-2 initially rated with an immunoreactivity score of 6 decreased to 2 after treatment with Px (200x).

There was no difference in COX-2 score in non-treated and treated samples according to T-stage and histologic grade (ANOVA, P>0.05). The same resulted from the Ki67 analysis (P>0.05). The mean and median of the scores and indexes are summarized in table 2.

Table 2. Median and Mean of COX-2 and Ki67, according to T-stage and histological grade (n = 15).

	N	COX-2		Ki67	
		Non-treated IS[a]	Treated IS[a]	Non-treated IP[b]	Treated IP[b]
T-stage					
T2	5	3 (2 – 6)	2 (1 – 6)	56.4 ± 13.9	46.6 ± 17
T3	6	6 (1 – 6)	2.5 (1 – 6)	58.3 ± 21.7	54.3 ± 12.4
T4	4	2 (1 – 3)	3 (1 – 4)	67.2 ± 15.5	56.2 ± 27.7
Lymph Nodes					
Normal	11	3 (1 – 6)	2 (1 – 6)	54.9 ± 17.43	51 ± 19.5
Metastatic	4	2.5 (1 – 6)	3 (2 – 4)	74.25 ± 2.75	55.7 ± 14.1
Histological Grade					
Grade I	9	3 (1 – 6)	3 (1 – 6)	57.2 ± 20.3	51.7 ± 18
Grade II	6	4 (1 – 6)	2.5 (1 – 6)	64.3 ± 11.5	53 ± 19.3
Overall	15	3 (1 – 6)	3 (1 – 6)	60 ± 17.2	52.2 ± 17.9

[a] = Score median; [b] = Index Mean;

In order to state an association between T-stage and histologic grade with an increase, decrease or stable COX-2 IS and Ki67 PI, we conducted several Chi-square/Fisher's test essays. There was no significative difference in distribution of patients that exhibit decrease or increase in immunolabelling and these clinicopathological variables (P>0.05). In fact, there was no association between COX-2 change after Px treatment with a decrease in Ki67 (P>0.05). However, 6 out of 15 patients (40%) had a decrease in both COX-2 and Ki67 (Table 3).

Table 3. Association between the difference of non-treated and treated COX-2 score and Ki67 proliferative index with clinicopathological variables in canine cutaneous SCC. (n=15).

	COX-2				Ki67		
	Increased	Stable	Decreased	P	Increased	Decreased	P
T-stage				0.1178[a]			0.8936[a]
T2	1	1	3		2	3	
T3	0	2	4		2	4	
T4	3	0	1		1	3	
Lymph node				1.000[a]			0.2308[b]
Normal	2	3	5		5	46	
Metastatic	2	0	2		0	4	
Histologic Grade				0.8856[a]			0.5804[b]
Grade I	2	2	5		4	5	
Grade II	2	1	3		1	5	
Ki67				0.5604[a]			
Increased	2	1	2				
Decreased	2	2	6				

[a] = Chi Square; [b]= Fisher's exact test;

Discussion

The present study aimed to access the effect of a short-term treatment with piroxicam prior to surgery, over some immunohistochemical parameters of cutaneous SCC in dogs. Px is widely used in low-dose continuous chemotherapy protocols to prevent or delay recurrence (Elmslie et al., 2008) and as an adjuvant treatment to chemotherapy (Knapp et al., 2016); however, to our knowledge, there is no previous

published study evaluating its use in canine cutaneous SCC. In fact, most studies report its use in transitional cell carcinoma of the bladder (Knapp et al., 1994), oral SCC (Schmidt et al., 2001), and inflammatory mammary carcinoma (de M Souza et al., 2009; Rossi et al., 2018), with favorable results. The choice for a 21-days treatment was made to mimic a continuous therapy with the objective to evaluate the *in vivo* effect of this drug over canine skin SCC. Research treating several types of tumors with Px reported that all antitumor responses were evident until the 28th day (Knapp et al., 1992).

All tumors expressed COX-2 and Ki67, as demonstrated previously (Pestilli de Almeida et al., 2001; Poggiani et al., 2012). Our patients with cutaneous SCC seem to have lower expression of COX-2 compared to mast cell tumors, which had an overall prevalence IS of 6 and 9 (Prada et al., 2012). Since all dogs had ulcerated surface tumors, sun exposure and considering the inflammatory component of the SCC, a higher expression of COX-2 was expected (Buckman et al., 1998; Bardagí et al., 2012; Millanta et al., 2016).

The COX-2 immunolabeling was associated with malignancy, the presence of ulceration and metastasis, tumoral recurrence, MI and even Ki67 immunostaining in mast cell tumor (Gregório et al., 2017), renal carcinoma (Carvalho et al., 2017) and melanocytic tumors (Martínez et al., 2011); However, none of these factors could be related to COX-2 expression in canine cutaneous SCC in our research and in humans (Thomas et al., 2005). Other studies also failed to find an association between COX-2 expression and clinicopathological variables of skin SCC (Millanta et al., 2016).

There was no difference in COX-2 expression after treatment with Px ($P>0.05$); however, more than half of the patients experienced a decrease in IS. A study treating canine actinic keratosis with firocoxib showed clinical improvement and decrease on COX-2 after 50 days of treatment (Albanese et al., 2013). These authors attributed the

poor response to treatment in a case to the evolution of pre-neoplastic lesions to malignant neoplasm, although the mechanism to drug failure in the neoplasm is unknown (Albanese et al., 2013).

COX-2 enzyme is required for the synthesis of prostaglandin E_2, a stimulant of keratinocyte proliferation (Albanese et al., 2013). The clinical benefit with the use of firocoxib in actinic keratosis suggests the antiproliferative effect of the COX-2 inhibitor treatment (Albanese et al., 2013). Our study showed a significant decrease in Ki67 immunostaining after treatment with Px ($P<0.05$), confirming that its antiproliferative effect occurs *in vivo* on cutaneous SCC. This phenomenon was demonstrated on xenograft model and *in vitro* assays with melanoma (Choisunirachon et al., 2015) and bladder carcinoma (McMillan et al., 2011; Knapp et al., 2016; Silva et al., 2017). The decrease in PI observed after treatment with Px can be evidence of the molecular benefit of the anti-COX-2 target therapy as an antiproliferative drug for canine cutaneous SCC.

The presence of antitumor effect in the absence of COX-2 inhibition, as observed in our study, with no decrease in COX-2 score was described on an *in vitro* study that concluded the non-steroidal anti-inflammatory, including Px, might act by a COX/prostaglandin-independent pathway (Yoshitake et al., 2017). The lack of COX-2 inhibition with a significant decrease in Ki67 *in vivo* may be supported by Yoshitake et al. (2017) data.

The necessity to exclude outliers of the Ki67 analysis lies on the concern of treatment bias, such as the poor owner compliance with drug delivery, uncontrolled sustained sun exposure, and the requirement of compounding the Px, which can lead to suboptimal dose, as demonstrated for other drugs used in continuous low-dose therapy (Burton et al., 2017).

Overall, non-treated samples had a high Ki67 expression, with a mean of 52%, ranging from 19% to 83%. Other tumors that had Ki67 as a standard prognostic factor showed a much lower cut-off. For example, the cut-off of Ki67 for human breast cancer was 20% (Tashima et al., 2015) and 25% for early-stage anal sac adenocarcinoma in dogs (Skorupski et al., 2018). Difficulty of comparing results lies on different methodologies to report Ki67, as some studies, such as with mast cell tumors, count the labeling as cells per grid and not as percentage of marked cells (Smith et al., 2017). Nonetheless, our study used a methodology described for veterinary patients and recommended by an international Ki67 working group for human breast cancer (Dowsett et al., 2011; Skorupski et al., 2018). The presence of a high density of cells in mitosis, even though the cutaneous SCC in dogs is a slow-growing tumor, reveals it is a very active tumor, at least in its borders, where the samples were harvest.

Conclusion

This is the first research addressing pre-operative Px effect in naturally-occurring cases (*in vivo)* of canine cutaneous SCC. The data supports that Px effectively decreases Ki67 expression independent of COX-2 decrease, rising optimism of its clinical benefit in these cases. Larger studies are necessary to evaluate the long-term administration of Px pre-operative in dogs with skin SCC.

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Conflict of Interest Statement

The authors declare no conflict of interest that could inappropriately influence the content of the research.

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2. Trabalho Científico 2

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Post-operative low-dose cyclophosphamide with piroxicam did not prevent recurrence of canine cutaneous squamous cell carcinoma

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Abbreviation: SCC, Squamous cell carcinoma; NSAID, Non-steroidal anti-inflammatory drugs; CYC, Cyclophosphamide; Px, Piroxicam; TNM, Tumor Node and Metastasis; CBC, Complete blood count; CR, Complete response; PR, Partial response; PD, Progressive disease; SD, Stable disease; DFI. Disease-free interval; MI, Mitotic Index;

ABSTRACT

This study was designed to evaluate the role of low-dose chemotherapy with cyclophosphamide (CYC) and piroxicam (Px) on the disease-free interval (DFI) of canine cutaneous squamous cell carcinoma (SCC). Nine dogs with surgically resected cutaneous SCC were continuously treated with Px (0.3 mg/kg, PO, q24h) combined with CYC (15

mg/m², PO, q24h) for 180 days and evaluated for recurrence every two months. This group was compared with a surgically treated retrospective control group (n = 9). No statistical difference in DFI ($p>0.05$) was verified when compared the treated (160 days) with the control group (145 days). Both groups were merged (n=18) for statistical purpose, to compare the DFI according to T-stage (DFI: T4 = 128 days, T3 = 205 days and T2 = 240 days), N-stage (DFI: Metastasis = 160 days; Free = 145 days), histological grade (DFI, moderately differentiated = 160 days; well differentiated = 145 days) and surgical margin status (DFI, compromised = 144 days; free = 160 days), but no differences were found. However, patients with T4-stage are 3.2-fold (95% CI 0.7529 – 13.99) and 4.8-fold (95% CI 1.9028 – 26.13) more likely to develop recurrence than T3 and T2-stage, respectively. Treatment was well tolerated for most patients, despite one that died of acute kidney disease, rising concerning about rational use of Px. Metronomic therapy with CYC and Px had no impact on the DFI of patients with cutaneous SCC.

Keywords: Dogs, spinocellular, piroxicam, cyclophosphamide, chemotherapy, metronomic.

INTRODUCTION

Squamous cell carcinoma (SCC) is a malignant tumor arising from epidermal keratinocytes with a slow-growing and locally aggressive biologic behavior (Hauck 2013, Millanta et al. 2016). The etiology of this neoplasm is multifactorial and ultraviolet solar radiation has an important role in actinic keratosis development, and malignant progression (Nikula et al. 1992). The early phase of the disease is characterized by a preneoplastic lesion known as actinic keratosis, that can progress to *in situ* SCC, sharing similar behavior and biologic features with malignant SCC (Röwert-Huber et al. 2007, Poggiani et al. 2012). Epidemiologic studies place the SCC as the first or second most common skin cancer in Brazil, an incidence of 7% to 15% of all cutaneous tumors (Souza et al., 2006; Fernandes et al., 2015). A similar study in the USA revealed a much lower incidence (1.25%) demonstrating this is a tropical disease related to sunlight exposure

(Villamil et al. 2011, Poggiani et al. 2012). Abdomen, other sites of hypotrichosis, and low pigmentation skin are the main sites of SCC (Poggiani et al. 2012).

As the tumor has low response rates to maximum tolerated dose chemotherapy, surgery is the gold-standard treatment for cutaneous SCC (Webb et al., 2009). Unlike traditional chemotherapy, the low-dose chemotherapy, also known as metronomic chemotherapy, is the high-frequency administration of oral chemotherapeutic agents aiming mainly an anti-angiogenic effect during the treatment (Biller et al. 2016, Natale & Bocci 2018). Other mechanisms of low-dose chemotherapy are the activation of the immune system, induction of dormancy and senescence, and modulation of growth factors (Mutsaers, 2009; Maiti et al. 2014).

The non-steroidal anti-inflammatory drugs (NSAIDs) are COX inhibitors, that besides analgesic and anti-inflammatory effects can also act as a chemopreventive drug (Alkan et al. 2012). Protocol combining cyclophosphamide (CYC) and piroxicam (Px), a non-selective COX-2 inhibitor, as low-dose chemotherapy protocol, decreased tumor recurrence of soft tissue sarcoma (Elmslie et al. 2008), provided overall survival time similar to other classic therapies in splenic hemangiosarcoma (Lana et al. 2008), stabilization of the disease, better quality of life and even a complete remission in dogs with spontaneous tumors (Marchetti et al. 2012).

Although continuously studied in a wide variety of tumors in animals (Lana et al. 2007, Elmslie et al. 2008, Lavalle et al. 2012, Marchetti et al. 2012, Schrempp et al. 2013), low-dose chemotherapy remains as an experimental treatment (Biller et al., 2014). The induction of stable disease is the aim of low-dose chemotherapy, and therefore, to maintain and improve long-term quality of life of patients with cancer (Mutsaers, 2009; Biller et al., 2014).

To our knowledge, no previous study has been published on the use of low-dose chemotherapy in canine cutaneous SCC. Therefore, we designed a prospective study to evaluate the effect of the use of low-dose chemotherapy based on CYC and Px for dogs with cutaneous SCC, considering disease-free interval. Adverse effects and other risk factors were also studied.

MATERIALS AND METHODS

This prospective study was approved by the Ethics Committee on Animal Use (CEUA/ School of Veterinary Medicine and Animal Science - São Paulo State University, UNESP) under protocol 5/2017. All owners were informed and consent before dogs were included in the experiment. For the treated group, client-owned canine patients with histologic confirmed cutaneous SCC, were clinically staged with three-view thoracic radiographic, abdominal ultrasound examination, urinalysis, complete blood count, and serum biochemistry profile (i.e. creatinine, urea, alanine aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, total protein, albumin, and globulin). Clinical staging was established according to the World Health Organization proposed TNM system for skin tumors (Owen 1980). In animals with multiple tumors, the highest stage was considered. Patients with ≥T2 stage regardless of lymph node involvement were prospectively included in the study. Animals with distant metastasis, previous treatment, T1 stage, minor residual disease or those whose owner decided to leave the study were excluded. The retrospective control group comprised the same inclusion and exclusion criteria from the treated group, but involved animal retrospectively treated between 2013-2016. The medical records were reviewed and patients without a consistent data over at least 6 months, complete exams before surgery or histologic confirmed recurrence were excluded. All dogs in the control group were treated with curative intent surgery without any other adjuvant treatment.

Nine dogs with naturally occurring SCC were eligible to receive the treatment with CYC and Px. From 120 dogs with cutaneous SCC diagnosed between 2013 and 2016, 9 dogs fulfilled all inclusion criteria to compose the control group.

For both groups, information regarding to age, gender, body weight, skin and fur colors, and clinical history was recorded. Macroscopic aspects of the evaluated tumors were noted and included two-dimension size (measured with digital caliper), anatomic location, distribution (i.e. focal, multifocal, and disseminated), morphological aspects (i.e. nodular, plaque, and ulcer), local invasiveness, presence of ulceration, infection, and actinic dermatosis, as well as lymph node involvement, were noted. Owners informed evolution time, considered the first-time owners noticed the mass, solar exposure, and comorbidities.

All dogs from the treated group received Px (0.3mg/kg, PO, daily) for at least 21 days until surgery. Curative intent surgery was performed with at least 1 cm lateral margins of macroscopic health tissue and an underlying fascia. In cases where disseminated neoplastic lesions were present around the main tumor, we resected with wide margins and proceeded with wound reconstruction with skin flaps, as necessary. Reactive or metastatic lymph nodes were resected as well. Skin closure occurred in a standard pattern.

After surgery, all dogs were treated with PO Amoxicillin and Clavulanic acid (22mg/kg, q12h), omeprazole (1 mg/kg, q24h) and dipyrone (25 mg/kg, q8h) for approximately seven days. At surgical discharge (about ten days postoperative), CYC (15 mg/m², PO, daily) and Px (0.3 mg/kg, PO, daily) were prescribed for continuous administration throughout the 6-months period. CYC (Genuxal®, Baxter Hospitalar Ltda, São Paulo, Brazil) and Px (Feldene®, Medley S.A. Indústria Farmacêutica, Campinas, Brazil) were compounded to dose at a human compounding pharmacy. These doses are

described as safe and effective (Burton et al., 2011, Harper et al. 2017), but to avoid sterile hemorrhagic cystitis owners were oriented to administer CYC early in the morning, provide free fresh water, and to encourage animals to urinate. Gastrointestinal protectants were not prescribed as standard as it is considered a risk factor for adverse events related to Px (Eichstadt et al. 2017).

Dogs treated with low-dose CYC daily were evaluated at two months interval for at least six months or until progressive disease/severe toxicity occurred. Reevaluation included full physical examination, tumoral recurrence or new tumors development mapping, CBC, serum biochemical analyses and urinalysis. At the sixth month postoperatively, triple thoracic radiographic views and abdominal ultrasound examination were performed. Lack of detectable tumor growth for >6 months was considered a positive response to treatment and then CYC and Px could either be suspended or continued according to the presence of pre-neoplastic lesions. Tumor response to treatment was classified as complete remission, partial remission, stable disease or progressive disease as established by Response Evaluation Criteria for Solid Tumors in Dogs Consensus (RECIST – VCOG; Nguyen et al., 2015). Briefly, complete remission (CR) was defined as disappearance of all target lesions; Partial remission (PR) when at least a 30% reduction in the sum of diameters of target lesion occurs; progressive disease (PD) when either the growth of one or more lesions or at least a 20% increase in the sum of diameters; and stable disease (SD) when less than 30% decrease or 20% increase in the sum of diameters of target lesions (Nguyen et al., 2015).

Low-dose chemotherapy was suspended when detected grade III or higher adverse event according to the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events (VCOG-CTCAE, 2011). Patients with toxicity grade I or II continued to receive medication after adverse effects have been treated.

The disease-free interval (DFI) was defined as the time from surgical resection of the tumor to the day dogs had a tumor recurrence or PD. Median DFI was calculated as the moment when 50% of the patients had a relapse or new tumors. Additionally, the authors subjectively evaluated the growth rate of tumor recurrence under the effect of CYC/Px over primary tumor growth rate reported by owners. The causes of death were described as either tumor-related or non-tumor related.

Tumor samples were fixed in 10% formalin for 48 hours, processed routinely, and embedded in paraffin for histologic evaluation. SCC was diagnosed in hematoxylin and eosin (HE) sections and classified according to the Broder's grading system (Goldschmidt, Shofer, 1992) as well differentiated, moderately differentiated and poor differentiated. Mitotic Index was defined as the total number of mitotic figures in 10 high power fields (400x). Surgical margins were evaluated in HE-stained sections as well (Kamstock et al., 2011).

The DFI curves were plotted using the Kaplan-Meier Analysis and compared using the log-rank test (Mantel-Cox and Mantel-Haenszel test). Differences in DFI were considered statistically significant for $p<0.05$. Data is shown as mean and standard deviation or median according to normality (Shapiro-Wilk test). Comparison between mitotic index among the groups was performed with Mann-Whitney test ($p<0.05$ considered significant). As there was no difference in variables among both groups, we merged control and treated group to evaluate the influence of clinical stages, histologic grade, margin and lymph node status on DFI curves. Data were analyzed using GraphPad Prism 5.0 software (San Diego, CA).

RESULTS

The mean age of the treated and control groups were 10 ± 3.0 years (range, 4-13 years) and 9.5 ± 4.0 years (range, 5 – 16 years), respectively. The mean weight of the animals were 21 ± 7.5 kg (range, 8-32 kg) and 12 ± 7.0 kg (range, 2 – 24 kg) on the treated and control group. There were five female and four male dogs on the treated group and six females and three males in the control group. Mixed breed dogs were most commonly affected with 50% (9/18), followed by Pitbull with 27.7% (5/18) and Cocker Spaniel, Boxer, poodle, and pinscher with 5.5% (1/18) each. While considering only treated group, the mixed breed was still the most common with 44%, Pitbull with 33%, Cocker Spaniel and boxer with 11% each. Control group had a prevalence of mixed breed dogs with 55%, followed by Pitbull (22%), poodle and pinscher (11% each). About skin and fur color in the treatment group, two animal had dark-colored skin and fur while seven had white skin and fur. All dogs were exposed to sunlight.

Tumors of the treated group and control had a median evolution time of 3.5 months (range, 1 – 8 months) and 2 months (range, 1 – 6), respectively. Data regard location, distribution, T-stage, N-stage, macroscopic appearance and invasiveness of the tumors are described (Table 1).

The longest diameter of the tumors had a mean of 6.7 ± 2.66 cm (range, 3.4 – 12 cm). The number of main nodules had a homogeneous median of 2 (range, 1 – 4) in both treated and control group. In addition, dogs with multifocal and diffuse SCC had countless small lesions near main tumors. Distant metastasis to lungs and abdominal organs was not found throughout treatment time in both groups. Table 2 summarize data from both groups.

Table 1. The summary of clinical features, macroscopic appearance and lymph node status of treated (n = 9) and control groups (n = 9).

	Treated Group		Control Group		Total	
	n	%	n	%	N	%
Location						
Ventral Abdomen	6	66	5	55	11	61.1
Members	2	22	1	11	3	16.6
Thorax			2	22	2	11.1
Cervical/Face	1	11	1	11	2	11.1
Distribution						
Focal	3	33	5	55	8	44.4
Multifocal	3	33	1	11	4	22.2
Disseminated	3	33	3	33	6	33.3
Ulcerated Surface	9	100	9	100	18	100
Infection						
Present	7	77	5	55	12	66.6
Absent	2	22	4	44	6	33.3
Invasiveness						
Present	2	22	2	22	4	22.2
Absent	8	88	8	88	16	88.8
T-Stage						
T4	3	33	2	22	5	27.5
T3	5	55	3	33	8	44.4
T2	1	11	4	44	5	27.5
Lymph Node						
Metastasis	4	44	2	22	5	27.7
Reactive Hyperplasia	2	22	0	0	2	11.1
Not enlarged	3	33	7	77	10	55.5

Table 2. Summary of the main characteristics of all patients enrolled in the study. Legend: TNM = Tumor, nodes and metastasis system of clinical staging; MI = Mitotic Index; DFI = Disease-free interval (days); Well = well differentiated; Mod = moderately differentiated; * Censored due to early death unrelated to tumor.

ID	Size (cm)	Local	Distribution	TNM	Histologic Grade	MI	Surgery	DFI
1T	8.0	Abdomen	Multifocal	$T_3N_{1b}M_0$	Well	26	Resection	330*
2T	7.5	Hindlimb	Focal	$T_4N_{1b}M_0$	Mod	5	Resection	160
3T	8.7	Forelimb	Focal	$T_4N_{1b}M_0$	Mod	11	Amputation	160
4T	6.7	Abdomen	Multifocal	$T_3N_{1a}M_0$	Well	3	Resection	448*
5T	3.4	Face	Focal	$T_3N_0M_0$	Mod	15	Resection	145
6T	12	Abdomen	Disseminated	$T_4N_{1a}M_0$	Well	22	Resection	128
7T	6	Abdomen	Disseminated	$T_3N_0M_0$	Well	46	Resection	71
8T	3.2	Abdomen	Disseminated	$T_2N_0M_0$	Well	7	Resection	96*
9T	4.9	Abdomen	Multifocal	$T_3N_{1b}M_0$	Well	12	Resection	266
1C	7	Abdomen	Multifocal	$T_3N_0M_0$	Well	4	Resection	412

2C	4	Abdomen	Focal	$T_3M_{1b}M_0$	Well	22	Amputation	145
3C	5	Thorax	Focal	$T_4N_0M_0$	Well	3	Resection	62
4C	3	Abdomen	Disseminated	$T_2N_0M_0$	Well	1	Resection	39
5C	4	Abdomen	Disseminated	$T_2N_0M_0$	Well	14	Resection	391
6C	1.5	Thorax	Focal	$T_3N_0M_0$	Well	2	Resection	89
7C	3.5	Neck	Focal	$T_2N_0M_0$	Poor	15	Resection	240
8C	2.5	Forelimb	Focal	$T_4N_{1b}M_0$	Well	7	Amputation	75
9C	4	Abdomen	Disseminated	$T_2N_0M_0$	Mod	6	Resection	182

In treated group, all dogs were submitted to tumor resection, one of them underwent forelimb amputation. Seven out of 9 (77%) patients in the treated group had its tumor removed with 1 cm margins, the other two with 2 cm and >3 cm (amputation) each. Reconstruction of the wound bed was necessary in 2 cases. Free margins could be obtained in 6 cases (66%), 5 of them after 1 cm wide surgical margins (55%). The other 3 patients had compromised lateral (2/9) or deep (1/9) margins, with 1 cm (both lateral margins) and 2 cm wide (deep) surgical margins. Two patients with compromised margins on the treated group had a recurrence. On the control group, 5 out of 9 (55%) had compromised margins.

The histologic evaluation identified 6/9 well differentiated (66%) and 3/9 moderately differentiated (33%) in the treated group. Regarding the control group, 7 out of 9 (77%) were considered well differentiated, only 1 (11%) moderately differentiated and 1 (11%) poorly differentiated. Mitotic index had wide variability among both histologic grades, with an overall median 9 (range, 1 – 46 mitotic figures). Well-differentiated tumors had a median 7 (range, 1 – 46 mitotic figures), while moderately differentiated had a median of 8.5 (range, 5 – 15 mitotic figures), but no statistical difference was found ($p>0.05$). In the treatment group, the mitotic index had an overall median of 12 mitotic figures (range, 3 – 46), with a median of 11 (range, 5 – 15) and 17 mitotic figures (range, 3 – 46) in well and moderately differentiated tumors, respectively.

No difference could be found between well and moderately differentiated mitotic index in the treated group ($p<0.05$). In the control group, well-differentiated tumors had a median of 4 mitotic figures (range, 1 – 22), while moderately and poorly differentiated had only one patient each, with 6 and 15 mitotic figures/10 fields, respectively.

In general, CYC and Px therapy was well tolerated by the study population. There was no impairment in wound healing process using low-dose chemotherapy, even in patients undergoing reconstructive surgery. Two animal developed adverse events related to treatment protocol, one of them evolved into renal toxicity (ID 8T in table 1). This 13-years-old dog had hypertension (280 mmHg) and grade III acute kidney injury (Urinary density: 1.012; Serum creatinine: 7.83 mg/dl) after 60 days of CYC and Px administration that lead to death despite treatment with fluid therapy, electrolytic replacement, and anti-hypertensive drugs. The other one developed grade I vomit and anorexia at the 30th day, that was treated with temporarily Px and CYC discontinued. Serum biochemical evaluation and hematologic parameters did not reveal any treatment-related changes throughout the therapy period. None of the dogs developed sterile hemorrhagic cystitis or hematuria.

In the treated group, 6 out of 9 dogs (66%) had tumoral recurrence during or after the treatment and 3 of them died of unrelated causes (33%) before recurrence. All animals in the control group showed recurrence sometime after surgery. The median DFI of the group treated with CYC and Px was 160 days (range, 71 – 448 days), with no statistical difference ($p=0.3289$) from the median DFI of 145 days (range, 39 – 412) of the control group (Figure 1A). However, at the 120th day 77% of the patients treated group were free of tumors against only 55% of the control group. The 1-year disease-free rate was of 22% (2 out of 9) for both treated and control group. Supposing all the living dogs of the treated group developed a recurrence by the time this study was finished, the predicted minimum

DFI would be 155 days. The mean time with CYC/Px therapy was 113 ± 50.9 days (range, 50 – 185 days). We were not able to calculate an overall survival curve since most dogs died from causes unrelated (3/9; 33%) to the tumor or are alive (3/9; 33%). The control group failed to report this information.

In addition, the DFI for dogs with SCC was compared according to TNM system. Since both groups were considered homogeneous and no difference was found in overall DFI, we grouped the patients for statistical purpose. When comparing the DFI of patients according to T-stage (DFI: T4 = 128 days; T3 = 205 days; T2 = 240 days), no difference was present between T4-T3 (p=0.11), T4-T2 (p=0.06) and T3-T2 (p=0.49) as assessed by Log-Rank analysis (Figure 1B). However, patients with T4 tumors were 3.2 and 4.8-fold more likely to develop tumors during the study time than T3 (Hazard Ratio (HR): 3.245; 95% confidence interval (CI): 0.7529 – 13.99) and T2 (HR: 4.857; 95% CI: 0.9028 – 26.13), respectively. No risk was observed between T3 and T2 dogs (HR: 0.6090; 95% CI: 0.1485 – 2.498). Pearson's correlation test between disease-free interval and the longest tumor diameter found a weak (r = 0.2632) but not significant (p=0.0692) correlation (Figure 1C). No correlation was found between mitotic index and DFI as well (r = -0.0687; p = 0.7865).

A similar analysis of DFI was performed for lymph node status and no difference was found (p=0.9758) when metastatic lymph nodes (DFI = 160 days) were compared with free or no affected lymph nodes (DFI = 145 days; Figure 1D). Dogs with local metastatic lymph nodes were not in higher risk to develop new tumors than N0 or N1a dogs (HR: 0.9824; 95% CI: 0.3134 – 3.080).

Moderately differentiated tumors had a DFI of 160 days and well-differentiated tumors had a DFI of 145 days, a statistically not significant difference in Log-Rank test (p=0.6962; Figure 1E). Patients with moderately differentiated tumors did not have a

greater chance of recurrence than well-differentiated tumors (HR: 0.7683; 95% CI: 0.2036 – 2.899). However, no patients with moderately differentiated tumors were free of tumors after 182 days, while 38.4% (5/13) of the well-differentiated tumors did not had recurrence yet. Finally, the DFI was not affected by histologic margins (p=0.2793; Figure 1F). Dogs with incompletely resected SCC had a DFI of 144 days, while those with free margins had a DFI of 160 days. Compromised margins were not a risk factor for the development of new SCC lesions (HR: 1.328; 95% CI: 0.4634 – 3.808).

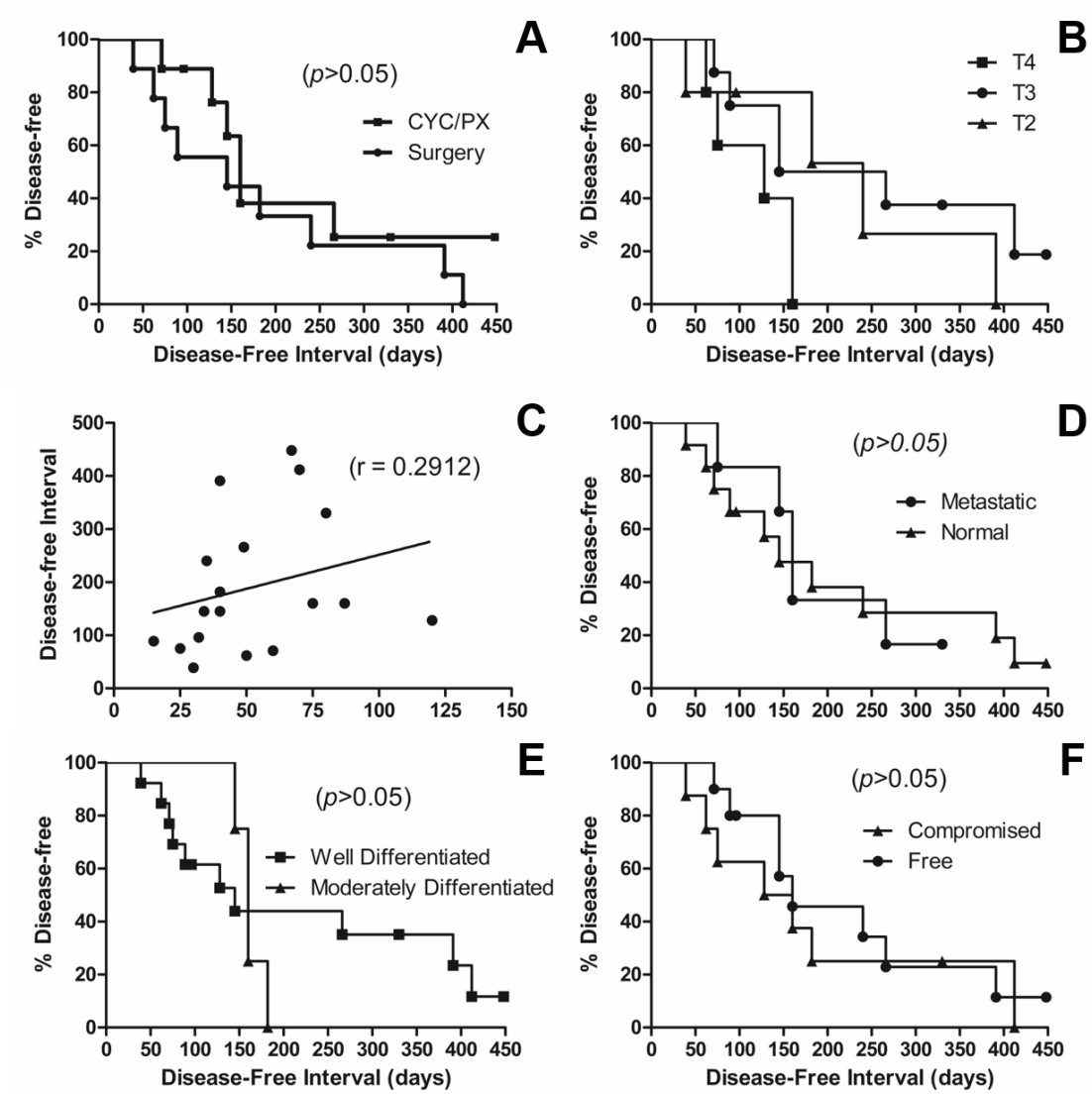

Figure 1. Disease-free interval (DFI) curves for cutaneous canine SCC. (A) DFI curve for treated (surgery/CYC/Px) and control (only Surgery) groups of dogs with SCC (Log-Rank test, p=0.32). (B) DFI curve for T-stage groups (P>0.05). (C) Correlation between DFI and tumor size (r=0.2912). (D) DFI curve for N-stage (p=0.97). (E) DFI curve according to histologic grade (p=0.16). The (F) DFI curve for incomplete resected SCC (p=0.59).

DISCUSSION

In a large study of canine skin tumors, SCC represented 1.25% of all skin tumors identified (n = 25,996). Dogs with age between 7 and 10 years and >10 years are in 2.59 (95% CI 2.05 – 3.28) and 4.67 (95% CI 3.73 – 5.85) times more likely to develop cutaneous SCC than control, respectively (Villamil et al. 2011). Our results corroborate with this data, as the mean age of tumor development in treated and control group were within that limit. Dalmatians and Basset Hound were especially at risk for SCC (Villamil et al. 2011), while in our study mixed breed dogs and Pitbull were the most prevalent. Pitbull usually have short white hair and pink skin features highly predisposed to sunlight mutation (Poggiani et al. 2012).

Human studies suggest the use of COX-2 inhibitors to decrease the incidence of actinic keratosis and SCC (Butler et al. 2005, Greenhough et al. 2009). Other veterinary studies suggest the same effect, as canine SCC also express strong labeling for COX-2 (Bardagi et al. 2012, Poggiani et al. 2012). The multi-target mechanism of action proposed to metronomic chemotherapy, increased our interest to study if these drugs could provide a better prognosis for cutaneous SCC tumors. We proposed that the association of Px as a COX-2 inhibitor and CYC would prevent recurrence of SCC as well. However, our results have demonstrated that the use of low-dose CYC and full-dose Px was not effective in preventing the formation of new neoplastic lesions and local recurrence, showing a low efficiency as a chemopreventive protocol in this population. The authors expected to see better results with this anti-angiogenic drug since cutaneous SCC exhibits a higher microvessel density in CD31 immunostaining in later stages (Maiolino et al., 2001).

This is the first study to address low-dose chemotherapy in canine cutaneous SCC. Dogs in treated group and control group had a similar DFI, with a high rate of recurrence

within 1 year (33% and 22%, respectively), making clear that, despite local control, other factors, such as actinic keratosis, *in situ* SCC and diffuse skin sensibilization to UV radiation may interfere with SCC formation of new lesions (Webb, 2009; Poggiani et al. 2012). Other author also failed to show the efficacy of similar protocols in osteosarcoma (Matsuyama et al. 2018). The resistance to anti-angiogenic drugs is one of the main causes of failed therapy in metronomic, low-dose regimens. This occurs as cancer cells can develop escape mechanisms or tolerance to low oxygen environments (Maiti et al. 2014). Additionally, the difficulty to minimize the continuous sunlight exposure and the cumulative aspect of solar radiation (Wu et al. 2014) on animals may stand out against the chemopreventive effect of CYC and Px.

The authors provided the drugs to compound and the owners were responsible to maintain and to give the medication to the animals at home. Since there is no way to make sure the medication was well stored and correctly administered by the owners, this configures the main fragility of the study. The bias of a home care might also contribute to sustained solar UV exposure, despite the owner were warned to keep dogs away from the sun.

Leach et al., (2011) had described recurrence after treatment with continuous low-dose chlorambucil; however, reported a slower rate of progression than seen prior to the treatment. Also, some neoplastic lesions grew until reaching a size and then remained stable (Leach et al., 2011). Despite not effective in preventing recurrence, it is impossible to determine with our data if CYC and Px decreased the growth rate or provided a stable size. To evaluate this phenomenon, it would be necessary a CYC/Px group without surgery for recurrence, which would deprive these patients of the surgical resection, the gold-standard treatment for SCC.

The treatment was well tolerated by most patients, and no classic adverse event usually related to CYC was seen. This probably occurred due to short-term administration of the protocol, the whole treatment did not overcome 185 days, with a mean of 113 days. Other studies had a longer survival/disease-free period, requiring a much longer treatment, and so relating a 40-49% rate of mild adverse events and 10-58% sterile hemorrhagic cystitis, although the median time to develop hemorrhagic cystitis was 90-110 days (Elmslie et al. 2008, Harper et al. 2017, Matsuyama et al. 2018).

One patient without previous reported disease developed severe arterial systemic hypertension, acute kidney injury and died, rising concerning over the use of COX-2 inhibitors in patients susceptible to kidney disease. Caution is recommended when prescribing this medication, especially in old animals, and frequent control of renal function should be performed, as well as surveillance of gastrointestinal signs of toxicity (Eichstadt et al. 2017).

Current literature fails to state an evidence-based recommendation for surgical margins in cutaneous SCC as occurs with other tumors. For cutaneous SCC in cats, a minimum of 5 mm of lateral margins is recommended (Murphy, 2013). An author suggests empirically at least 2 cm margin for invasive canine SCC (Nimwegen & Kirpensteijn, 2018). Due to irregular and poorly defined edges, it is a challenge to define clinical limits of cutaneous SCC in humans, a primary factor that directly influences surgical margin (Gualdi et al., 2015). This issue can be seen in dogs as well. A general recommendation is to resect low and high-risk human cutaneous SCC with 4 to 5 mm and 6 to 13 mm, respectively, to provide 95% of complete resection (Gualdi et al., 2015). We aligned our decision to use at least 1 cm margins with this current human recommendation.

The *en bloc* resection of cutaneous SCC plus at least 1 cm of healthy tissue provided free lateral margins in 78% of the treated group (7/9). In dogs with nasal plane SCC, 1 cm margin of healthy tissue, provided 66% of free margins, and all incompletely resected tumors recurred (Lascelles et al., 2000).

Cutaneous SCC is locally aggressive and low metastatic, increasing the importance of local control with surgery (Webb et al., 2009). Although free of macroscopic neoplastic lesions and free lateral margins in most of the animals, the presence of actinic keratosis, pre-neoplastic lesions such as Bowenoid carcinoma *in situ*, and solar-induced carcinogenesis may have interfered in the results (Poggiani et al., 2012). These effects are not present in other types of tumors and can overcome the low-dose continuous chemotherapy effect. Nevertheless, the presence of those conditions in most patients in this study proves that CYC/Px does not positively affect patients at risk of or with pre-neoplastic lesions. SCC induced by UV sunlight may have a *TP53* mutation in other sites and so new lesions can grow despite local control (Webb et al., 2009).

To our knowledge, there is no retrospective or prospective study published describing the DFI of canine cutaneous SCC. In fact, information about SCC in other locations can easily be found. A series of clinical (i.e. TNM-stage) and histologic (i.e. margins and grade) risk factors were evaluated (n = 18), but all failed to show any difference in DFI. Despite not being significant, T-stage DFI curve showed a much shorter time for patients in T4. Due to low statistical power, a study with a greater number of dogs probably would show significance in this parameter. Patients bearing a T4-stage tumor are in 3.2-4.8-fold higher risk to develop local tumor recurrence or new tumor within the time of the study. To be staged as T4, tumors must invade subcutaneous tissue and deeper layers, such as fascia, muscle, bone or cartilage, and are often >5 cm in size (Owen, 1980). A 3.8-fold increase in local recurrence was found in humans with

cutaneous SCC greater than 20mm, a result similar to our study (Mullen et al. 2006). Unfortunately, this cut-off from humans does not seem to be useful for dogs, since all cases in this study, except for one, had a >2 cm tumor. Recurrent cutaneous SCC in humans is more likely to occurs as greater maximum diameter and high-risk features (Clayman et al. 2005).

There was no difference in DFI according to histologic grade ($p<0.05$). Moderately and well-differentiated tumors had a median DFI (160 days against 145 days, respectively). Although the median DFI was very close, the moderately differentiated Kaplan-Meier curve showed a sharper and earlier decrease than the well-differentiated one. The small number of patients can explain the absence of difference between histologic grade. In humans, poorly differentiated cutaneous SCC is a risk factor (HR 4.26; 95% CI 2.31 – 7.85) for metastasis (Brougham et al. 2014), but only one poorly differentiated tumor was diagnosed in our study. The mitotic index was not evaluated as a risk factor since it was inconsistent within short and long DFI, and a cut-off could not be proposed. The mitotic index could not be used as a prognostic factor for canine cutaneous SCC.

An invasive front grading system, using the deep invasive edge of the tumor was proposed as more reliable than the surface or center of neoplasm (Nagamine et al. 2017). Tumor thickness and depth of invasions are the most important prognostic factors for metastasis in humans with cutaneous SCC, with a 2 mm cutoff is no metastatic, for 2 – 6 mm a metastatic rate of 3.8 – 4.5% and 15 – 15.6% for that thicker than 6mm (Breuninger et al. 1990, Brantsch et al. 2008).

Finally, there was no evidence to support the use of post-operative low-dose continuous chemotherapy with CYC and Px for dogs with cutaneous SCC. Future clinical trials should be focused on combining other drugs or treatment modalities. Additionally,

T-stage was the only known risk factor for recurrence described in dogs with cutaneous SCC that can predict high-risk patients and allow a reliable prognosis.

CONCLUSIONS

The combined therapy of low-dose CYC and Px in dogs undergoing surgery for resection of cutaneous squamous cell carcinoma did not alter the prognosis of these patients. In addition, T-stage appears to be the only clinical risk factor for new lesions. A long-term research with numerous cases should be carried out to elucidate the clinical benefit of this therapy for SCC.

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Conflict of interest statement

The authors declare no conflicts of interest.

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CAPÍTULO 3

1. Considerações Finais

Os estudos do tipo coorte prospectivo tem um alto grau de dificuldade e diversos fatores limitantes. Entre eles, pode-se destacar o alto custo financeiro e pessoal dedicado a pesquisa. As limitações tradicionais deste tipo de estudo parecem se acentuar quando se lida com animais tutorados, pois a relação médico veterinário e tutor deve ser profunda e periódica para que se evite a evasão do estudo.

Ainda sobre a evasão, esta foi a principal dificuldade de se completar o número de amostras do delineamento inicial, seguido da quebra dos critérios de inclusão nas etapas iniciais de seleção. No entanto, de um modo geral, os tutores dos animais que efetivamente participaram do estudo se mostravam dispostos a contribuir com a pesquisa e receber a medicação no período pós-operatório.

A ausência de um grupo controle prospectivo recebendo placebo se justifica pela dificuldade de se obter casos de CCE dentro dos critérios de inclusão no tempo disponível. Em dois anos de estudo cerca de 25 casos passaram pelo estudo, sendo que somente 15 completaram o tratamento. A inclusão do grupo controle significaria o declínio no número de indivíduos no grupo tratado.

Além disso, questões técnicas envolvendo a marcação imunohistoquimica prejudicaram o objetivo inicial de avaliar o efeito anti-angiogênico do piroxicam juntamente com o efeito anti-inflamatório e anti-proliferativo. Felizmente acredita-se que estas dificuldades ainda serão superadas.

Apesar das dificuldades encontradas, inerentes a qualquer estudo clínico prospectivo, os dados encontrados são inéditos e preenchem de certa forma uma lacuna existente na literatura tanto no acompanhamento e prognostico de pacientes com CCE, quanto na indicação de quimioterapia metronômica nestes pacientes. Entretanto, é sabido que um estudo utilizando um número amostral maior de animais com CCE ainda é necessário para validar os resultados.

ANEXO 1

Resumo das características e resultados de todos os pacientes estudados

ID	Sex	Age (years)	Breed	Local	Distribution	T	N	M	Tumor Size (cm)‡	HG (Grade)	Treated MI (MF/10hpf)	Control COX-2 (IS)	Treated COX-2 (IS)	Control Ki67 (%)	Treated Ki67 (%)	DFI (days)	ST (days)
1	Male	10	PitBull	Abdome	Multifocal	T3	N1b	M0	110	I	26	6	3	71	61	330*	329*
2	Female	6	PitBull	Member	Focal	T4	N1b	M0	130	II	5	2	3	77	73	160	404*
3	Male	13	Mixed	Member	Focal	T4	N1b	M0	174	II	11	1	4	73	48	160	377
4	Female	10	Mixed	Abdome	Multifocal	T3	N1a	M0	129	I	3	1	1	18	38	448*	391*
5	Male	12	Cocker	Face	Focal	T3	N0	M0	68	II	15	6	6	57	55	145	192
6	Female	4	PitBull	Abdome	Disseminated	T4	N1a	M0	232	I	22	3	1	75	21	128	120
7	Male	6	Mixed	Abdome	Focal	T4	N0	M0	157	I	16	2	3	44	83	†	†
8	Female	13	Mixed	Abdome	Disseminated	T3	N0	M0	120	I	46	6	3	74	61	71	280*
9	Female	12	Mixed	Abdome	Disseminated	T2	N0	M0	71	I	7	3	3	64	61	96†	96*
10	Male	10	Boxer	Abdome	Multifocal	T3	N1b	M0	97	I	12	3	2	76	41	266	264*
11	Female	8	PitBull	Abdome	focal	T2	N0	M0	64	II	3	2	1	51	19	226*	226*
12	Male	8	Mixed	Abdome	Focal	T3	N0	M0	77	II	54	6	2	54	70	324*	32*
13	Female	8	Mixed	Abdome	Multifocal	T2	N0	M0	3,7	II	21	6	2	74	53	246*	246*
14	Female	15	Mixed	Abdome	Multifocal	T2	N0	M0	50	I	41	3	1	56	58	227*	227*
15	Male	11	Dalmata	Abdome	Focal	T2	N0	M0	62	I	39	3	6	37	42	217*	217*

Grey Lines = Patients included only in study number 1; White Lines = Patients included in both studies; * = Data censored; † = Patient lost post-operative evaluation; ‡ = The sum of bidimensional axis of the tumor;