

UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CAMPUS DE JABOTICABAL

**Comparison of two different doses of bleomycin in
electrochemotherapy protocols for feline cutaneous
squamous cell carcinoma nonsegregated from ultraviolet
light exposure**

Denner Santos Dos Anjos

Médico Veterinário

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light exposure**

Discente: Denner Santos Dos Anjos

Orientador: Prof. Dr. Andriago Barboza De Nardi

Coorientador: Prof. Dr. Carlos Eduardo Fonseca-Alves

Tese apresentada à Faculdade de
Ciências Agrárias e Veterinárias –
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CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: COMPARISON OF TWO DIFFERENT DOSES OF BLEOMYCIN IN ELECTROCHEMOTHERAPY PROTOCOLS FOR FELINE CUTANEOUS CELL CARCINOMA NONSEGREGATED FROM ULTRAVIOLET LIGHT EXPOSURE

AUTOR: DENNER SANTOS DOS ANJOS

ORIENTADOR: ANDRIGO BARBOZA DE NARDI

COORIENTADOR: CARLOS EDUARDO FONSECA ALVES

Aprovado como parte das exigências para obtenção do Título de Doutor em MEDICINA VETERINÁRIA, área: Clínica Médica Veterinária pela Comissão Examinadora:

Andrigo Barboza de Nardi

Prof. Dr. ANDRIGO BARBOZA DE NARDI (Participação Virtual)
Departamento de Clínica e Cirurgia Veterinária / FCAV / UNESP - Jaboticabal

Andrigo Barboza de Nardi

Prof. Dr. BRUNO COGLIATI (Participação Virtual)
FMVZ/USP / São Paulo/SP

Andrigo Barboza de Nardi

Prof. Dr. RAFAEL RICARDO HUPPES (Participação Virtual)
Departamento de Clínica e Cirurgia Veterinária / UNICESUMAR - Maringá/PR

Andrigo Barboza de Nardi

Prof. Dr. JORGE LUIZ COSTA CASTRO (Participação Virtual)
Pontifícia Universidade Católica do Paraná / Curitiba/PR

Andrigo Barboza de Nardi

Profa. Dra. PAOLA CASTRO MORAES (Participação Virtual)
Departamento de Clínica e Cirurgia Veterinária / UNESP / FCAV - Jaboticabal

Jaboticabal, 31 de março de 2021

ABOUT THE AUTHOR

DENNER SANTOS DOS ANJOS –born in Rondonópolis, Mato Grosso, on January the 19th of 1990, child of Alexandre Mario dos Anjos and Pérola Mendes Santos dos Anjos. He studied Veterinary Medicine in Federal University of Mato Grosso do Sul, Campo Grande, and took his degree in December 2012 under the supervision of Dr. Veronica Jorge Babo-Terra. He worked as a resident of internal medicine at the same university (UFMS), from 2013 to 2015. He performed international externships in the oncology department of *University of Madrid*, and *Aúna Especialidades Veterinarias (Valencia, Spain)*. He also performed international externship in internal medicine department of *Hospital Veterinario de Santiago*, Chile. He started his Master of Science (MSc.) studies in 2015 in the University of Franca (UNIFRAN), Franca, São Paulo, area of Animal Science with emphasis in oncology, under the supervision of Profa. Dra. Sabryna Gouveia Calazans. In March 2017, he started his Doctoral studies (PhD.) in Faculty of Agricultural and Veterinary Sciences under the Graduate Program of Veterinary Medicine, area of Veterinary Medical Clinics of the São Paulo State University – UNESP, Campus of Jaboticabal under the supervision of Prof. Dr. Andrigo Barboza De Nardi. During his Postgraduate studies, he was elected as a monitor in the *lato sensu* Posgraduate course of Veterinary Oncology in the Bioethicus Institute, Botucatu, SP from 2016 to 2017. He has published several articles and scientific abstracts in international and national journals and annals of congress. He is a collaborator researcher from Biopulse company, Naples, Italy. Nowadays, he is taking *lato sensu* Posgraduate course in Endocrinology and Metabolic in Small Animal Medicine in the Anclivepa Institute, São Paulo.

“Não podemos acrescentar dias à nossa vida, mas podemos acrescentar vida nos
nossos dias”

Cora Carolina

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Câmpus de Jaboticabal



CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Eficácia comparativa entre dose convencional e reduzida de bleomicina na eletroquimioterapia em felinos com carcinoma de células escamosas cutâneo"**, protocolo nº 016279/19, sob a responsabilidade do Prof. Dr. Andriago Barboza De Nardi, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 18 de dezembro de 2019.

Vigência do Projeto	01/01/2020 a 01/07/2020
Espécie / Linhagem	Felinos
Nº de animais	40
Peso / Idade	5-10 Kg / 1 -15 anos
Sexo	Machos e Fêmeas
Origem	Serviço Eletro-Onkovet

Jaboticabal, 18 de dezembro de 2019.

Fabiana Pilarski
Profª Drª Fabiana Pilarski
Coordenadora – CEUA

Comparação de duas doses diferentes de bleomicina em protocolos de eletroquimioterapia para carcinoma de células escamosas cutâneo felino não segregados da exposição à luz ultravioleta

RESUMO- O carcinoma de células escamosas cutâneo (CCEc) é um dos tumores mais comuns em gatos devido à exposição crônica à luz ultravioleta. Tratamentos locais como a eletroquimioterapia (ECT) promovem o controle da doença ou mesmo a remissão completa. Nossa hipótese é que os gatos poderiam se beneficiar de tratamentos com bleomicina em dosagens reduzidas. Um estudo prospectivo não randomizado simples-cego avaliou os parâmetros clínicos, local de lesão, estadiamento, intervalo livre de doença (DFI) e tempo de sobrevida comparando a dose padrão de bleomicina (15.000 UI / m²) (n = 22) com uma dose reduzida (10.000 UI / m²) (n = 34) em gatos com CCEc que foram submetidos à ECT como única modalidade de tratamento. Nenhuma diferença estatisticamente significativa em DFI ou sobrevida global foi observada entre os 2 grupos. Um maior DFI foi encontrado em gatos com tumor de tamanho pequeno em comparação com gatos com tumor de tamanho grande (P = 0,045) (mediana de 335 vs. 60 dias, respectivamente). Além disso, foi observado um tempo de sobrevida global reduzido para gatos com estágio clínico avançado no grupo padrão SG (T3 e T4) (P = 0,004) quando comparado aos gatos com estágio clínico inicial (T1 e T2). Em conclusão, a ECT usando ambas as doses de bleomicina pode atingir a mesma taxa de resposta em termos de resposta geral, DFI e sobrevida global.

Palavras-chave: bleomicina, gatos, carcinoma cutâneo, eletroporação, dose reduzida, tamanho do tumor

Comparison of two different doses of bleomycin in electrochemotherapy protocols for feline cutaneous squamous cell carcinoma nonsegregated from ultraviolet light exposure

ABSTRACT- Cutaneous squamous cell carcinoma (cSCC) is one of the most common tumors in cats due to chronic exposure to ultraviolet light. Local treatments such as electrochemotherapy (ECT) promote disease control or even complete remission. We hypothesize that cats could benefit from treatments using bleomycin at reduced dosages. A prospective nonrandomized single-blind study evaluated the clinical parameters, site lesion, staging, disease-free interval (DFI) and survival time by comparing the standard dose of bleomycin (15,000 UI/m²) (n=22) with a reduced dose (10,000 UI/m²) (n=34) in cats with cSCC that underwent ECT as the sole treatment modality. No statistically significant difference in DFI or overall survival was observed between the 2 groups. A higher DFI was found in cats with a small tumor size compared with that for cats with a large tumor size ($P = 0.045$) (median 335 vs. 60 days, respectively). Furthermore, a reduced overall survival time for cats with a higher stage in the standard group SG (T3 and T4) ($P = 0.004$) was observed when compared to that for cats with a lower stage (T1 and T2). In conclusion, ECT using both doses of bleomycin may achieve the same response rate in terms of the overall response, DFI, and overall survival.

Keywords: bleomycin, cats, cutaneous carcinoma, electroporation, reduced dose, tumor size

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CAPÍTULO 1 – Considerações gerais

1. INTRODUÇÃO

O carcinoma de células escamosas cutâneo (CCEc) corresponde a 10% das neoplasias da pele em felinos, acometendo principalmente felinos de pele clara expostos a radiação ultravioleta de forma crônica (Murphy, 2013). Dentre as terapias indicadas para o CCEc citam-se a cirurgia, criocirurgia, radioterapia, terapia fotodinâmica ou manejo medicamentoso. Dentre elas, a terapia de maior êxito ainda é a ressecção cirúrgica para lesões em região de pavilhão auricular, pálpebras e plano nasal em estágios mais avançados (T3 ou T4). Contudo, uma das limitações do procedimento cirúrgico é o resultado cosmético final, não sendo bem aceito por alguns tutores (Murphy, 2013).

A eletroquimioterapia (EQT) é um tratamento que associa a administração sistêmica ou local de fármacos antineoplásicos (normalmente lipofóbicos) à aplicação de pulsos elétricos permeabilizantes com amplitude, duração e ondas específicas (Spugnini et al., 2012). Seu uso em humanos está atualmente direcionado ao tratamento de neoplasias cutâneas incluindo o tratamento paliativo de metástase de tumores de pele (Benevento et al., 2012; Mali et al., 2013). A EQT também tem se destacado na medicina veterinária devido à sua eficácia, facilidade de aplicação e baixa toxicidade tornando-se, para alguns tipos de neoplasias, a primeira linha de tratamento em cães e gatos (Spugnini et al., 2012; Spugnini e Baldi, 2014).

Contudo, diferentes estudos têm utilizado doses variadas de bleomicina (15-30 mg/m²) em gatos diagnosticados com CCEc (Tozon et al., 2014; Spugnini et al., 2015; Spugnini e Baldi, 2019) e em humanos na dose de 10-15 UI/m² (Groselj et al., 2017) mostrando eficácia semelhante. Frente a isso, e aliado ao fato de que alguns países como o Brasil tiveram a medicação suspensa pela indústria farmacêutica, hipotetizamos que a dose reduzida de bleomicina (10 UI/m²) apresentaria o mesmo resultado que a dose convencional (15 UI/m²).

Objetivou-se realizar um estudo prospectivo clínico comparativo não-randomizado cego com dois protocolos diferentes de bleomicina (dose convencional versus dose reduzida) em gatos com CCEc submetidos à EQT como terapia única.

2. Revisão de literatura

2.1 Carcinoma de células escamosas cutâneo (CCEc)

O CCE, também denominado de carcinoma espinocelular ou carcinoma epidermoide, origina da diferenciação dos queratinócitos correspondendo a 10% dos tumores cutâneos em gatos (Walder, 1995; Murphy, 2013). Comparativamente, é uma das neoplasias malignas mais comuns em pessoas e estima-se que cerca de duas mil pessoas morrem anualmente nas Américas em decorrência do CCEc (Buckman et al., 1998).

Tanto em pessoas quanto em animais a prevalência para este tipo de enfermidade aumenta com a idade e não existe predileção sexual ou racial (Buckman et al., 1998; Salashe, 2000; Graf et al., 2016; Muzic et al., 2017). Em gatos, o CCEc é localmente invasivo demonstrando maior acometimento em áreas com menor quantidade de manto piloso como pinas, pálpebra, região nasal e região temporal da face (Murphy, 2013). Sua etiologia principal em felinos decorre pela exposição solar crônica a radiação ultravioleta, sendo que felinos brancos ou felinos com áreas despigmentadas apresentam maior risco para seu desenvolvimento neoplásico (Dorn et al., 1971; Murphy, 2013). Além disso, o CCEc também pode se desenvolver devido à infecção do papilomavírus observado em 84% dos gatos protegidos da luz UV e 40% dos gatos expostos a luz UV (Munday et al., 2011).

Mais de 80% dos casos desenvolvidos de CCEc em humanos eram provenientes de lesões prévias à dermatite actínica (Mittelbronn et al., 1998) ilustrando o processo da carcinogênese anterior ao desenvolvimento do CCEc.

Os CCEc são neoplasias localmente agressivas com baixa taxa de metástase (13%) (Withrow e Straw, 1990; Kirpensteijn et al., 1994; Rogers et al., 1995; Lascelles et al., 2000), semelhantemente ao que ocorre em humanos, com cerca de 5% de metástase regional pelo CCEc (Brougham et al., 2012; Schmults et al., 2013; Karia et al., 2014). Como a maioria dos CCEc não se metastatizam, o tratamento local efetivo como cirurgia, crioterapia, radioterapia, terapia fotodinâmica, quimioterapia e eletroquimioterapia, fornece aumento do tempo de sobrevida na maioria dos caso (Muller e Kirk, 2013; Spugnini et al., 2016).

O tratamento cirúrgico, pinectomia e/ou nosectomia, apresenta efetividade, porém o resultado cosmético pode não ser satisfatório para alguns tutores (Withrow & Straw, 1990; Ruslander et al., 1997; Lascelles et al., 2000), porém apresentam qualidade de vida.

Estudos anteriores relataram tempo livre de doença de 365 dias em casos de carcinoma de células escamosas cutâneo avançado em felinos, pelo qual foi necessário realizar abordagem de cirurgia reconstrutora (Schmidt et al., 2005; Hunt, 2006). Já a crioterapia pode fornecer taxas de respostas de 83% para felinos com CCEc em região auricular, palpebral e nasal. Contudo, recomenda-se que essa terapia seja indicada para lesões pequenas, pois trata-se de uma terapia superficial (Muller e Kirk, 2013).

A radioterapia também é mais efetiva para lesões superficiais. A média de tempo livre de doença para felinos com CCEc em plano nasal tratados com ortovoltagem ou teleterapia de cobalto variou de 12 a 16 meses (Lana et al., 1997). Um dos efeitos adversos da radioterapia em pacientes felinos é o risco de desenvolver triquíase e lesão de córnea secundária como ceratite ulcerativa (Lana et al., 1997; Pinard et al., 2012). A taxa de resposta relatada com o uso da radioterapia hipofracionada em felinos com CCEc é de 52% (correspondendo a 40% remissão completa e 12% remissão parcial) com média de sobrevida de 224 dias e tempo livre de doença de 271 dias (Pinard et al., 2012).

A terapia fotodinâmica também apresenta melhor taxa de sucesso para lesões pequenas (menores que cinco centímetros ou não invasivas). Lesões maiores podem apresentar remissão, porém observa-se maior risco de recidiva (Hahn et al., 1998; Muller e Kirk, 2013). Bexfield et al. (2008) observaram taxa de resposta de 85-90% em 55 gatos submetidos a terapia fotodinâmica.

Já a quimioterapia sistêmica para CCE apresenta eficácia limitada, porém, a quimioterapia intralesional tem sido utilizada com melhor resposta tanto em gatos quanto em cães com CCEc (Kitchell et al., 1995; Theon et al., 1996).

Por fim, a eletroquimioterapia (EQT) tem se destacado nos últimos anos na medicina veterinária e na medicina (Cadossi et al., 2014).

Seu objetivo é aumentar a eficácia de agentes antineoplásicos lipofóbicos, como bleomicina e cisplatina através do aumento de moléculas dentro do citoplasma da célula tumoral após a exposição do campo elétrico externo através de pulsos elétricos permeabilizantes na membrana celular (Spugnini e Baldi, 2019). Estudos comprovam a eficácia do tratamento utilizado em cães e gatos com CCEc em região nasal, periocular e de cabeça, resultando melhor efeito cosmético, bem como sua eficácia como tratamento adjuvante em sarcomas de tecidos moles excisados de forma incompleta obtendo maior controle local (Spugnini et al., 2007; Tozon et al., 2014; Spugnini et al., 2015; Dos Anjos et al., 2019).

2.2 Eletroquimioterapia

A EQT é um tratamento que associa a administração sistêmica ou local de fármacos antineoplásicos (normalmente lipofóbicos) com a aplicação de pulsos elétricos permeabilizantes com amplitude, duração e ondas específicas (Spugnini et al., 2012). Se destacado na medicina veterinária devido a sua eficácia, facilidade de aplicação e baixa toxicidade, tornando-se, para alguns tipos de neoplasias, a primeira linha de tratamento em cães e gatos (Spugnini et al., 2012; Spugnini e Baldi, 2019).

O princípio da EQT consiste na aplicação de pulsos elétricos curtos e de alta intensidade sobre as células neoplásicas, promovendo a exposição do campo elétrico sobre a membrana celular, induzindo um aumento transitório da permeabilidade à várias moléculas na membrana plasmática. Isso facilita a passagem de fármacos lipofóbicos como a bleomicina e cisplatina e, por conseguinte, aumentando sua citotoxicidade (Spugnini e Baldi, 2019). Esses campos elétricos externos induzem uma alta voltagem transmembrana (superando o limite crítico fisiológico da membrana celular), levando a formação de poros aquosos na superfície da membrana celular (Miklavcic et al., 2014) (Figura 1).

Os fármacos que têm sido utilizados mais frequentemente em EQT são a bleomicina (BLM) e a cisplatina (cis-diaminodicloroplatina-II) (CDDP), tanto em humanos, quanto em medicina veterinária. Ambos os fármacos têm o DNA como seu alvo e apresentam baixa capacidade em atravessar a membrana celular, pela sua polaridade (Escoffre e Rols, 2012).

Outros fármacos como netropsina, carboplatina, 2-n-metil-9-hidróxielipticinio, vincristina, actinomicina D, citarabina, mitomicina C, vimblastina, paclitaxel, doxorubicina, metotrexato, melfalan, gencitabina e gencitabina, já foram testados e apresentaram baixa ou insignificante potencialização (Orlowski et al., 1988; Gehl et al., 1998; Jaroszeski et al., 2000). Kulbacka et al. (2014), avaliaram o efeito da eletroporação quanto ao transporte de doxorubicina em células de adenocarcinoma gastrintestinal com super expressão de glicoproteína P, e observaram maior distribuição da doxorubicina na célula, confirmando aumento da sua citotoxicidade.

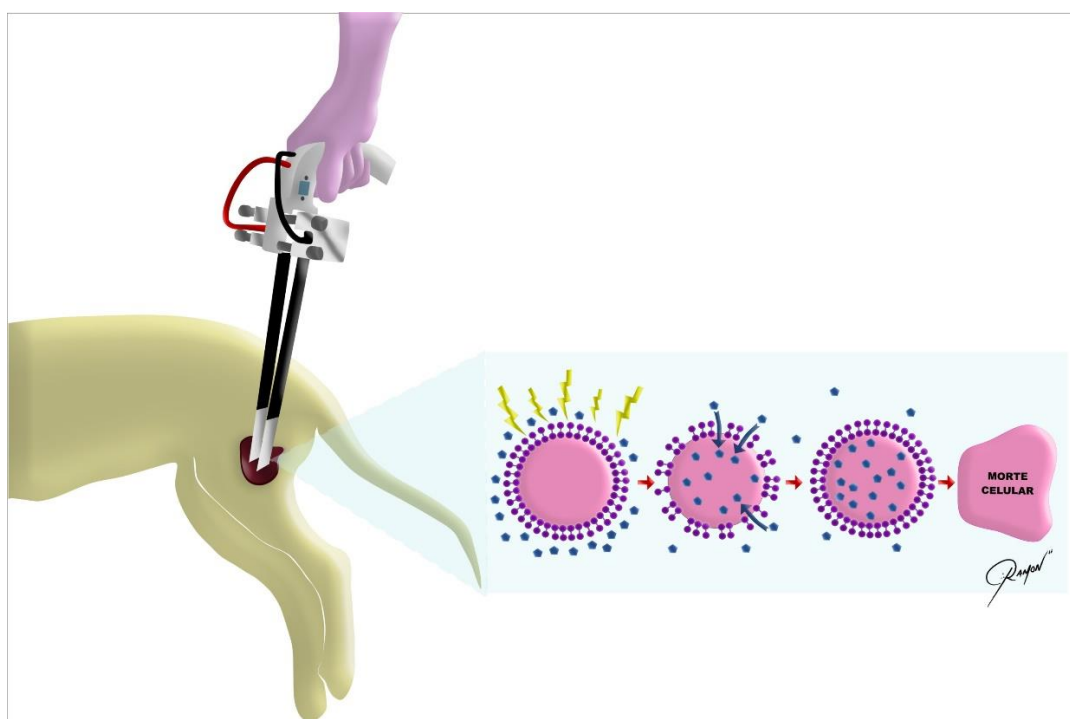


Figura 1. Imagem ilustrativa de um dos mecanismos de ação da eletroquimioterapia (aumento da permeabilidade da membrana celular). Observa-se da esquerda para a direita, a aplicação com eletrodo em placa sobre o tecido tumoral, pelo qual o campo elétrico, através dos pulsos elétricos, com amplitude e voltagem específica, induzirá a formação de poros aquosos na superfície da membrana celular. Após a formação dos poros, maior quantidade de moléculas lipofóbicas como bleomicina ou cisplatina, migrarão para o interior do citoplasma. Uma vez que o campo elétrico é cessado, ocorrerá o fechamento da membrana plasmática, com maior quantidade de moléculas antineoplásicas em seu interior, aumentando consequentemente seu efeito citotóxico, e por fim, morte celular (Dos Anjos et al. 2020).

2.3 Mecanismo de ação

Após a exposição dos pulsos elétricos, uma cascata de eventos intracelulares são ativadas como a ativação de bombas transportadoras de íons, produção e consumo de adenosina trifosfato (ATP), ativação de proteases, liberação de espécies reativas de oxigênio (ROS), liberação de antígenos tumorais, liberação de moléculas associadas ao dano celular (DAMPs), ativação de apoptose e necrose culminando na morte celular (Calvet et al., 2014; Spugnini e Baldi, 2019; Dos Anjos et al., 2019).

Além do aumento da permeabilidade da membrana celular via formação de poros aquosos e maior quantidade do fármaco intracelular; a EQT também apresenta efeitos anti-angiogênicos e o estímulo do sistema imunológico (Miklavcic et al., 2014; Spugnini e Baldi, 2019). Como dito anteriormente, um dos mecanismos principais da EQT é o aumento da permeabilidade da membrana celular pela exposição das células ao campo elétrico, consequentemente aumentando a passagem de BLM e CDDP para o interior da célula. O aumento da citotoxicidade da BLM e CDDP foi observada *in vitro*, demonstrada pelo aumento de sua potencialização (Mir et al., 1991; Sersa et al., 1998; Jaroszeski et al., 2000) e *in vivo* em diferentes tumores e modelos animais (Sersa et al., 1995; Heller et al., 1995; Jaroszeski et al., 1997; Pendas et al., 1998; Hyacinthe et al., 1999). Atualmente, a BLM é o fármaco predominantemente utilizado em EQT, porém também pode ser utilizada cisplatina, e menos comumente doxorrubicina, carboplatina, mitoxantrona e cálcio (Spugnini e Baldi, 2019; Staresinic et al., 2018; Dos Anjos et al., 2019; Dos Anjos et al., 2019b)

A EQT também apresenta alguns efeitos vasculares distintos. Além de aumentar o consumo do fármaco nas células estromais, a EQT também atua nas células endoteliais dos vasos sanguíneos tumorais induzindo a morte celular endotelial (apoptose) e consequentemente a supressão do fluxo sanguíneo tumoral, causando um efeito de dano vascular local (Sersa et al., 2008). Outro efeito é a vasoconstrição das arteríolas, demonstrada nos tumores em vasos sanguíneos normais e tumorais (Markelc et al., 2013). Esse efeito de bloqueio vascular (denominado *vascular lock*) promove a permanência do fármaco no interior do tumor por tempo prolongado, proporcionando melhor ação do quimioterápico (Jarm et al., 2010). Isso significa, que este fenômeno também impede o influxo do fármaco nos tumores se este for administrado após a aplicação dos pulsos elétricos (Miklavcic et

al., 2014).

O envolvimento da resposta imunológica do organismo também poderá influenciar a eficácia da EQT. Evidentemente, devido à heterogeneidade das células tumorais, em relação a sua disposição, tamanho e distribuição irregular no tumor, nem sempre todas as células são efetivamente erradicadas pela EQT (Sersa et al., 1997). Isso se deve ao fato de algumas células não serem eletroporadas e/ou o fármaco não estar disponível para todas as células neoplásicas (Muftuler et al., 2006).

Isso ocorre devido à influência da vascularização intratumoral. Um estudo ao avaliar a vascularização em carcinomas e melanomas, observou-se que a eficácia da EQT foi mais proeminente em carcinomas que melanomas, devido a maior distribuição de pequenos vasos no interior do tumor. Essa característica histopatológica ilustra a importância do microambiente tumoral e a resposta pela EQT (Groselj et al., 2018).

Entretanto, semelhantemente a outros métodos físicos como, por exemplo, a radioterapia, a fração de células residuais quando suficientemente baixas, podem ser eliminadas pelo sistema imunológico do organismo. Nestes casos, existe a necessidade de um organismo devidamente imunocompetente para a erradicação completa da neoplasia após a EQT, como demonstrado por Sersa et al. (1997), pela qual a taxa de cura em camundongos imunodeficientes foi significativamente mais baixa que nos imunocompetentes.

Pesquisas destacam o recrutamento de células apresentadoras de antígenos, infiltração de linfócitos T CD8⁺ e diminuição de células T regulatórias após o tratamento com EQT em pacientes humanos, murinos e cães com melanoma (Gerlini et al., 2012; Ursic et al., 2018; Milevoj et al., 2019).

Portanto, as diferentes respostas podem ser explicadas por fatores como permeabilidade da membrana celular (Spugnini e Baldi, 2019), distribuição do fármaco, que também depende da vascularização do tumor (Jarm et al., 2010; Groselj et al., 2018) e imunogenicidade tumoral (Sedlar et al., 2012; Gerlini et al., 2012; Ursic et al., 2018; Milevoj et al., 2019).

2.4 Indicações

Baseado no mecanismo de ação da EQT, a atividade antitumoral deve ser observada em todos os tipos de tumores, independentemente de sua origem histológica, mas sua eficácia parece variar, dependendo do tipo tumoral (Cemazar et al., 2001). A EQT pode ser indicada no controle de melanoma oral, tanto como terapia única quanto em associação à citorredução cirúrgica em cães (Spugnini et al., 2006; Milevoj et al., 2019).

Atualmente, o uso da EQT na espécie humana está sendo voltado ao tratamento de neoplasias cutâneas incluindo o tratamento paliativo de metástase cutânea de tumores mamários (Benevento et al., 2012; Mali et al., 2013; Cabula et al., 2015), tumores cutâneos como carcinoma vulvar (Perrone et al., 2019) e de controle de recidiva e metástase cutânea em região de pescoço por carcinoma papilar de tireoide (Grau et al., 2016). Contudo, estudos também indicam um futuro promissor na utilização da EQT para o tratamento de carcinomas hepatocelulares (Djokic et al., 2018) e adenocarcinoma pancreático (Trueba-Arguiñarena et al., 2015).

Dada à segurança e aos mínimos efeitos colaterais, a EQT é um tratamento seguro para tumores superficiais (Heller et al., 1998; Marty et al., 2006; Larkin et al., 2007; Sersa et al., 2008b). Alguns efeitos tardios podem ocorrer, como eritema, edema e necrose. Todos esses efeitos costumam ser transitórios, locais e bem tolerados pelos pacientes (Miklavcic et al., 2014; Dos Anjos et al., 2019). Em medicina veterinária, citam-se dor transitória após o procedimento e necrose (consequência de resposta eficaz) (Tozon et al., 2005).

Inúmeros estudos têm sido realizados em veterinária, avaliando o emprego da EQT em diferentes tipos histológicos, assim como a sua utilização como terapia adjuvante ou neoadjuvante em cães e gatos com neoplasias em estágio avançado (Spugnini et al., 2007a; Silveira et al., 2010; Tozon et al., 2010; Spugnini et al., 2011a; Spugnini et al., 2011b; Spugnini et al., 2012; Spugnini et al., 2015; Spugnini et al., 2016). O emprego da EQT pode ser limitado em casos de tumores constituídos por tecidos fibrosos, o que dificulta a penetração dos eletrodos com as agulhas, prejudicando a passagem do pulso elétrico (Campana et al., 2009).

Mir et al. (1997) observaram extensa necrose induzida pela EQT em gatos com sarcoma de aplicação caracterizado por infiltração difusa de macrófagos, linfócitos e eosinófilos peritumorais. Em um estudo com 127 animais, dentre eles cães e gatos acometidos por tumores como melanoma oral, sarcoma de tecidos moles, mastocitoma e carcinoma de células escamosas, observou-se que, durante a fase inicial da EQT, houve resposta inflamatória aguda com presença de neutrófilos, linfócitos e plasmócitos, seguida por extensa necrose e apoptose. Já durante a fase final da EQT, duas semanas após a aplicação, observou-se ausência de resposta inflamatória, com presença de necrose, tecido cicatricial e apoptose das células tumorais residuais. O padrão apoptótico nessa segunda fase pode ser uma consequência não só da EQT, mas também pela imunidade celular mediada, dependente da presença de população mista de linfócitos T e B (Spugnini et al., 2007a).

Dos Anjos et al. (2019), avaliaram após o tratamento com EQT a proliferação celular através do Ki67 e proteínas pró- e anti-apoptóticas como Bax e Bcl-2 em cães com CCEc, e uma redução significativa da proliferação celular foi observada após o tratamento. Entretanto, no 21^a dia, não houve diferença das proteínas relacionadas a apoptose quando comparada ao início do tratamento, sugerindo que outros mecanismos possam estar envolvidos na morte celular.

Além disso, Spugnini et al (2007a) observaram três casos de sarcoma de aplicação em gatos que não apresentaram controle local com a EQT, recidivaram com um tipo histológico menos agressivo, semelhante a neurofibroma. De acordo com o mesmo estudo, os níveis de necrose e apoptose após EQT apresentaram correlação significativa com o tempo de sobrevida dos pacientes, assim como demonstrado em carcinoma pulmonar em humanos e carcinoma de células escamosas, melanoma oral e sarcoma em animais, sendo considerado um fator prognóstico importante (Groeger et al., 2004; Spugnini et al., 2007b, c, d).

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CHAPTER 2. Comparison of two different doses of bleomycin in electrochemotherapy protocols for feline cutaneous squamous cell carcinoma nonsegregated from ultraviolet light exposure¹

Denner S. Dos Anjos^{1*}, Oscar R. Sierra¹, Enrico P. Spugnini², Andriago B. De Nardi^{†1}, Carlos E. Fonseca-Alves^{†*3,4}

¹ Department of Veterinary Clinic and Surgery, São Paulo State University (UNESP), Jaboticabal, Brazil

² Biopulse srl, Via Toledo 256, Naples 80132, Italy

³ Department of Veterinary Surgery and Animal Anesthesiology, São Paulo State University - UNESP, Botucatu-SP, Brazil.

⁴ Institute of Health Sciences, Paulista University-UNIP, Bauru-SP, Brazil.

*Correspondence: denner.anjosoncology@gmail.com; +556798144-8191

†These authors contributed equally to this work.

Abstract: Cutaneous squamous cell carcinoma (cSCC) is one of the most common skin tumors in cats due to chronic exposure to ultraviolet light. Local treatments such as electrochemotherapy (ECT) promote disease control or even complete remission. We hypothesize that cats could benefit from treatments using bleomycin at reduced dosages. A prospective nonrandomized single-blind study evaluated the clinical parameters, site lesion, staging, disease-free interval (DFI) and survival time by comparing the standard dose of bleomycin (15,000 UI/m²) (n=22) with a reduced dose (10,000 UI/m²) (n=34) in cats with cSCC that underwent ECT as the sole treatment modality. No statistically significant difference in DFI or overall survival was observed between the 2 groups. A higher DFI was found in cats with a small tumor size compared with that for cats with a large tumor size (P = 0.045) (median 335 vs. 60 days, respectively). Furthermore, a reduced overall survival time for cats with a higher stage in the standard group SG (T3 and T4) (P = 0.004) was observed when compared to that for cats with a lower stage (T1 and T2). In conclusion, ECT using both doses of bleomycin may achieve the same response rate in terms of the overall response, DFI, and overall survival.

Keywords: bleomycin, cats, cutaneous carcinoma, electroporation, reduced dose, tumor size

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Introduction

Cutaneous squamous cell carcinoma (cSCC) accounts for 10% of skin tumors in cats due to chronic exposure to ultraviolet (UV) light [1,2]. The lesions usually develop in pale or unpigmented skin with white areas being at the greatest risk [3]. Since fur is a physical barrier to UV radiation, the most commonly areas affected by cSCC are the ears, eyelids, nasal planum, lips and temporal areas [4-6]. Most of these tumors have an insidious progression characterized by nonhealing scabbing lesions that tend to progress over time to ulcers. As a result, these cats are mostly diagnosed in late stages, showing rapid tumor growth with advanced disease that requires aggressive treatment such as surgery combined with radiation therapy [1,7].

Among treatments indicated for cSCC (surgery, cryosurgery, radiotherapy, photodynamic therapy or medical management), surgical excision is the most successful way of treating advanced stage lesions (T3 or T4) of the pinna, eyelids, and nasal planum. However, the major limitation of surgery is the cosmetic outcome being unacceptable for some owners [1].

Chemotherapy has been used for controlling local disease or downstaging gross disease, but its use has been limited in feline cSCC due to the poor response rate [1,8-10]. However, the use of lipophobic agents has been used combined with electroporation, promoting cell death after enhancing the uptake of these drugs within neoplastic cells [1,6]. This therapy, known as electrochemotherapy (ECT), has been investigated over the past 15 years as an additional treatment modality for local control of solid tumors [4-6,11]. In the veterinary oncology field, it can be used as palliative, adjuvant, or neoadjuvant treatment or administered at the time of surgery (intraoperative ECT) [11].

ECT uses either bleomycin or cisplatin (both lipophobic agents), and its efficacy has been shown in different types of tumors with different outcomes [12]. Bleomycin has been considered the cornerstone drug for ECT since its uptake is facilitated after permeabilization of the cell membrane by electric pulses, resulting in cytotoxicity improvement by 700-fold [11]. Bleomycin fragments the DNA, which results in G2-M phase arrest of the neoplastic cycle [11,13-16].

In addition, studies have used different doses of bleomycin ranging between 15 to 30 mg/m² in cats diagnosed with cSCC with varied results [5,6,11].

In humans, a reduced dose has been proposed (10,000 UI/m²) to show the same efficiency as the standard dose (15,000 UI/m²) [17] in elderly patients with nonmelanoma head and neck skin cancer. Furthermore, other authors have also used a reduced dose of 10 mg/m² and found good results in humans [18]. Likewise, some countries have experienced a serious shortage of this drug secondary to decreased production by manufacturers. Since the reduced dosage presented good results in humans, we hypothesize that cats could benefit from treatments using the reduced dosage. Thus, this study aimed to evaluate the clinical parameters, site lesion, staging, disease-free interval, and survival time by comparing the standard dose of bleomycin (15,000 UI/m²) with a reduced dose (10,000 UI/m²) in cats with cSCC that underwent ECT as the sole adjuvant treatment modality.

Materials and Methods

Ethical approval. This study was performed in accordance with the National and International Recommendations for the Care and Use of Animals (National Research Council) [37]. This study was approved by the Ethics Committee on Animal Use (CEUA) of the Veterinary Teaching Hospital of University Estadual Paulista “Júlio de Mesquita Filho”, Jaboticabal, Brazil (CEUA/UNESP, protocol #016279/19).

Study design. To improve the precision of reported clinical studies on ECT, we strictly followed the design of Campana et al. [38], who developed 47 quality criteria clustered into four domains (trial design, description of the patient population, treatment delivery and outcome assessment and analysis of results and their interpretation). Furthermore, we also followed the checklist by Cemazar et al. [39] that outlines a proposal for proper nomenclature and guidance for reporting of materials and methods related to the use of electroporation of biological samples in *in vivo* applications. This was a prospective nonrandomized single-blinded comparative clinical trial evaluating cats with cSCC that underwent ECT. Informed consent to perform the treatment was obtained from the cats’ owners. Follow-up was dated until the last examination of the patient with a minimum one-month follow-up. We defined the term “patients” as cats, and no human participants or human data were involved in the study.

Inclusion criteria. All cats enrolled in this study had to fulfill the following criteria: cytologically (fine-needle aspiration) or histopathologically confirmed cSCC diagnosis (some cats lacked histopathological examinations); absence of distant metastases; compliance of the owner with follow-up after four weeks; normal physical exam; and clinical staging based on the tumor, nodes and metastasis (TNM) system from the World Health Organization [40].

Patients. A power analysis was performed to determine the minimum sample of patients to be enrolled for adequate study power. We used a type 1 error of 5% and a power of 80% and determined that at least 12 subjects would be needed in each group using a sample size calculator (<https://clincalc.com/stats/samplesize.aspx>).

Then, between July 2018 and December 2019, 56 cats with cSCC were included. Six cats had more than one affected site for a total 66 lesions submitted to ECT. Twenty-two cats were included in the ECT standard group (SG) (15,000 UI/m²), and 34 cats were included in the ECT reduced group (RG) (10,000 UI/m²).

The allocation of subjects to treatment groups was accomplished by alternatively including the cats in each of the ECT groups (first in the SG, second in the RG, third in the SG, fourth in the RG, and so on). When we achieved a considerable number of cats in the SG (22 cats), we arbitrarily decided to include cats only in the RG afterwards until 34 cats in total were included (leading us to have a minimal follow-up of four weeks).

We established an observational period of one month (30 days) as the minimum observational period and 20 months the maximum observational period. All cats were mixed breed cats except for one Persian in the SG; their ages ranged from 4 to 17 years (median, 12 years) and from 5 to 20 years (median, 10 years) in the SG and RG, respectively. Feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) were not tested for in the included cats. There were 14 spayed females and 8 castrated males in the SG, whereas in the RG, there were 25 spayed females and 9 castrated males.

The most commonly affected sites observed in the SG were the nasal planum (n=12) followed by the palpebral region (n=5), labial region (n=3), periocular region (n=2), auricular region (n=1) and ocular region (n=1).

In the RG, the most affected sites were the nasal planum (n=21) followed by the palpebral region (n=10), auricular region (n=5), periocular region (n=3), labial region (n=1), ocular medial canthus (n=1) and jaw region (n=1).

None of the patients had nodal metastasis at presentation. In the ECT SG, 13 cats had stage T1, four had stage T2, two had stage T3, and three had stage T4 disease. In contrast, in the ECT RG, 18 had stage T1, 12 had stage T2, three had stage T3, and one had stage T4 disease. The tumor size ranged from 0.02 cm³ to 28.8 cm³ in the SG (median, 0.815 cm³), whereas in the RG, the tumor size ranged from 0.0005 to 57.8 cm³ (median, 0.267 cm³).

In the SG, one cat had history of cryosurgery, and one had been treated with corticosteroids prior to referral, whereas the other cats had no prior treatments. In the RG, one cat had a history of two previous surgeries. Patients had received previous treatment approximately three to six months before ECT. Therefore, ECT was not considered adjuvant therapy. All neoplastic lesions had been present, accordingly to the cats' owners, for at least 6 months before referral. No other concomitant oncological treatment was performed in cats from this study.

Electrochemotherapy protocol. We attempted to follow the updated standard operating procedure (SOP) for ECT of cutaneous tumors in humans and animals [41,42]. Bleomycin (Bleocris, Quality Pharma, LKM Laboratory, Argentina) was diluted in 5 mL of saline solution and administered intravenously (IV). The ECT protocol was divided into two groups: ECT SG (15,000 UI/m²) and ECT RG (10,000 UI/m²). In both groups, 5 minutes after the administration of bleomycin, one train of 8 biphasic pulses at a voltage per centimeter of 800-1,300 V/cm and 1-Hz frequency lasting 50 + 50 μ s with a 300- μ s interpulse (total treatment time per train, 3.2 ms) was administered by means of a certificated clinical electroporator for veterinary application (Onkodisruptor, Biopulse S.r.l., Naples, Italy) using a plate or a single pair of needle array electrodes until complete coverage of the lesion. The decision to use both a plate and needle was based on the tumor size; namely, larger volumes was treated with combination electrodes (> T2 stage). Good contact between the electrodes (plates) and the skin was assured by depilation and application of a conductive gel to the treatment area.

The number of sessions ranged from 1-4 sessions (30-day interval between sessions). All ECT procedures were performed under general anesthesia; for induction, propofol was administered (Provive, União Química - Farmacêutica Nacional S/A. São Paulo, SP, Brazil) (5 mg/kg) followed by endotracheal intubation; anesthesia was maintained with isoflurane (Isoforine, Cristália Produtos Químicos Farmacêuticos Ltda. Itapira, SP, Brazil). All animals received postoperative analgesia including an IV injection of dexamethasone (Dexamethasone 0.2%, Hypopharma, Minas Gerais, Brazil) and tramadol (Tramal União Química - Farmacêutica Nacional S/A, São Paulo, SP, Brazil) (2 mg/kg).

Furthermore, all cats received a post-ECT oral antibiotic (amoxicillin trihydrate and clavulanate potassium at dosage of 20 mg/kg orally q12h), corticosteroids (prednisolone 1 mg/kg orally q24h) for one week due to ulceration and transitory edema and tramadol (3 mg/kg for three consecutive days q12h) for analgesia (Cronidor, Agener União, São Paulo, SP, Brazil).

Electrodes. These were either (1) Onkodisruptor M1 (M1 Clamp) adjustable clamp electrodes with a distance between 9 and 45 mm (we set them at 10 mm) between stainless-steel plates (dimensions: 10 mm width and 60 mm length) or Onkodisruptor spherical probes, which include a pair of two needle-shielded electrodes (80 mm length) with a special insulating resin shield that guarantees the application of voltage only in the last 5 mm of the electrode (<https://www.onkodisruptor.com/electrode-holders/>). Furthermore, two needle-shielded electrodes were inserted into the tumor while the clamp electrode was used superficially. A conductive gel was used for the M1 Clamp between electrodes to achieve good contact.

Tumor response. We estimated the tumor volume using the following formula: $V = ab^2\pi/6$ (where “a” is the larger diameter of the tumor nodule, and “b” is the diameter of the tumor nodule perpendicular to “a”). Moreover, we used the Response Evaluation Criteria in Solid Tumors (RECIST) system to measure and assess the response of tumor lesions [43,44]. CR was defined as total reduction in the measured tumor volume, and PR was defined as a $\geq 30\%$ reduction in tumor volume. PD was defined as a $\geq 20\%$ increase in tumor volume or new lesions, and SD was defined as a $< 30\%$ reduction in tumor volume or a $< 20\%$ increase in tumor volume [39]. In cats that had more than one site lesion, the worst lesion (worst tumor stage) was considered for statistical evaluation.

Statistical analysis. For statistical purposes, we calculated the median value of each clinical parameter (tumor size and tumor stage) and classified the value as “low” when it was lower than the median and “high” when it was greater than the median. Then, we compared the survival rates of cats with low and high values of each parameter using the log-rank test and Kaplan-Meier curves. To evaluate the association of the tumor stage and DFI with survival time between the ECT standard and reduced groups, Fisher’s exact test was applied. For the survival curve, animals that died from other diseases or that were lost to follow-up were censored. When the subjects died from the disease, they were classified as uncensored. Commercial software (GraphPad Prism 8.1.0 – GraphPad Software) was used for the statistical analysis. P values < 0.05 were considered significant.

Results

Toxicity

Local toxicity – Standard group (SG)

After ECT treatment (n=22), local adverse effects such as the development of crusts, hyporexia, ulceration, nasal edema/obstruction, palpebral edema, and sneezing were noted. In the SG, 12 cats had cSCC located on the nasal planum, but only two received tube feeding for 7-14 days post-ECT for nutritional support due to nasal edema/obstruction generating anosmia.

In the remaining 10 cats with cSCC located on the nose, tube feeding was not based on the owners' decisions, and transitory hyporexia for up to 7 days was observed in all of them. Supporting care including gentle force (hand) feeding was done by the owners. In other locations (periocular, auricular, peripalpebral, and lips), hyporexia was not observed among the cats (data not shown).

Local toxicity – Reduced group (RG)

As observed in the SG, local adverse effects (n=34) such as development of crusts, hyporexia, ulceration, nasal edema/obstruction, palpebral edema, and sneezing were noticed. In the RG, 21 cats had cSCC located on the nasal planum, but only one received tube feeding for 14 days post-ECT for nutritional support. In other locations (peripalpebral, auricular, periocular, auricular, jaw, and lips), hyporexia was not observed among the cats (data not shown).

Systemic toxicity – Standard and reduced groups

No systemic toxicity was observed in either group treated with ECT. However, one cat in the RG experienced sudden death 40 days post-ECT from a suspicious thromboembolism; we cannot infer if it was due to tumor lysis syndrome.

Response to treatment

Standard group: In the ECT SG group, 10/24 lesions had complete remission (CR) (41.6 %), 11/24 had partial remission (PR) (45.8%%) and 3/24 had stable disease (SD) (12.5%) at four weeks post-ECT. No progressive disease (PD) was observed. At the end of the observation period (10 cats excluded due to death and the remaining 14 cats alive), 10/14 lesions had CR (71.43%), 1/14 had SD (7.15%), and 3/14 had PD (21.42%).

Reduced group: In the ECT RG group, 16/42 lesions had CR (38 %), and 26/42 had PR (61.90%) at four weeks post-ECT. Neither SD nor PD was observed at four weeks post-ECT. At the end of the observation period (10 cats excluded due to death and the remaining 29 cats alive), 21/29 lesions had CR (72.41%), 3/29 had SD (10.34%), and 5/29 had PD (17.24%).

Altogether, CR was achieved in 26/66 (39.4%) lesions, PR in 37/66 (56%) lesions, and SD in 3/66 (4.5%) lesions at four weeks post-ECT. At the end of the study (20 cats excluded due to death and the remaining 43 cats alive), 31/43 lesions had CR (72%), 8/43 had PD (18.6%), and 4/43 (9.3%) had SD (Supplementary Table 1). Supplementary Figures 1 and 2 show the response to treatment of some cats in both groups before and after ECT treatment.

Among the 20 cats that died during follow-up, death in 13 cats was attributed to PD of cSCC, whereas the remaining cats died from other comorbidities (hypertrophic cardiopathy, chronic kidney disease, secondary tumors, thromboembolism, and unrelated causes).

In the SG, the median disease-free interval (DFI) and overall survival time corresponded to 240 days (60 – 485 days) and 300 days (65 – 610 days), respectively. In the RG, the median DFI and overall survival time were 210 days (30 – 395 days) and 210 days (40 – 485 days,) respectively.

No statistically significant difference in the Kaplan-Meier curve for DFI was observed between the SG and RG groups ($P = 0.346$) (240 and 210 days, respectively), nor was there a difference in overall survival ($P = 0.689$) (300 and 210 days, respectively) (Figure 1).

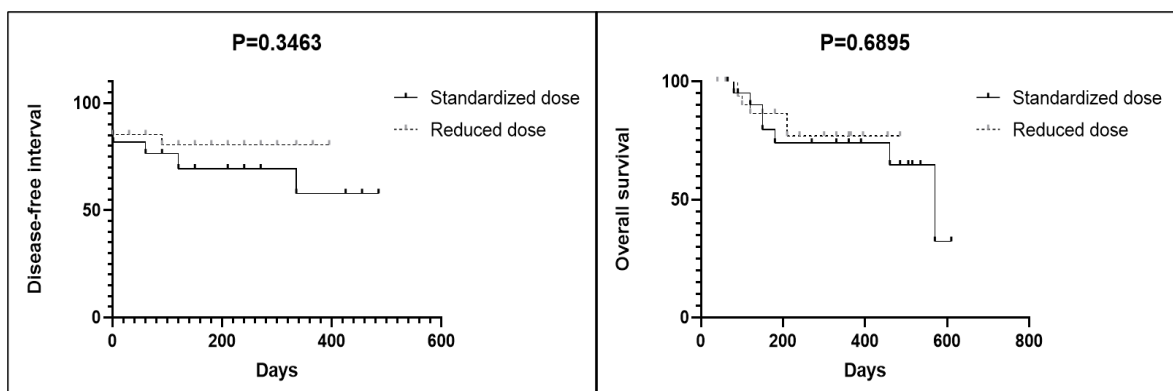


Figure 1. Kaplan-Meier curve for the disease-free interval and overall survival between the 2 bleomycin treatment groups of cats with cSCC that underwent ECT. No statistically significant difference in DFI ($P = 0.346$) (A) or overall survival ($P = 0.689$) (B) was observed between the 2 groups.

The cutoff for tumor size was based on the median of all lesions, which corresponded to 0.33 cm³. Then, tumors were classified as “low” when they were smaller than the median size, and “high” when they were larger than the median size. With regard to the impact of tumor size on DFI in the two cohorts in the RG and SG, we observed a significant difference in the former group ($P = 0.045$), with higher DFI in cat with a small tumor size (less than 0.33 cm³) compared with those with large tumor size (Fig. 2B). However, no statistically significant difference was observed between DFI and tumor size ($P = 0.157$) in the latter group (Fig. 2A).

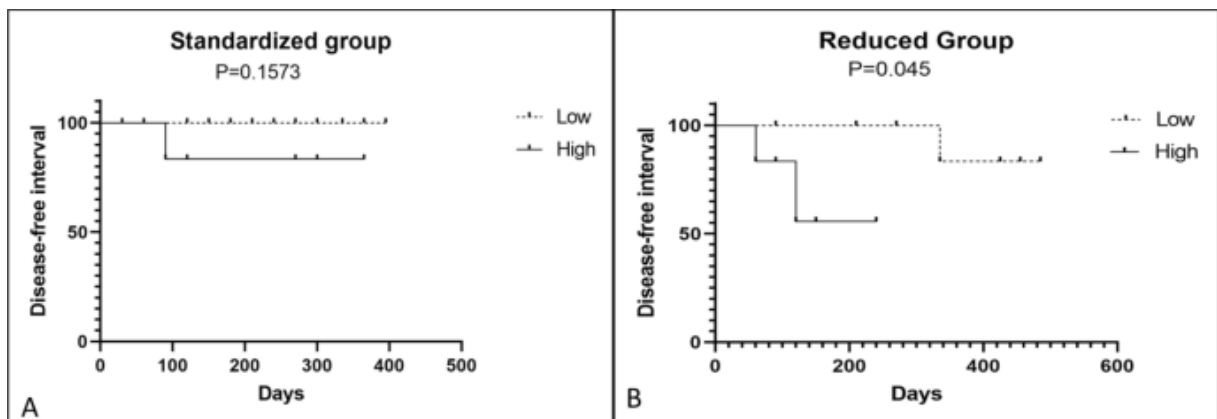


Figure 2. Kaplan–Meier curve for disease-free interval according to tumor size (cutoff of 0.33 cm³) between the 2 bleomycin-treated groups of cats with cSCC that underwent ECT. A statistically significance difference in the DFI according to tumor size was observed in the reduced group ($P = 0.045$) (B). In contrast, no statistically significant difference was observed in the DFI according to tumor size in the standardized group ($P = 0.1573$) (A).

The overall survival time was not statistically affected by tumor size in the SG or the RG ($P = 0.499$ (SG) and $P = 0.700$ (RG)) (Supplementary Figure 3 A, B). We observed a statistically significantly reduced overall survival time for cats with a higher stage in the SG (T3 and T4) ($P = 0.004$) when compared to cats with a lower stage (T1 and T2) (Fig. 3A). Nevertheless, in the RG, no statistically significant difference was observed in tumor stage ($P = 0.495$) (Fig. 3B).

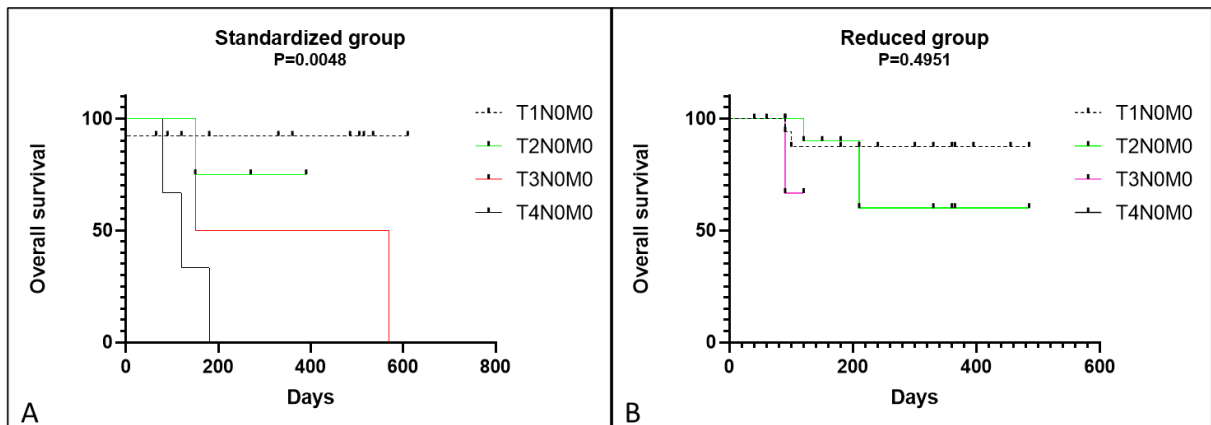


Figure 3. Kaplan-Meier curve for overall survival and tumor stage between the two groups of cats with cSCC that underwent ECT. A statistically significant difference in the standardized dose group was observed with a decrease in overall survival mostly in cats with more advanced stages of the disease (T3 and T4) ($P = 0.004$) (Fig. 4A). Nevertheless, in the reduced dose group, no statistically significant difference was observed between tumor stage and overall survival ($P = 0.495$) (Fig. 4B).

Based on the RECIST evaluation criteria, we evaluated the overall survival of patients that experienced CR, PR and SD at 4 weeks post-ECT. An expected statistically significant difference was observed in overall survival for cats that achieved a CR in the SG ($P = 0.013$). No difference was found in the RG ($P = 0.191$) (Figure 4A, B) (Supplementary Table. 1).

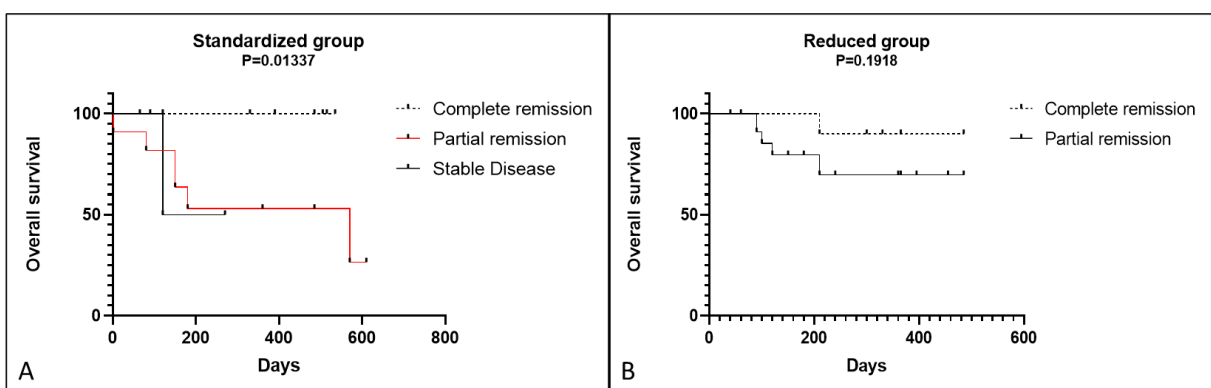


Figure 4. Kaplan-Meier curve for overall survival based on the RECIST criteria between the 2 groups of cats with cSCC that underwent ECT. A statistically significance difference was observed in the CR, PR and SD rates in overall survival ($P = 0.013$) (A). In contrast, no statistically significant difference was observed in the

reduced dose group among CR and PR ($P = 0.191$) (B).

Considering the number of ECT sessions, we did not observe a statistically significant difference between the two groups ($P > 0.05$) (Supplementary Fig. 4). The median number of sessions in the SG and the RG was one (1-4) and one (1-2), respectively.

Discussion

This study demonstrated a similar efficacy between the SG and RG in terms of bleomycin treatment in cats with cSCC that underwent ECT without differences in DFI and overall survival. In addition, the ECT resulted in an overall response (CR + PR) of 95.4% at four weeks post-ECT. However, at the end of the observation period (1-20 months), the overall response rate was 72% (CR + PR), while the PD and SD rates were 18.6% and 9.3%, respectively. Notably, this clinical response may be due to possible residual neoplastic lesions in the site treated that progressed over time or due to persistent chronic solar exposure even after treatment (since all cats had outdoor access). In routine practice, most owners do not accept collection of a new biopsy after treatment, precluding the evaluation of residual lesions.

A previous report observed responses with ECT with CR in 81.8% (9/11) cats with cSCC and a DFI ranging from 2-42 months [5], similar to our results of a 72% CR rate (31/43) and a DFI ranging from 1-16 months. Another report also observed an overall response rate of 89% (CR + PR) among cats with acceptable adverse effects with a median time to progression of 30.5 months [6]. The treatment for cSCC is primarily surgical when feasible; however, to achieve clean surgical margins to gain good local control, an aggressive surgical excision is usually indicated [7, 19-21]. In a series report of 61 cats treated with surgery, radiotherapy, and cryosurgery, the surgery group had the longest median DFI of 19.8 months in cats with noninvasive disease [22]. However, a surgical approach is not always feasible due to the anatomical region involved, such as the nasal planum and eyelid. Previous reports observed a DFI of 12 months after reconstruction of the lower eyelid using a flap [7,21].

Furthermore, cats undergoing radiation therapy may suffer from adverse effects such as trichiasis and secondary corneal disease (ulcerative keratitis) [22,23]. In a study evaluating the efficacy of a hypofractionated radiation protocol for feline cSCC, 52% of cats responded to the treatment (40% CR and 12% PR) with a mean overall survival time and DFI of 224 and 271 days, respectively [24]. The efficacy rate of hypofractionated radiation in this previous study was much lower than that in cats in this study that underwent the ECT protocol with 72% responding to treatment (72% CR and 0% PR). However, the mean overall survival time (310 and 230 days in the SG and RG, respectively) and DFI (260 and 205 days in the SG and RG, respectively) were similar to those in our study.

In comparison with other nonsurgical approaches such as photodynamic therapy (PDT), a previous report observed a response rate of 85-90% in 55 cats with SCC, with a median time to recurrence of 157 days (109-205 days) [25]. This was a similar response rate to our response rate of 95.4% CR. However, PDT showed lower DFI results than those in our ECT study, which achieved a DFI of 210 days.

Based on this anatomical limitation and some secondary adverse effects of radiation therapy, one of the advantages of the ECT modality is its good antitumor efficacy, cosmetic effects and noninvasiveness, resulting in better outcomes in patients in whom radical surgery may not be an option [4-6, 11]. Previous studies have shown the efficacy of ECT treatment in incompletely resected soft tissue sarcomas in dogs and cats, showing a long DFI and improved local control when compared with surgery alone [26-30]. In this way, ECT coupled with bleomycin or using bleomycin and cisplatin simultaneously (injected intratumorally) may result in efficient tumor local control, and their use should be considered [29, 30].

After an extensive literature review of international databases, we found only one study with a considerable number of cats (47 cats) diagnosed with cSCC that were treated with ECT [6]; this study demonstrated a longer median time to progression of 30.5 months than that in our study (median time to progression of 7 months). In this previous study [6], the longer response could also be explained by the segregation of the cats according to the hours with the greatest ultraviolet (UV) exposure (11 AM until 4 PM).

Unfortunately, in our study, cats were not segregated according to the timing of greatest UV exposure, which may have contributed to the lower DFI that that in the previous study. We suggest the possibility of using a UV screen in outdoor cats or retinoids or vitamin A supplementation as strategies to strengthen local control.

Other studies were identified; however, they presented few animals (9-11 cats,) [4,5]. In the preliminary study by Spugnini et al. [4], nine cats with SCC were enrolled and treated with ECT and showed a 77.7% CR rate. In the study by Tozon et al. [5], 11 cats were treated with ECT, showing an 81.8% CR rate (9/11) with a DFI of 2-42 months. In a nonrandomized prospective controlled study, Spugnini et al. [6] showed an overall response rate of 89% (23/26) in cats with advanced carcinoma of the head and periocular carcinoma in whom a surgical approach was not an option.

In this study, patients who received a dose of 15,000 mg/m² intravenous bleomycin showed a long median time to progression of approximately 24.2 months (periocular cohort) and 20.6 months (advanced head SCC cohort). In our study, when we considered only patients with a tumor stage of T3 and T4 in both groups, we observed a better median overall survival in the SG of 150 days (80-540 days) and in the RG of 105 days (90-120 days) than in other research studies [4] that observed a DFI for cats with T3- and T4-stage disease (5/11) that ranged from 60-390 days. This reiterates the fact that ECT may be an alternative modality for the treatment of cats with advanced carcinoma.

Another point to highlight is the number of sessions cats underwent; most of them only underwent one session, and few cats received more than one session. In the ECT SG, 15/22 cats received one session, 5/22 received two sessions, 1/22 received three sessions, and 1/22 received four sessions, while in the ECT RG, 26/34 received one session, and 8/34 received two sessions.

The number of sessions could influence the response rates of the patients when considering the possibility of residual lesions. However, another study observed an 81.8% CR rate after a single ECT session [5], while Spugnini et al. [6] observed an 81% CR rate with the number of treatment sessions ranging from 2-9 (median, 4).

When considering these findings, our CR rate was inferior to those from previous reports with a 50% CR rate with a single ECT session at the end of observation period. Regarding the DFI according to tumor size, we observed a higher

DFI in patients with a small tumor size in the SG ($P = 0.045$) but not in the RG. These results are expected since cats with a larger tumor size may have disease with a more infiltrative biological behavior, and they may have higher TNM staging [1]. The fact that the RG did not showed a significance difference may be due to heterogenous tumor sizes among cats since the median tumor size in the SG was higher than that in the RG (0.815 versus 0.267 cm³). The majority of publication on ECT used in humans and veterinary medicine oncology used different cutoff to evaluate effectiveness of ECT, i.e., tumors less than 3 cm size (cutaneous tumor) or less than 1 cm³ (canine perianal tumors). Usually, in the different cutoff, it was observed a better tumor control and efficiency of ECT in smaller tumors when compared with larger tumors [31,32,33]. However, since we prospectively included patients from routine practice, it was difficult to achieve a homogenous group in terms of tumor size. Thus, we opted to perform a median value for all tumors (including animal from RG and SG) and used a median value as a cutoff value for both tumors.

Another important aspect to consider is that, in our study, cats with large tumors (T3 and T4) showed decreased overall survival when compared with those with small tumors (T1 and T2). This was also observed by Tozon et al. [5] and Spugnini et al. [6], who observed that cats with T3- and T4-stage disease showed poorer responses to ECT since these cats had highly invasive tumor growth. This poorer response may be due to bone microenvironment involvement as a cause of treatment failure since bone may act as a sanctuary for cancer cells and dictate tumor cell survival and recurrence [34,35].

Although it was not a main goal of our research, clinical stage is an important prognostic factor for the treatment of feline cSCC, and this result was also found by other authors [4,6]. Furthermore, this difference was only noted in the SG compared with the RG; thus, this difference may be due to heterogenous stage numbers between the two groups. Further studies with a greater number of cats in each clinical stage could be important to affirm that clinical stage is strongly associated with patient prognosis. In the ECT SG, 13 cats had T1-stage disease (median DFI, 302 days), four had T2-stage disease (median DFI, 105 days), two had T3-stage disease (120 days), and three had T4-stage disease (no DFI observed).

In contrast, in the ECT RG, 18 had T1-stage disease (median DFI, 225 days), 12 had T2-stage disease (median, DFI 90 days), three had T3-stage disease (no DFI observed), and one had T4-stage disease (no DFI observed).

One of the limitations of our study is that we did not have access to histological features or further biopsies after treatment to perform a comparison of histological changes. This approach could reveal some interesting information regarding what ECT with different dosages induces in tumor cells including the apoptosis rate, number of mitoses and tissue necrosis. However, due to complete remission in some cases and owner decisions in other cases, we were not able to perform this evaluation. Previously, our research group observed that this therapy induced a decrease in the proliferation index and tumor volume 21 days after treatment in dogs with cSCC treated with ECT [36]; future studies evaluating tissue samples in feline cSCC could provide new valuable information.

We conclude that ECT with both doses of bleomycin may achieve the same overall response rate, DFI, and overall survival rate. In this way, ECT can be considered a potentially affordable alternative treatment for cats with cSCC even in advanced stages when surgery is not an option. Additional studies with a larger number of patients are warranted to elucidate an extended duration of local control, especially with biopsies taken after treatment to evaluate possible residual lesions.

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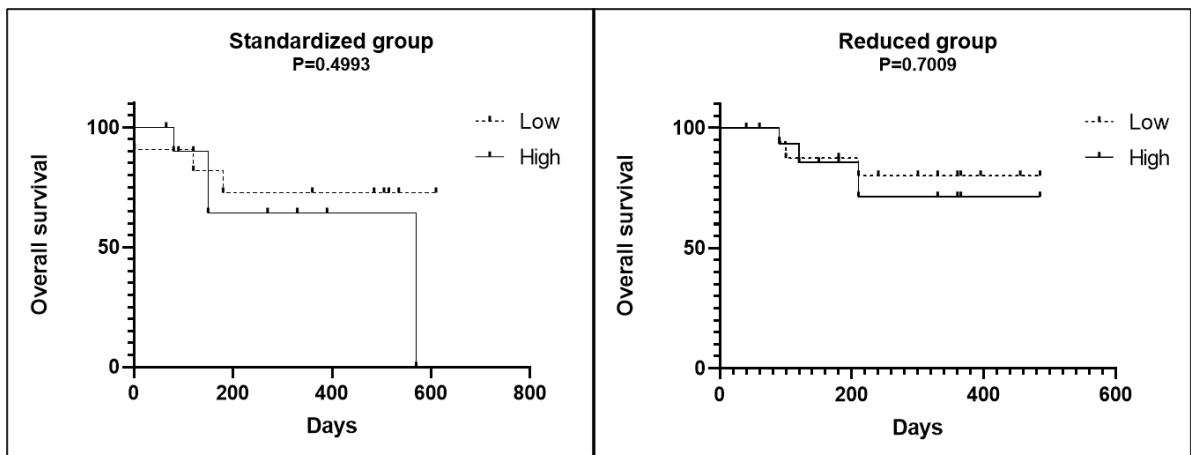
Supplementary data



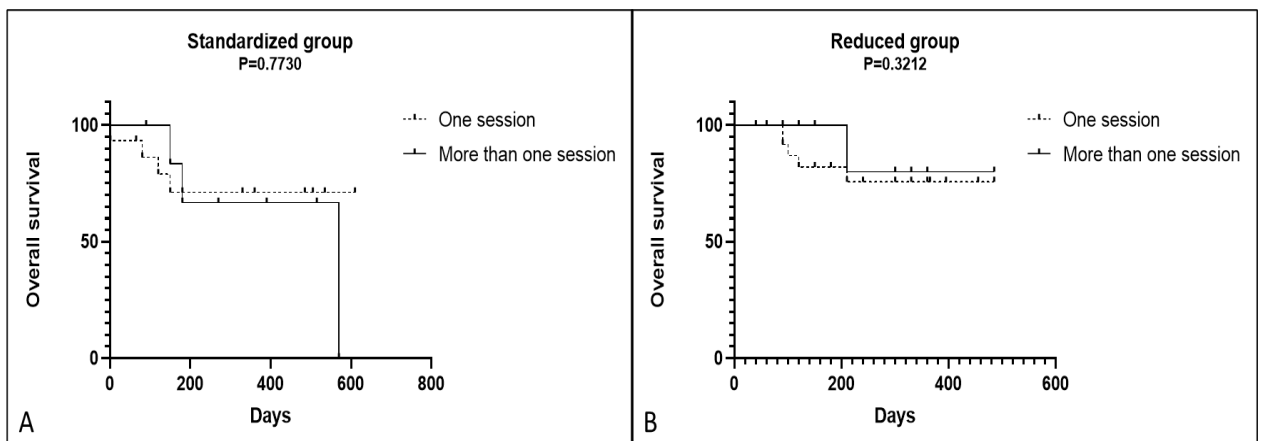
Supplementary figure 1. Cats diagnosed with cSCC that underwent ECT in the standardized group (15,000 UI/m²). Patient 1 (A) with advanced carcinoma on the nose and upper lip prior ECT treatment and with complete remission after two sessions (B). Patient 2 with cSCC on the nose before ECT (C) and after 30 days with complete remission after one session (D). Patient 3 with cSCC in upper right lip before ECT (E) and after 30 days with complete remission after one session (F)



Supplementary figure 2. Cats diagnosed with cSCC that underwent ECT in the reduced group (10,000 UI/m²). Patient 1 (A) with cSCC on the nose prior ECT treatment and with complete remission after one session (30 days) (B). Patient 2 with advanced carcinoma on the nose before ECT (C) and after 60 days with complete remission after one session (D). Patient 3 with cSCC on the nose and cutaneously on left side of the face before ECT (E) and after 30 days with complete remission after one session (F).



Supplementary figure 3. Kaplan-Meier curve for overall survival according tumor size (small and large) between the 2 treatment groups of cats with cSCC that underwent ECT. No statistically significant difference between the 2 treatment groups was observed in the SG ($P = 0.499$) (A) or RG ($P = 0.700$) (B).



Supplementary figure 4. Kaplan-Meier curve for overall survival according to the number of ECT sessions between the 2 treatment groups of cats with cSCC. No statistically significance difference was observed in survival among groups that received one or more than one session ($P > 0.05$).

Supplementary Table 1. Outcome of 66 lesions (56 cats) at 4 weeks post-ECT and at the end of the observation period (45 lesions).

RECIST at 4 weeks	STANDARD DOSE				REDUCED DOSE			
Variable	No(%)	CR (%)	PR (%)	SD (%)	No(%)	CR (%)	PR (%)	SD (%)
All lesions	24	10	11	3	42	16	26	0
Tumor size (cm)								
< 2	16	9	5	2	30	13	17	0
2-5 cm	7	1	5	1	11	3	8	0
> 5	1	0	1	0	1	0	1	0
End of the observation period (1 - 20 months)	STANDARD DOSE				REDUCED DOSE			
Variable	No(%)	CR (%)	PD (%)	SD (%)	No(%)	CR (%)	PD (%)	SD (%)
All lesions	14	10	3	1	29	21	5	3
Tumor size (cm)								
< 2	12	10	2	0	20	18	4	0
2-5 cm	2	0	1	1	8	5	0	3
> 5	0	0	0	0	1	0	1	0