Electrochemotherapy with bleomycin associated with doxorubicin induces tumor regression and decreases the proliferative index in canine cutaneous squamous cell carcinomas

Electrochemotherapy in canine cutaneous squamous cell carcinomas

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29 ABSTRACT

30 Canine cutaneous squamous cell carcinoma (cSCC) is the most common skin cancer in
 31 dogs, and due to its low metastatic rate, local treatments such as electrochemotherapy (ECT)
 32 promote disease control or even complete remission and increase the survival time in most
 33 cases. This study aimed to evaluate the expression of BAX, Bcl-2, and Ki67 and clinical
 34 parameters in dogs with cSCC subjected to ECT. A prospective clinical nonrandomized study
 35 was performed in dogs with naturally occurring cSCC treated with ECT. Eighteen lesions
 36 (from 11 dogs) were selected, independent of breed, sex and age. The ECT protocol consisted
 37 of bleomycin plus doxorubicin followed by electric pulses characterized by 8 biphasic electric
 38 pulses lasting 100 ms, 1 Hz and 1000 V/cm. Among the 18 lesions, the lesion volume
 39 significantly decreased after treatment ($p=0.04$). The tumor size at D0 had no impact on
 40 survival time or prognosis ($P>0.05$). A decreased mitotic index was observed at compared
 41 with D0 ($P=0.019$). We also observed more intratumoral necrosis at D21 compared to D0
 42 ($P=0.041$). The median expression level of Ki67 was 277.96 at D0 and 193.92 at D21. Thus,
 43 tumor samples had a lower proliferative index after ECT (D21) ($P=0.031$). The survival times
 44 of subjects with Ki67 values lower and higher than the Ki67 median value were not
 45 significantly different ($P>0.05$). Regarding apoptotic markers, there was no significant
 46 difference in BAX or Bcl-2 expression between D0 and D21 ($P>0.05$) or in overall survival
 47 between subjects with different levels of apoptotic markers. Furthermore, a positive
 48 correlation was observed between BAX and Bcl-2 before ECT (D0) ($P=0.0379$, $r=0.5067$). In
 49 conclusion, there was no change in BAX and Bcl-2 protein expression levels in response to
 50 ECT at the time points evaluated, and ECT was able to reduce tumor volume and cellular
 51 proliferation in cSCC.

52 **Key-words:** Apoptotic markers, cutaneous carcinoma; electroporation, proliferative index,
 53 tumor size.

54

55 **Introduction**

56 Canine cutaneous squamous cell carcinoma (cSCC) is the most common skin cancer in
57 dogs, and the most important etiological factor is chronic sunlight exposure. On the other
58 hand, canine papillomavirus infection, chronic inflammation and immunosuppression might
59 be involved in the development of cSCC [1-4]. cSCC is the second most common cutaneous
60 tumor in dogs [5], with an incidence between 3-20% [6,7]; however, the incidence of cSCC
61 depends on the geographic area [5]. In humans and dogs, actinic keratosis is an SCC precursor
62 lesion with more than 80% of the SCC cases in humans being derived from previous actinic
63 keratosis [8, 9].

64 Generally, cSCCs are locally invasive tumors with a low metastatic rate (13%) [10-
65 13], with is similar to the behavior of SCCs humans, in whom the metastatic rate is 5%;
66 metastasis mainly occurs in locoregional lymph nodes [14-16]. Due to this low risk of
67 metastasis, local treatments such as surgery, cryotherapy, radiotherapy, photodynamic therapy
68 and electrochemotherapy (ECT) promote disease control and increase the survival time in
69 most cases [1,17].

70 ECT is able to induce an inflammatory response, necrosis, scar tissue and apoptosis in
71 tumors [18-20]. This process is a genetically programmed process for the elimination of
72 damaged cells, and its onset is controlled by numerous interrelating processes that are
73 influenced by extrinsic and intrinsic signals that converge in an effector pathway. Alterations
74 in these pathways are an important in the process of tumorigenesis, leading to the persistence
75 of neoplastic cells and the promotion of progression and metastasis [21,22].

76 BAX (Bcl-2 associated X protein) and Bcl-2 are important proteins in the BCL-2
77 family, which consists of pro- and anti-apoptotic proteins. The BAX/Bcl-2 cross-regulation
78 controls apoptosis, cell survival or cellular proliferation [23,24]. Several studies have

determined the expression level of a single apoptosis-associated protein, such as BAX and/or BCL-2, by immunohistochemistry or flow cytometry and have correlated its expression with the prognosis of mammary tumors and lymphoma [25,26]. However, conflicting data have been observed among the studies [25-27]. In feline cutaneous SCCs, neither BAX nor BCL-2 expression was detected. However, in basal cell tumors, BCL-2 expression was higher (23/24) than that of BAX, which was expressed only in seven out of 24 tumors. For the tumors that expressed both BAX and BCL-2, the BAX:BCL-2 ratio was low [28].

Another important factor to be evaluated in cSCC is cellular proliferation, which is measured by the level of Ki67. It is a nuclear protein that is used to detect proliferative cells because it is present in proliferating cells during the late G1, S, G2 and M phases of the cell cycle, and it is correlated with poor prognosis in dogs [29-32]. It is known that the high expression level of Ki67 is correlated with a poorer prognosis in dogs with several tumor types as well as in humans, such as mammary tumors, mast cell tumors, perianal tumors, oral and cSCCs [29-37].

ECT has gained popularity in recent years in veterinary medicine as well as in human medicine [38,39]. ECT is a combination of chemotherapy and the localized delivery of electric pulses to the tumor nodule. ECT aims to increase antineoplastic drug diffusion into the cell after cell membrane electroporation, thus increasing its cytotoxicity [17].

To the best of the authors' knowledge, this is the first study to evaluate the expression of BAX, Bcl-2, and Ki67 in dogs with cSCC that underwent ECT. This study aimed to evaluate the clinical parameters, proliferative index, and expression levels of BAX and Bcl-2 in dogs with cSCC that underwent ECT.

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106 **Materials and Methods**

107 **Ethical approval**

108 This study was performed in accordance with the National and International
109 Recommendations for the Care and Use of Animals. All procedures were performed after
110 receiving the approval of the Institutional Animal Ethics Committee (CEUA/UNIFRAN,
111 #033/15).

112

113 **Study design**

114 A prospective clinical nonrandomized study was performed in dogs that presented to
115 the Veterinary Teaching Hospital of Sao Paulo State University (UNESP) and the Veterinary
116 Teaching Hospital of University of Franca (UNIFRAN) with naturally occurring cSCC and
117 that were treated with ECT. Consent was obtained from the dogs' owners to perform the
118 treatment. The recommendations made by Campana et al. [40] for reporting clinical studies on
119 ECT were followed.

120

121 **Inclusion criteria and animal selection**

122 All dogs enrolled in this study fulfilled the following characteristics: a
123 histopathologically confirmed diagnosis of stage T1 cSCC (according to the World Health
124 Organization) [41]; the absence of distant metastases; the compliance of the owner with
125 follow-up after 21 days; the owner's permission to perform biopsies before (D0) and after
126 ECT (D21).

127 Eighteen lesions (from 11 dogs) were selected, independent of breed, sex and age. The
128 eligibility inclusion criteria were as follows: dogs with clinically staged cSCC and complete
129 physical examinations, laboratory exams, and fine needle aspiration of regional lymph nodes.
130 Three-view thoracic radiography and abdominal ultrasonography were also performed.

131

132 **Electrochemotherapy protocol**

133 Bleomycin (Cinaleo[®]-Meizler, Barueri-SP) was diluted in 5 mL of saline solution and
134 administered intravenously (IV) at a dose of 15,000 UI/m². Five minutes after the
135 administration of the bleomycin, doxorubicin (Cloridrato de Doxorubicina[®] - Eurofarma
136 Laboratórios S.A. Ribeirao Preto – SP) was diluted in 25 mL of saline solution and
137 administered intravenously at a dose of 30 mg/m², followed by sequences of 8 biphasic
138 electric pulses lasting 100 ms each, with a frequency of 1 Hz, 1000 V, generated by an LC
139 BK-100 portable electroporator and delivered by six needle electrodes with 0.3 mm distance
140 between them; the needle electrodes were arranged in rows (parallel array) until they achieved
141 complete coverage of the tumor. The procedure lasted until 28 minutes, according to a
142 previous report [42,43].

143 All ECT procedures were administered under general anesthesia using propofol (5
144 mg/kg) IV followed by endotracheal intubation; anesthesia was maintained with isoflurane.
145 All animals received postoperative analgesia, including the IV injection of meloxicam (0.2
146 mg/kg) and tramadol (2 mg/kg).

147

148 **Tumor response**

149 The total volume of the neoplasm was calculated by the following formula:
150 $\pi \times length \times width \times height / 6$; tumor volume was measured at D0 and D21 using a digital
151 pachymeter [29,30]. The same evaluator measured tumor volume to exclude measurement

152 bias. Complete remission (CR) was defined as a total reduction in measured tumor volume,
153 while partial remission (PR) was defined as $\geq 30\%$ reduction in tumor volume. Progressive
154 disease (PD) was defined as $\geq 20\%$ increase in tumor volume or new lesions, and stable
155 disease (SD) was defined as $\leq 30\%$ reduction in tumor volume or $\leq 20\%$ increase in tumor
156 volume [43]. CR and RP were considered “favorable” responses, while PD and SD were
157 considered “unfavorable” responses.

158

159 **Histopathological evaluation**

160 The first sample was collected immediately before the first session of ECT (D0). The
161 second sample was obtained on day 21 (D21) after ECT. A 6-mm punch biopsy was used to
162 obtain the tumor samples. All samples were immediately placed in 10% formalin for 24
163 hours, followed by 70% alcohol until paraffinized sections were prepared. All tumor samples
164 were classified according to Gross et al. [5], as follows: 1) well-differentiated SCC,
165 presenting centralized accumulatio of compact laminated keratin or keratin pearls, with
166 keratinization progressing through the granular cell layer as in the normal epidermis and the
167 keratinized centers of the lobules undergoing necrosis and becoming infiltrated by neutrophils
168 or 2) poorly differentiated SCC presenting smaller epithelial structures, cords and nests rather
169 than large islands of squamous epithelial cells, moderate to high mitotic activity, and no
170 keratin pearls.

171

172 **Immunohistochemistry**

173 Immunohistochemical staining for BAX, BCL-2 and Ki67 antibodies was performed
174 in all cSCCs in the original biopsy samples (D0) and in the biopsy specimens collected 21
175 days after the first ECT (D21). Immunohistochemical staining was performed using the
176 peroxidase method and 3,3’ diaminobenzidine tetrachloride (DAB). The slides were dewaxed

177 in xylol and rehydrated in graded ethanol. For antigen retrieval, the slides were incubated in
178 citrate buffer (pH 6.0) in a pressure cooker (Pascal, Agilent Technologies, Santa Clara, CA,
179 USA). The endogenous peroxidase was blocked with a commercial solution (Protein Block,
180 Agilent Technologies, Santa Clara, CA, USA), and the samples were incubated with primary
181 antibodies overnight at 4°C. Anti-BAX (mouse monoclonal, Santa Cruz Biotechnology,
182 Dallas, TX, USA) was detected with a monoclonal antibody at a 1:200 dilution overnight.
183 Bcl-2 (mouse monoclonal, Santa Cruz Biotechnology, Dallas, TX, USA) was a monoclonal
184 mouse antibody used at a 1:400 dilution overnight, and anti-Ki67 (MIB-1, Agilent
185 Technologies, Santa Clara, CA, USA) with a monoclonal mouse antibody used at a 1:50
186 dilution, applied overnight. After incubation with the above antibodies, the slides were placed
187 on an autostainer platform (Agilent Technologies, Santa Clara, CA, USA). Then, the sections
188 were counterstained with Harris's hematoxylin, dehydrated, and mounted. The anti-Ki67
189 antibody cross-reactivity with canine tissue was provided by the manufacturer in the antibody
190 datasheet. For the anti-Bcl-2 and anti-BAX antibodies, we performed western blotting to
191 show the antibody reactivity.

192 Negative controls were run for all antibodies with a mouse universal negative control
193 (Dako, Carpinteria, CA, USA) according to the manufacturer's instructions. The positive
194 control consisted of normal lymph nodes for all antibodies according to Protein Atlas
195 guidelines (www.proteinatlas.org).
196

197 **Immunohistochemical evaluation**

198 The immunohistochemical slides were examined with a light microscope using an
199 ocular grid 26-mm in diameter (Leica Microscopy DMLB, HC, PLAN 10x/20, 4"x5") at
200 400X magnification. The immunoexpression levels of BAX, Bcl-2 and Ki67 were established
201 by counting the number of stained cells and considering the number of positive cells in

relation to the total number of cells inside the ocular grid per five random high-power fields (400x). The samples were classified as 0 (absence of stained cells), 1 (< 10% stained cells), 2 (10-25% stained cells), 3 (26-50% stained cells) and 4 (> 50% stained cells) according to Fonseca-Alves et al. [44]. For the evaluation of the scores, cytoplasmic staining for BAX and Bcl-2 and nuclear staining for Ki67 were considered.

Western blotting

The western blot analysis was performed to validate the protein cross-reactivity with canine tissue. The procedures were performed according to a previously published method [45]. Briefly, two cSCC tissue biopsies were collected and homogenized (Polytron homogenizer, Kinematica, Lucerne, Switzerland) for 30 seconds at 4°C in RIPA Buffer (Millipore, Burlington, MA, USA). Total protein was extracted and quantified as described by Bradford [46]. A total of 70 µg of protein was subjected to electrophoresis and then transferred to nitrocellulose membranes (Sigma Chemical Co., St. Louis, MO). The blots were blocked with 3% bovine serum albumin in TBS-T for 2 hours and incubated overnight with BAX (mouse monoclonal, Santa Cruz Biotechnology, Dallas, TX, USA) and Bcl-2 (mouse monoclonal, Santa Cruz Biotechnology, Dallas, TX, USA) antibodies. Goat anti-β-actin antibody (1:1,000; sc-1615, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a control. A horseradish peroxidase-conjugated secondary antibody was incubated with the blots for 1 hour, and the blots were visualized via chemiluminescence (Amersham ECL Select Western Blotting Detection Reagent, GE Healthcare).

Statistical analysis

227 For statistical purposes, we calculated the median value of each clinical,
228 histopathological and immunohistochemical parameter and classified the value as “low” when
229 it was lower than median and “high” when the value was greater than the median. Then, we
230 compared the survival between dogs with low and high expression values of each parameter
231 through the Kaplan-Myer method. To evaluate the association between the tumor stage ($\leq T2$
232 x $>T2$) before treatment and after ECT, Fisher’s exact test was applied. Furthermore,
233 Spearman’s correlations between the expression levels of BAX, Bcl-2 and Ki67 were
234 performed to compare the levels at two time points, namely, before and after ECT.
235 Commercial software (GraphPad Prism[®] 6.0 – GraphPad Software, Inc. 2015) was used for
236 the statistical analysis. P values <0.05 were considered significant.

237

238 **Results**

239 **Clinical data**

240 Mixed breed dogs (n=5) were the dogs most commonly affected by cSCC, followed
241 by American Pit Bulls (n=3), Boxers (n=2) and English Pointers (n=1). All subjects had
242 sparse fur and lightly pigmented skin. The mean age of the animals was 7.5 ($\pm$ 2.29) years,
243 with females being the more common sex (n=10). Five animals had more than one lesion.
244 Thus, we investigated 18 lesions in 11 subjects.

245 The most commonly affected sites observed were the abdominal region (n=10),
246 followed by the thoracic region (n=5), axillar region (n=1), preputial region (n=1) and tibial
247 region (n=1). Among the 11 subjects, only three had regional lymph node involvement at the
248 time of diagnosis. There was no difference in survival time between subjects with and without
249 lymph node involvement (S Fig 1A) ($P > 0.05$).

250

251 **Suppl Fig. 1. Survival analysis according to lymph node involvement, tumor size, and**
 252 **apoptotic markers in dogs with cSCC.** (A) There was no difference in survival time
 253 between subjects with and without lymph node involvement ($P > 0.05$).

254

255 Among the 18 lesions, the volume before the treatment ranged from 0.14 to 112.9
 256 cm^3 (median of 4.64), and the volume decreased significantly after treatment ($p=0.04$),
 257 ranging from 0.11 to 118.2 cm^3 (median of 1.49) (Table 1) (Fig 1A).

258 **Table 1. Records from the subjects that underwent ECT regarding the clinical data, tumor localization, stage and tumor size.**

ID	Breed	Age	Sex	Number of tumors	Tumor localization	Histopathological evaluation	TNM	Tumor size at D0 (cm ³)	Tumor size at D21 (cm ³)	Outcome	Number of sessions
1	Pitbull	8	M	2 (a, b)	Axillar (a) Prepuccial (b)	poorly differentiated well differentiated	T2N0M0 T2N0M0	3.75 19.23	0.91 6.44	PR PR	3 / S
2	Pitbull	9	F	1	Abdominal	well differentiated	T2N0M0	5.04	0.73	PR	2
3	Boxer	2	F	1	Thorax lateral	well differentiated	T3N0M0	68.49	65.94	SD	2 / S
4	Mixed	10	F	2 (a, b)	Thorax lateral (a) Abdominal (b)	poorly differentiated well differentiated	T2N0M0 T3N0M0	2.3 14.15	0.278 1.68	PR PR	1 / S
5	Mixed	7	F	1	Abdominal	well differentiated	T3N0M0	93.57	118.24	PD	2
6	Pitbull	9	F	4 (a, b, c, d)	Thorax lateral (a) Abdominal (b) Abdominal (c) Abdominal (d)	well differentiated poorly differentiated well differentiated well differentiated	T1N0M0 T3N0M0 T1N0M0 T2N0M0	1.14 112.92 0.81 22.36	0.175 48.2 0.533 5.014	PR PR PR PR	2 / S
7	Mixed	NA	F	1	Abdominal	well differentiated	T2N1M0	104.6	104.6	SD	2
8	Mixed	NA	F	2 (a, b)	Abdominal (a) Thorax lateral (b)	well differentiated well differentiated	T3N0M0 T2N0M0	4.24 0.22	10.36 1.046	PD PD	2
9	English Pointers	8	F	1	Abdominal	well differentiated	T3N1M0	76.93	30.03	PR	2 / S
10	Boxer	8	F	2 (a, b)	Thorax lateral (a) Tibial (b)	well differentiated well differentiated	T1N0M0 T1N0M0	0.314 0.141	0.113 0.28	PR PD	2
11	Mixed	7	F	1	Abdominal	well differentiated	T4N1M0	1.2	1.31	SD	1

M, male; F, female
 PR: partial remission; SD: stable disease; PD: progressive disease
 S: surgery
 NR: death not related to cancer
 NF: no follow-up
 NA: not available

Fig. 1. Evaluation of tumor volume, clinical response according to tumor volume, mitotic index, intratumoral necrosis and Ki67 expression before and after treatment with ECT in dogs with cSCC. (A) The volume before the treatment ranged from 0.14 to 112.9 cm³ (median of 4.64), and the volume significantly decreased after treatment (p=0.04), ranging from 0.11 to 118.2 cm³ (median of 1.49).

Based on the volume measurements, 11 (61.1%) lesions underwent PR, 4 (22.2%) underwent PD, and 3 (16.6%) underwent SD. We evaluated the tumor size at D0 as a prognostic factor according to the TNM recommendation for human SCCs, and we grouped the subjects according to whether they had tumors smaller or larger than 5 cm³. The volume at D0 had no impact on survival time (Fig 1B) and had no prognostic value (P>0.05).

Fig. 1. Evaluation of tumor volume, clinical response by tumor volume, mitotic index, intratumoral necrosis and Ki67 expression before and after treatment by ECT in dogs with cSCC. (B) Evaluation of tumor size at D0 as prognostic factor in dogs with tumors smaller and larger than 5 cm³. The volume at D0 had no impact on survival time and had no prognostic value (P>0.05), and the response to ECT was not significant (p=0.332).

In addition, the association between the clinical stage before treatment (D0) and the response to ECT was not significant (p=0.332) (Fig 1B). The median survival time of the 11 dogs was 180 days (32 to 570 days) (Fig 2A).

Fig. 2. Overall median survival time of the 11 dogs and survival time based on mitotic index. (A) The median survival time of the 11 dogs was 180 days (32 to 570 days).

The additional clinical results are shown in Table 1. The number of sessions that each subject underwent ranged from one to three. Five subjects underwent a surgical procedure after D21 (four due to PD and one due to the owner request).

Histopathological features

Regarding the histopathological grade, 16 lesions (83.3%) were classified as well-differentiated SCC (Suppl Fig 2A), and three (16.6%) were classified as poorly differentiated SCC (Suppl Fig 2B). Interestingly, only well-differentiated tumors progressed, and all (3/18) poorly differentiated cSCC experienced PR.

The median mitotic index in the tumor group at D0 was 4.94 (0 to 34). We found a relationship between the mitotic index and overall survival. Subjects with mitosis numbers lower than 4.9 at D0 experienced a longer survival time ($P=0.009$) (Fig 2B).

Suppl Fig 2. Histopathological evaluation of squamous cell carcinoma (SCC) in dogs that underwent electrochemotherapy (ECT). (A) Well-differentiated SCC with the presence of mononuclear inflammatory infiltrate before ECT. (B) SCC before ECT. It is possible to see tissue disorganization with the presence of apoptotic cells showing basophilic nuclei.

Fig. 2. Overall median survival time of the 11 dogs, and the survival time based on the mitotic index. (B) Subjects with mitotic index values lower than 4.9 at D0 experienced a longer survival time than subjects with mitotic index values greater than 4.9 ($P=0.009$).

Additionally, we found decreased mitotic index values at D21 compared to D0 (median 1.5, ranging from 0 to 20) ($P=0.019$) (Fig 1C). We also assessed intratumoral

317 necrosis in the tumor samples at D0 and D21. It is not surprising that we found more necrosis
318 in tumors at D21 than at D0 ($P=0.041$) (Fig 1D). We evaluated necrosis at D0 and the survival
319 time, and we did not find significant differences ($P>0.05$).

320 **Fig. 1. Evaluation of tumor volume, clinical response by tumor volume, mitotic index,**
321 **intratumoral necrosis and Ki67 expression before and after treatment by ECT in dogs**
322 **with cSCC.** (C) A decreased mitotic index at D21 (median 1.5, ranging 0 to 20) ($P=0.019$)
323 was observed. (D) Greater intratumoral necrosis was observed in tumor samples at D21 compared
324 to those at D0 ($P=0.041$).

325

326 **Protein expression**

327 Immunohistochemical expression was evaluated in 15 tumors (15/18) at two different
328 time points (D0 and D21). Three out of 18 samples were excluded due to the absence of
329 neoplasticism in tumor samples from D0 or D21. The median expression of Ki67 in D0 was
330 277.96 (113.4 to 511.4), and D21 was 193.92 (15 to 494). We found decreased proliferative
331 expression between D0 and D21 (Fig 3 A and B). Thus, tumor samples after ECT (D21) had a
332 lower proliferative index than those before ECT ($p=0.031$) (Figure 1E). Using the
333 proliferative index at D0, we evaluated the survival time between subjects with Ki67 values
334 lower and higher than the median Ki67 value, and we did not find a significant difference
335 ($P>0.05$).

336

337 **Figure 3. Photomicroscopy of cSCC in dogs with Ki67 immunolabeling.** (A) High
338 nuclear immunostaining of neoplastic cells at D0 (score 3). (B) Low nuclear immunostaining
339 at D21 (score 1). The 4"x5" area, used for counting the cells, is observed in the images
340 (immunocytochemistry, Envision, DAB, counterstaining with Harris hematoxylin, x400).

341 **Fig. 1. Evaluation of tumor volume, clinical response by tumor volume, mitotic**
 342 **index, intratumoral necrosis and Ki67 expression before and after treatment by ECT in**
 343 **dogs with cSCC.** (E) Lower proliferative index ($p=0.031$) was observed at D21 when
 344 compared to D0.

345

346 For BAX and Bcl-2, cytoplasmic expression was identified (Fig 4A). We found
 347 median BAX expression levels of 21.02% (ranging from 8.34 to 70.56) at D0 and 24.53%
 348 (ranging from 10 to 74.47) at D21. There was no significant difference in BAX expression
 349 between D0 and D21 ($P>0.05$) (Fig 4B). There was no significant difference in overall
 350 survival between subjects with low and high BAX expression levels ($P>0.05$) (Supl Fig 1C).

351

352 **Fig. 4. BAX and Bcl-2 immunoexpression levels and the correlation between both**
 353 **apoptotic markers in dogs with cSCC before and after ECT.** (A) Cytoplasmic expression
 354 of BAX (arrows). (B) There was no significant difference in BAX expression between D0 and
 355 D21 ($P>0.05$).

356

357 **Supl Fig. 1. Survival analysis according to lymph node involvement, tumor size, and**
 358 **apoptotic markers in dogs with cSCC.** (C) There was no significant difference in overall
 359 survival between subjects with high and low levels of BAX ($P>0.05$).

360

361 Regarding Bcl-2 expression (Fig 4C), we found medians of 20.62% (ranging from
 362 2.76% to 48.37) at D0 and 26.42% at D21 (ranging from 0.65 to 77.23). There was no
 363 statistically significant difference in Bcl-2 expression between D0 and D21 ($P>0.05$) (Fig
 364 4D). When we evaluated the survival time between samples with low Bcl-2 expression versus
 365 those with high expression, we did not find a significant difference ($P>0.05$) (Supl Fig 1D).

Furthermore, a positive correlation was observed between BAX/Bcl-2 expression levels before ECT (D0) ($p=0.0379$, $r=0.5067$) (Fig 4E). However, after treatment (D21), we did not find a significant Gaussian approximation ($p=0.7370$, $r=0.0911$) (Fig 4F). Regarding the correlation between BAX/Ki67 and Bcl-2/Ki67, there was no significant difference among them at different time points ($p>0.05$).

371

Fig. 4. BAX and Bcl-2 immunoexpression and the correlation between both apoptotic markers in cSCC before and after ECT. (C) Cytoplasmic expression of Bcl-2 (arrows). (D) There was no statistically significant difference in Bcl-2 expression between D0 and D21 ($P>0.05$). (E) Positive correlation between BAX and Bcl-2 before ECT (D0) ($p=0.0379$, $r=0.5067$). (F) Twenty-one days after treatment, no significant Gaussian approximation was observed ($p=0.7370$, $r=0.0911$)

Western blot analysis

We found a 26-kDa band for Bcl-2 and a 21-kDa band size for BAX. Both antibodies showed only one band each in the western blot analysis (Fig 5).

381

Discussion

cSCC is a very common tumor in tropical regions, and one of the biggest therapeutic challenges for tropical regions is removing the animal from exposure to sunlight during treatment. Because cSCC has a low level response to chemotherapy and a low metastatic rate, a local approach is required to achieve long-term disease-free interval and overall survival. Surgery is the primary treatment for cSCC. However, many patients have multiple tumors at the time of diagnosis, making surgical approaches difficult. Therefore, new techniques are required to improve the outcomes of canine patients with cSCC.

390 In this study, we evaluated ECT as a primary therapy for cSCC. We determined the
391 association of different parameters with overall survival and tumor response to ECT. We
392 demonstrated a reduction of the tumor size after ECT (D21), even in subjects with poorly
393 differentiated tumors. Four lesions (in four different patients) presented progressive disease
394 (PD). Of these four lesions, two had large volumes (ID 5 and 8), indicating that ECT may be a
395 treatment option for small tumors. Unfortunately, we have no studies in Veterinary Medicine
396 indicating the association of a tumor size cutoff with prognosis. In a large series study about
397 the use of ECT in basal cell carcinoma (BCC) in humans, a 50% CR rate was observed after a
398 single ECT cycle in primary BCC. Interestingly, in the same study, a second ECT cycle
399 increased the CR rate from 50 to 63%, and retreatment was more advantageous in patients
400 with local BCC. Thus, retreatment with ECT seems a reasonable option in patients with small
401 BCCs to reduce the response duration [47]. In accordance with this human study, our results
402 indicated that ECT could be a good therapeutic option for small tumors, and large may need
403 more than one round of ECT. A previous case report by our study group observed complete
404 remission of a digital trichoblastoma after three sessions of ECT, reaffirming the usefulness of
405 repeated sessions of ECT for larger tumors [20].

406 Among the limiting factors of our study, there was an absence of a cutoff point for
407 tumor size in cutaneous neoplasms in dogs, unlike in human medicine. In addition, the staging
408 adopted in veterinary medicine does not take into account the size of the subject, which may
409 influence the behavior of a tumor of the same size in dogs of different sizes. The small sample
410 size is also a limitation of our study. However, we performed a G power analysis to guarantee
411 that our tumor group had a sufficient number of samples.

412 Interestingly, lymph node involvement was not a prognostic factor in dogs with cSCC.
413 The number and size of the lesions seems to be more important than the involvement of local
414 lymph nodes. This can be explained by the invasive behavior of cSCC [5]. Usually, these

415 tumors are more locally invasive than metastatic tumors [5,34,48]. On the other hand, the
416 number of mitoses was correlated with overall survival in our study. Because the mitotic
417 index is a routine evaluation, it will be important to evaluate the sensitivity and specificity of
418 these indices to predict outcomes in dogs with cSCC. However, a large sample size is
419 necessary. We also found more necrosis in samples after ECT, indicating that this therapy
420 induces both apoptosis and necrosis. Thus, the greater amount of necrosis identified at D21
421 was due to the therapy.

422 One of the most interesting results of our research was the decreased proliferative
423 index in tumors after ECT. Because ECT induces tumor apoptosis and necrosis, the number of
424 proliferating cells was reduced. Furthermore, we did not find any difference in cell apoptosis
425 between the two time points (D0 and D21), indicating that another mechanism may be
426 involved in inhibiting tumor proliferation after ECT. Because ECT is a recent technique in
427 Veterinary Medicine, a limited number of studies have demonstrated the ECT antitumoral
428 effect in cSCC.

429 In general, the high expression level of Ki67 observed in SCC, especially in poorly
430 differentiated SCC, is related to an aggressive potential [9,34,37,49]. However, in the lesions
431 studied, the proliferation index could also be considered high despite the predominance of
432 well-differentiated SCC (83.3%), suggesting the aggressive potential, despite the degree of
433 differentiation. Additionally, there is no established cutoff point for Ki67 to be considered an
434 indicator of recurrence or metastasis, as occurs in cases of cutaneous mast cell tumor, which is
435 23 positive cells in a 10 mm x 10 mm / 400x area [50]. It should be noted that the complete
436 evaluation of proliferation markers may be more effective in predicting the biological
437 behavior of the tumor. In this context, Auler et al. [49] reported the case of a dog with well-
438 differentiated SCC in the foreskin with inguinal lymph node metastasis. This animal showed

439 high Ki67 positivity in the tumor tissue, in addition to the high expression of the growth
440 factors HER-2 and EGFR (epidermal growth factor receptor).

441 A previous study observed the same pattern in mast cell tumors as the pattern
442 identified in this study with regard to the proliferative index, which was observed in fewer
443 positive cells for Ki67 among the neoplastic cells 28 days after treatment [19].

444 Regarding the apoptotic markers, all cSCC lesions showed cytoplasmic positivity for
445 BAX. These results differ from those of Madewell et al. [28], who observed less than 2%
446 BAX expression in cSCC in felines. However, other human studies have observed the
447 expression of BAX in cutaneous and pulmonary SCC, which ranged from 53-100% [51,52].
448 A recent study observed that the expression of BAX in normal keratinocytes in the epidermis
449 of dogs ranged from very weak to moderate, elucidating their importance in the pathogenesis
450 of diseases [53]. To avoid any unspecific cross-reaction with canine tissue and the primary
451 antibody, we performed western blotting for both antibodies using cSCC samples. Both
452 antibodies presented only one specific band, confirming the reactivity of both antibodies with
453 canine tissue.

454 Likewise, no significant difference was observed between the expression of the anti-
455 apoptotic Bcl-2 protein before and after treatment with ECT. This is the first study to evaluate
456 whether the immunohistochemical expression of Bcl-2 protein is altered by ECT in cSCC. A
457 previous report evaluated the expression in mast cell tumors and observed increased
458 expression of anti-Bcl-2 28 days after ECT and then decreased expression at D46 [19]. In our
459 study, all cSCC samples showed immunolabeling of Bcl-2. However, other studies have
460 shown different results and only one evaluated Bcl-1 by IHC in dogs with cSCC (n = 5),
461 among which only one showed positivity [54]. In humans, Puizina-Ivic et al. [55] observed an
462 absence of Bcl-2 positivity in SCC (n = 20), whereas Abu Juba et al. [56] observed positivity
463 in 50% of cSCC samples (n = 10).

464 It must be noted that the correlation between BAX/Bcl-2 markers was significant
465 before treatment, but no difference was observed afterwards, and no difference was observed
466 between BAX/Ki67 and Bcl-2/Ki67 at both time points. In human studies, the Bax/Bcl-2 ratio
467 can act as a rheostat that determines cell susceptibility to apoptosis [56], and lower levels of
468 this ratio may lead to resistance of human cancer cells to apoptosis. In colorectal tumors,
469 BAX and Bcl-2 expression levels were the most predictive of outcome when the Bax/Bcl-2
470 expression ratio was used [57]

471 Although the chemotherapeutic agents used have the capacity to cause cell damage
472 that induces cell death by apoptosis, the BAX and Bcl-2 markers in the samples were not
473 altered at the time point evaluated (D21). It is possible that other proteins involved in the
474 apoptosis pathways exert a greater influence on the process of cell death induced by ECT. In
475 addition, it cannot be ruled out that, in view of the dynamism and complexity of the apoptosis
476 process, the expression of BAX and Bcl-2 may have been influenced by the ECT prior to the
477 assessed moment, but not on the 21st day of treatment. A limitation in scoring a single protein
478 is that the expression of a single protein may not reflect the level of apoptosis because
479 apoptosis is a dynamic, complex process.

480 Regarding the proliferative index, 21 days after the application of ECT, it was possible
481 to observe a significant reduction in proliferative indices in the lesions sampled. The same
482 behavior was also observed in mammary neoplasms of human patients submitted to systemic
483 chemotherapy because there was a significant reduction in the level of Ki67 in the tumor at 21
484 days of treatment [58,59]. However, in the veterinary literature, no research was found
485 regarding the proliferative index before and after treatment in malignant neoplasms.

486

487 **Conclusions**

ECT was able to reduce tumor volume and cellular proliferation in cSCC. Furthermore, our results showed that the BAX and Bcl-2 proteins did not show alterations in their expression levels in response to ECT at the time points evaluated. Therefore, studies involving the serial evaluation of these proteins, as well as the investigation of other proteins involved in apoptosis, may contribute to the understanding of the effect of ECT on apoptosis in canine cSCC.

Disclosure

All the authors have declared that they have no competing interests.

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