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Evaluation of cell proliferation and apoptosis markers as predictive factors for electrochemotherapy in cutaneous squamous cell carcinoma of cats[1]

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ABSTRACT.- Ribeiro R.C.S., Anjos D.S., Pazzini J.M., Bertolo P.H.L., Carra G.J.U. & De Nardi A.B. 2023. **Cell proliferation and apoptosis markers as predictive factors for electrochemotherapy in cutaneous squamous cell carcinoma of cats.** *Pesquisa Veterinária Brasileira 43:e06518, 2023*. Departamento de Clínica e Cirurgia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus Jaboticabal, Via de Acesso Paulo Donatto Castellane s/n, Jaboticabal, SP 14884-900, Brazil. E-mail: roanacecilia_rc@hotmail.com

Determining cell proliferation rates and tumor apoptosis through immunohistochemistry allows the evaluation of the biological behavior of the tumor, optimizing the patient's clinical course. This study aimed to analyze the immunohistochemical expression of Ki-67, COX-2 and caspase-3 and correlate them with the type of response to ECT in feline cutaneous squamous cell carcinoma (SCC), thus determining the predictive potential of these variables. For this, 13 samples of feline cutaneous SCC were evaluated before ECT, and statistical analyses of the correlation intensity between the variables were performed using the Spearman correlation coefficient, with a significance level of 95%. The results indicate a significant negative correlation between histopathological grade and response to ECT (ρ=-0.6; *p*=0.03); there was no significant correlation between Ki-67, COX-2 and caspase-3 immunoexpression with the response to ECT (ρ=-0.18; *p*=0.54/ρ=-0.23; *p*=0.44/ρ=-0.12; *p*=0.69, respectively). Therefore, the study shows that the histopathological grade, tumor size and staging, degree of cellular pleomorphism and degree of inflammatory infiltrate can be considered negative prognostic factors for cutaneous SCC and negative predictors for response to ECT. However, the markers Ki-67, COX-2 and caspase-3 are not considered predictive factors for the type of response to ECT. In addition, no relationship between these immunoexpressions and greater tumor aggressiveness was observed. The SCCs evaluated in this study showed significant COX-2 labeling, indicating a potential therapeutic target. ECT has been shown to be safe and effective for local control of feline cutaneous SCC but with reduced effectiveness in larger and invasive lesions.

INDEX TERMS: caspase-3, COX-2, Ki-67, squamous cell carcinoma, electrochemotherapy, cats.

RESUMO.- [**Marcadores de proliferação celular e de apoptose como fatores preditivos à eletroquimioterapia em carcinoma de células escamosas cutâneo de gatos.**] A determinação das taxas de proliferação celular e apoptose tumoral por meio da imuno-histoquímica, permite avaliar o comportamento biológico tumoral, com otimização da evolução clínica do paciente. Este trabalho teve como objetivo analisar as expressões imuno-histoquímicas de Ki-67, COX-2 e caspase-3 e correlacioná-las com o tipo de resposta à EQT em carcinoma de células escamosas (CEC) cutâneo de felinos; assim, determinar o potencial preditivo destas variáveis. Para tanto, foram avaliadas 13 amostras de CEC cutâneo de felinos

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antes da EQT e as análises estatísticas quanto à intensidade de correlação entre as variáveis foram realizadas utilizando o coeficiente de correlação de Spearman, com nível de significância de 95%. Os resultados indicam que houve correlação negativa significativa entre o grau histopatológico e a resposta à EQT (ρ=-0,6; *p*=0,03); não houve correlação significativa entre as imunoexpressões de Ki-67, COX-2 e caspase-3 com a resposta à EQT (ρ=-0,18; *p*=0,54/ρ=-0,23; *p*=0,44/ρ=-0,12; *p*=0,69, respectivamente). Portanto, este estudo evidenciou que as variáveis grau histopatológico, tamanho e estadiamento tumorais, grau de pleomorfismo celular e grau do infiltrado inflamatório foram consideradas fatores prognósticos negativos para o CEC cutâneo e preditivos negativos para a resposta à EQT. Entretanto, os marcadores Ki-67, COX-2 e caspase-3 não foram considerados fatores preditivos para o tipo de resposta à EQT, assim como não foi observada relação entre essas imunoexpressões com maior agressividade tumoral. Os CECs avaliados neste estudo apresentaram importante marcação para COX-2, indicando um potencial alvo terapêutico. A EQT mostrou-se segura e efetiva para o controle local dos CECs cutâneos dos felinos, porém com efetividade reduzida em lesões maiores e invasivas.

TERMOS DE INDEXAÇÃO: caspase-3, COX-2, Ki-67, carcinoma de células escamosas, eletroquimioterapia, gatos.

INTRODUCTION

Squamous cell carcinoma (SCC) is a malignant tumor arising from the squamous epithelium (Murphy 2013). It is the most frequent skin tumor in cats (Cunha et al. 2014). It is a disease of elderly cats, with a median age of 10 to 12 years (Pinheiro 2010, Murphy 2013, Tozon et al. 2014). These tumors are usually secondary to exposure to ultraviolet light. Glabrous areas such as the nasal plane, pinnae and eyelids are the most affected, and white cats are more prone to the tumors than pigmented cats (Spugnini et al. 2009, Hauck 2013, Cunha et al. 2014, Tozon et al. 2014). They seldom cause metastases; these occur in more advanced stages, mainly in the regional lymph nodes and rarely in the lungs (Pinheiro 2010, Rosolem et al. 2012). However, this carcinoma is highly invasive, often progressing to ulcerations and manifesting as wounds that do not heal and cause pain (Spugnini et al. 2009, Cunha et al. 2014). The diagnosis is defined by biopsy and histopathological examination (Murphy 2013).

Electrochemotherapy (ECT) has recently been used as the therapy of choice for most cutaneous SCCs in cats (Spugnini et al. 2012). It is a technique that combines the administration of hydrophilic antineoplastic agents intravenously and/or intratumorally with the application of high voltage electrical pulses of short duration and appropriate waveform, aiming to increase the uptake of chemotherapeutic drugs by tumor cells (Mali et al. 2013, Brunner 2015) by creating transient hydrophilic pores in the cell membrane. This process is called electroporation, which potentiates the cytotoxic action of chemotherapy drugs (Cemazar et al. 2008, Silveira et al. 2011, Brunner 2015).

Given the above, it was hypothesized in the present study that the determination, through immunohistochemical analysis, of cell proliferation and apoptosis rates in cutaneous SCCs of cats could predict the degree of response to ECT and clinical evolution. The objective of this study was to investigate the immunohistochemical expression of Ki-67, COX-2 and caspase-3 in samples of cutaneous SCCs from felines submitted to EQT and to correlate them with macroscopic and histopathological aspects and with clinical evolution. By this means, we determined the predictive potential of these variables for the ECT response to obtain more objective information on the biological behavior of the tumor concerning ECT.

MATERIALS AND METHODS

Selection of animals. The present study was submitted and approved by the Committee on Ethics in the Use of Animals of the "Faculdade de Ciências Agrárias e Veterinárias (Faculty of Agricultural and Veterinary Sciences – FCAV) of Unesp/Jaboticabal. The tutors who joined this project received guidance and a consent form for signing the consent.

A prospective study was carried out in an initial heterogeneous population of 16 cats. Data on age, sex, coat color, tumor location and volume, time of evolution and previous treatments were obtained. The animals came from the Oncology Service of the Veterinary Hospital of Unesp, Jaboticabal campus, SP, Brazil, and from private clinics in this city and region from 2017 to 2018. Surgical interventions or other previous therapeutic approaches were allowed for inclusion in the study, as well as multiple lesions in the same animal. The lesion with the largest volume was used to collect the fragment for animals with more than one lesion.

At the first consultation, tumor staging was performed using laboratory tests (hemogram, ALT and creatinine); an aspiration biopsy of regional lymph nodes was also performed when significant changes were observed during clinical care. Complementary imaging tests were performed, such as chest X-rays (in the right lateral, left lateral and ventrodorsal projections) to search for pulmonary metastasis and skull radiographs, when necessary, to assess local tumor extension. Since the potential for distant metastasis from cutaneous SCC is low, abdominal ultrasonography was not performed. Tumor staging (TNM – tumor, lymph node, metastasis) was partially determined according to World Health Organization (WHO) guidelines (Owen 1980).

In addition, an incisional biopsy of the lesions was performed with a 4 mm thick dermatological punch to confirm the diagnosis of cutaneous SCC before the application of electrical pulses. Three of the 16 cats included in the study were rejected due to the small size of the collected fragment, with a preponderant presence of inflammatory infiltrate, making the conclusive diagnosis of cutaneous SCC impossible. The macroscopic characteristics were evaluated, and the tumor volume was determined using a caliper. The rotational ellipse method was used to calculate the tumor volume: V (cm^3) = length x height x width x π/6 (Gass 1985). The population was divided into three subgroups according to tumor size (1 = <2cm; 2 = 2-3cm; 3 = >3cm).

Afterward, the ECT protocol was performed with the patient under general anesthesia. The preanesthetic medications used were chlorpromazine (0.5mg/kg, IM) and methadone (0.2mg/kg, IM), and anesthetic induction was performed with propofol (5mg/kg, IV). 3% isoflurane diluted in 100% oxygen was used to maintain inhalational anesthesia. The chemotherapy used was bleomycin sulfate at a dose of 15UI/m2 of body surface (Mir et al. 2006), intravenously applied since it is described as the preferred route for ulcerated lesions (Silveira et al. 2010) and because it reduces environmental contamination.

The electrical pulses were distributed over the entire tumor extension five minutes after the application of bleomycin, according to the recommendations of the European Standard Operating

Procedures of Electrochemotherapy (ESOPE) (Marty et al. 2006). For electroporation, the LC BK100® electroporator device was used, composed of an electrode containing six stainless steel needles arranged parallel and equidistant (0.7cm) from each other. The electrical pulses had a voltage of 1000V/cm in a unipolar square wave, lasting 100μs, totaling eight cycles, with a repetition frequency of 1Hz; therefore, each pulse caused a muscle contraction. They were applied within a maximum of 28 minutes, as recommended by Mir et al. (2006). Good contact between the electrodes and the skin was ensured by shaving and applying a neutral conductive water-soluble gel to the treated area for imaging exams. Electric pulses were also applied to the 5 mm margin surrounding the tumor in apparently healthy tissue, with presumed local infiltration.

Microscopic evaluation. The collected fragments were kept in 10% buffered formaldehyde for 24 hours. Subsequently, the material was transferred to 70% alcohol and sent to the Veterinary Pathology Laboratory of FCAV-Unesp. Routine processing consisted of making 4μm-thick sections distended on glass slides for histochemical and immunohistochemical reactions.

Histopathological evaluation and classification, using routine hematoxylin and eosin (HE) staining, were performed by an experienced pathologist from the Laboratory of Veterinary Pathology at FCAV-Unesp. In the light microscopy analysis, the following microscopic characteristics were evaluated: the presence of horny pearls, degree of cellular pleomorphism, and degree and type of inflammatory infiltrate, which were scored as 1 = mild, 2 = moderate, and 3 = intense. The mitotic index (MI) was determined by counting the number of mitotic figures present in ten random fields at the highest magnification (40x). For the histopathological classification, the cutaneous SCCs were classified as well-differentiated, moderately differentiated and poorly differentiated, as proposed by Gross et al. (2007).

For the immunohistochemical reactions (IHC), carried out at the Veterinary Immunohistochemistry Laboratory of FCAV-Unesp, 4μm sections were extended on silanized polarized glass slides (poly-L-lysine adhesive). The sections were dewaxed in an oven at 67°C overnight, cleared in xylol and hydrated in increasing dilutions of alcohol to carry out specific reactions with the antibodies (Table 1). The Novolink polymer detection system was used as prescribed by the manufacturer. Primary antibodies were incubated for 2 hours in a humid chamber at room temperature. Counterstaining was performed with Harris hematoxylin. For each IHC reaction, negative and positive controls (healthy skin) were included.

For Ki-67 protein, positive cells were identified with nuclear brown immunostaining; for COX-2 and caspase-3, positive cells were identified with cytoplasmic brown immunostaining with different intensities.

Positivity for Ki-67 was expressed as the average number of immunopositive cells per 100 total cells, evaluated in five fields with the highest magnification (40x), selected by the hot-spot method, totaling 500 cells, as described by Vascellari et al. (2012). Scores were assigned [0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%), 4 (>75%)], according to De Nardi (2007).

For COX-2 and caspase-3, a score was assigned considering the percentage of distribution and the intensity of immunostaining of positive cells, evaluated in five fields of higher magnification (40x), also selected by the *hot-spot* method. The scores, regarding distribution, were 0 (0%), 1 (<30%), 2 (30-60%) and 3 (>61%); referring to the intensity of immunostaining, they were distributed as 0 (absence), 1 (weak), 2 (moderate), and 3 (strong). Finally, the positivity index for COX-2 and caspase-3 was calculated by multiplying the distribution score by the intensity score, obtaining the final score (0-9) according to the methodology described by Vascellari et al. (2012).

To determine the percentage of distribution of cells immunostained for COX-2 and caspase-3, a microscope coupled to a digital camera was used, which emits images for the computational program for capture. The percentage was obtained by the ImageJ program (version 1.50b, Java 1.8.0_60), which determines the proportion of positively colored pixels concerning the total number of pixels in the image.

Evaluation of response to electrochemotherapy. Evaluation of the effects of EQT on the tumor was performed three weeks (D21) after the ECT procedure, according to the Response Evaluation Criteria in Solid Tumors (RECIST) (Nguyen et al. 2015). Complete remission (CR) was defined as the complete disappearance of the lesion, with total re-epithelialization. Partial remission (PR) was defined as when at least a 30% reduction in tumor volume was detected. Stable disease was defined as when less than a 30% reduction or up to a 20% increase in tumor volume was observed. Progressive disease was defined as the appearance of new lesions or an increase of at least 20% of the tumor volume. Therefore, no response (NR) was considered when stable or progressive disease occurred. The response was determined by visual assessment by three observers and not by performing a new biopsy.

Statistical analysis. Spearman's correlation coefficient, a nonparametric test, was used to define the intensity of the correlation between the variables studied: Ki-67, COX-2, caspase-3 immunostaining; histopathological grade; tumor size; degree of cellular pleomorphism; degree of inflammatory infiltrate; mitotic index; WHO staging and type of response to EQT. In the nonparametric tests, the original data are replaced by their respective ranks; that is, the data are classified in ordinal scales in ascending or descending order. Spearman's correlation provides a measure of the association between two variables expressed by the coefficient ρ (rho), with limits -1 (highest negative correlation, represented in the matrix by the dark red color) and +1 (highest positive correlation, represented

Table 1. Antibodies, clones, companies, dilutions and antigenic recoveries used for immunohistochemistry in feline cutaneous SCC samples. FCAV-Unesp, 2018

Antibody	Clone	Dilution	Antigenic recoveries
Anti-Ki-67[a]	MIB-1 (mouse monoclonal)	1:125	Novocastra Epitope Retrieval Solutions®[c] – pH 6 Microwave (3 cycles of 4 minutes at maximum power)
Anti-COX-2[a]	CX-294 (mouse monoclonal)	1:500	Novocastra Epitope Retrieval Solutions®[c] – pH 9 Water bath 95°C, 30 min.
Anti-caspase-3[b]	31A1067 (IgG1 mouse monoclonal)	1:300	Novocastra Epitope Retrieval Solutions®[c] – pH 6 Microwave (3 cycles of 4 minutes at maximum power)

SCC = squamous cell carcinoma, FCAV-Unesp = "Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista 'Júlio de Mesquita Filho'"; [a] Dako, Agilent Pathology Solution – Agilent Technologies Ltda., USA, [b] Santa Cruz Biotechnology, Inc., USA, [c] Novocastra Epitope Retrieval Solutions®: Leica Biosystems New Castle Ltda., United Kingdom.

in the matrix by the dark blue color). If ρ=0, the two variables are not associated (Petrie & Watson 2013). In Spearman's correlation matrix, the ellipse format means greater correlation intensity, with the ellipse directed to the right indicating a positive correlation and to the left indicating a negative correlation. Statistical calculations were performed using the R® software program (version 3.4.0, 2017), with a significance level of 95% (p<0.05).

RESULTS

The clinical characteristics of the animals included in this study are presented in Table 2. Ten cats had one lesion, one had two lesions, and two had three lesions, for a total of 18 lesions. Six lesions were located on the nasal plane (33.3%), seven on the periocular region or the eyelids (38.9%), two on the pinnae of the ears (11.1%), two on the temporal region (11.1%) and one in the external auditory canal (5.6%). Some animals had been submitted to other previous therapies, including antibiotic, antifungal, anti-inflammatory, cryosurgery and conchectomy. Enlargement of the right submandibular lymph node (1/13) was observed, and cytology diagnosed metastasis. No changes were observed on chest and/or skull X-rays, evidencing the absence of distant metastases. According to the WHO TNM classification, five cats were classified as stage T1N0M0 (38.4%), six cats as T2N0M0 (46.2%), one cat as T4N0M0 (7.7%) and one cat as T4N1M0 (7.7%). The microscopic characteristics (Fig.1-6) of the evaluated SCCs are shown in Table 3.

Table 2. General clinical characteristics of cats diagnosed with cutaneous SCC. FCAV-Unesp, 2018

Sample	Breed	Sex	Age	Coat	Evolution	Location	Staging
1	Mixed breed	M	8 years	White and yellow	1 year and 3 months	Nasal planum and left pinna	T2N0M0
2	Mixed breed	M	10 years	White and black	1 year	Periocular and left eyeball	T4N0M0
3	Mixed breed	M	11 years	White and yellow	1 year	Leff and right eyelid and nasal planum	T2N0M0
4	Mixed breed	F	10 years	White and yellow	2 years	Nasal planum with right oral mucosa and palate involvement	T4N1M0
5	Mixed breed	F	4 years	White	3 years	Eye left lateral canthus	T1N0M0
6	Mixed breed	M	9 years	White	6 months	Left lower eyelid	T1N0M0
7	Mixed breed	F	15 years	White	6 months	Temporal region	T2N0M0
8	Mixed breed	M	8 years	White and yellow	6 months	Nasal planum	T1N0M0
9	Mixed breed	F	8 years	White	1 month	Right pinna	T1N0M0
10	Mixed breed	M	14 years	White	1 year	Nasal planum	T2N0M0
11	Mixed breed	M	7 years	White and black	2 months	Right external ear canal	T1N0M0
12	Mixed breed	M	12 years	White and yellow	1 year	Right and left lower eyelid and nasal planum	T2N0M0
13	Persa	F	16 years	Yellow	6 months	Left temporal region	T2N0M0

SCC = squamous cell carcinoma, FCAV-Unesp = "Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista 'Júlio de Mesquita Filho'", M = male, F = female.

Table 3. Histopathological characteristics of the cutaneous SCC of the cats included in the study, with inflammatory infiltrate scores and cellular pleomorphism, FCAV-Unesp, 2018

Samples	Corneal pearls	Inflammatory infiltrate	Pleomorphism	Mitotic index	Histopathological grade
1	Positive	3 (neutrophils)	1	5	Well-differentiate
2	Positive, with necrotic center	3 (chronic-active)	2	7 (atypical mitosis)	Moderately differentiated
3	Positive	1 (neutrophils)	1	3	Well-differentiate
4	Negative	3 (chronic-active))	2	36 (atypical mitosis)	Poorly differentiated (extensive areas of necrosis)
5	Negative	2 (neutrophils)	2	2	Poorly differentiated
6	Positive	3 (neutrophils)	1	6	Well-differentiate
7	Positive	2 (neutrophils)	1	9	Well-differentiate
8	Positive	3 (chronic-active)	2	6	Well-differentiate
9	Negative (presence of dyskeratosis)	1 (lymphocytes and plasmacytes)	3	25	Poorly differentiated
10	Negative (presence of dyskeratosis)	3 (neutrophils)	3	12 (atypical mitosis)	Poorly differentiated (binucleate cells)
11	Positive	2 (neutrophils)	1	7	Well-differentiate
12	Negative (presence of dyskeratosis)	3 (neutrophils)	3	27	Poorly differentiated (areas with mild necrosis)
13	Positive (a few)	3 (neutrophils)	1	5	Moderately differentiated

SCC = squamous cell carcinoma, FCAV-Unesp = "Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista 'Júlio de Mesquita Filho'"; Scores: 1 = mild, 2 = moderate, 3 = severe.

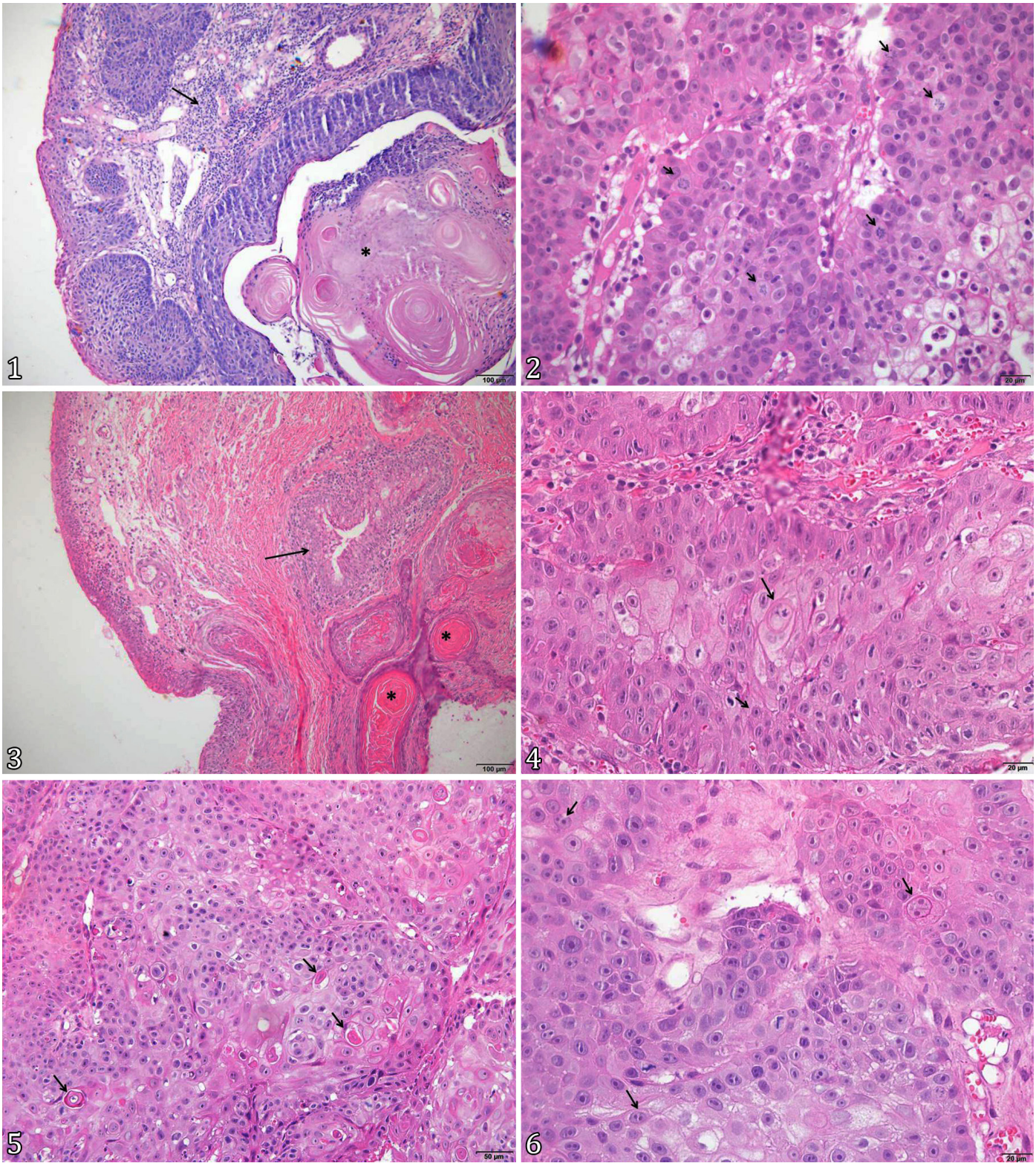

Fig.1-6. Photomicrographs of histopathological examinations of feline cutaneous squamous cell carcinoma (SCC) samples analyzed through HE histochemical reactions. (**1**) Sample 1: well-differentiated SCC, horny pearls (asterisk) and intense inflammatory infiltrate. HE, obj.10x. (**2**) Sample 4: poorly differentiated SCC, showing five atypical mitotic figures in the field (arrows). HE, obj.40x. (**3**) Sample 6: well-differentiated SCC; observe the invasion of a nest of neoplastic cells in the dermis (arrow), in addition to horny pearls (asterisks). HE, obj.10x. (**4**) Sample 9: poorly differentiated SCC, showing intense cellular pleomorphism (arrows). HE, obj.40x. (**5**) Sample 10: poorly differentiated SCC, with intense cellular pleomorphism and dyskeratoses (arrows). HE, obj.20x. (**6**) Sample 10: poorly differentiated SCC, showing binucleated cells (arrows). HE, obj.40x. FCAV-Unesp, Jaboticabal/SP, 2018.

The distribution percentages of SCC samples concerning Ki-67, COX-2 and caspase-3 scores are shown in Figure 7-9. Ki-67, COX-2 and caspase-3 immunoreactivity patterns are shown in Figure 10, 11-13 and 14-16, respectively. Table 4 shows the immunostaining evaluated in this study.

Ten of the 13 cats in the evaluations responded to ECT when evaluated on the 21st day after therapy, demonstrating a 76.9% overall response rate. Eight cats (61.5%) demonstrated complete remission, and two (15.4%) demonstrated a partial response. Three cats (23.1%) did not respond to ECT, with two of these cats having stable disease and one feline having progressive disease (Table 4). In the three cats that showed partial or no response, subsequent lesion progression required other therapeutic approaches or repetition of the ECT session. Figure 17-24 presents the data of the cats evaluated in this study and their respective responses.

Among the adverse effects of ECT, acute local alterations such as erythema and edema were highlighted, which were resolved in approximately one week. In most cats, tumor necrosis and crusting at the site were observed within two weeks post-treatment and were well tolerated by patients.

According to Spearman's correlation matrix for the variables studied (Fig.25), a significant positive correlation was observed between the histopathological grade and the degree of pleomorphism (ρ=0.81, p<0.01); a significant negative correlation between the histopathological grade and the response to ECT (ρ=-0.6; p=0.03); a significant positive correlation between tumor size and stage (ρ=0.92; p<0.01); a moderate negative correlation, but without significance, between tumor size and ECT response (ρ=-0.50; p=0.09); and a significant positive moderate correlation between pleomorphism and MI (ρ=0.57; p=0.04). A moderate negative correlation was observed between the degree of inflammatory infiltrate and the type of response to ECT, but it was not significant (ρ=-0.50; p=0.078). A moderate negative, nonsignificant correlation was observed between tumor staging and the type of response to ECT (ρ=-0.50, p=0.08). There were no significant correlations between Ki-67 (ρ=-0.18; p=0.54), COX-2 (ρ=-0.23; p=0.44) and caspase-3 (ρ=-0.12; p=0.69) and the type of response to ECT, but there was a tendency toward a negative correlation between these variables.

Table 5 shows the relationships between the histopathological grade and the Ki-67, COX-2 and caspase-3 scores. There was no tendency for an increase or decrease in the immunostaining scores according to tumor undifferentiation.

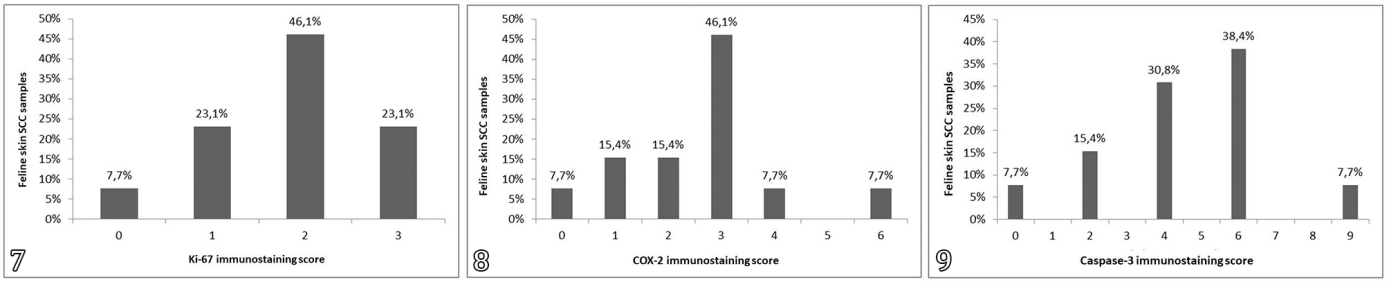

Fig.7-9. Percentage distribution of cutaneous squamous cell carcinoma (SCC) samples from cats included in the study in relation to (**7**) anti-Ki-67, (**8**) anti-COX-2 and (**9**) anti-caspase-3 labeling scores. FCAV-Unesp, Jaboticabal/SP, 2018.

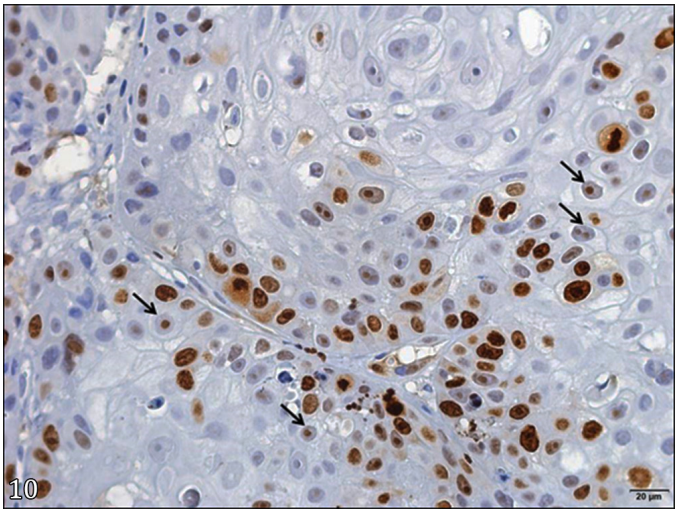

Fig.10. Photomicrograph of Ki-67 immunostaining of a poorly differentiated squamous cell carcinoma (SCC) of Sample 10, with a marking percentage of 57.2%/score 3. Note the variation in staining intensity in different neoplastic cells and the isolated staining of nucleoli (arrows). IHC, obj.40x. FCAV-Unesp, Jaboticabal/SP, 2018.

DISCUSSION

Similar to reports by Thompson (2007) and Rosolem et al. (2012), the cutaneous SCCs in cats in this study, presented as erosive or proliferative lesions, prevailed in older animals, were located in glabrous and clear areas even in cats with a mixed coat in which the lesions were found in depigmented regions. No sexual predisposition was observed. They showed low metastatic capacity in more advanced stages in the regional lymph node. This study's histopathological characteristics of cutaneous SCC in cats agree with Gross et al. (2007) and Rosolem et al. (2012).

The evaluation of the clinicopathological characteristics and the immunohistochemical expression of Ki-67, COX-2 and caspase-3 in cutaneous SCC in cats in this study proved important to elucidate aspects regarding the biological and clinical behavior of the tumor. Similar studies were carried out by De Nardi (2007) and Zuccari et al. (2008) in mammary neoplasms in bitches, Silva (2010) in mammary neoplasms in cats, Calderón (2008) and Vascellari et al. (2012) in canine cutaneous mast cell tumors, and Melzer et al. (2006) in cutaneous SCC of cats.

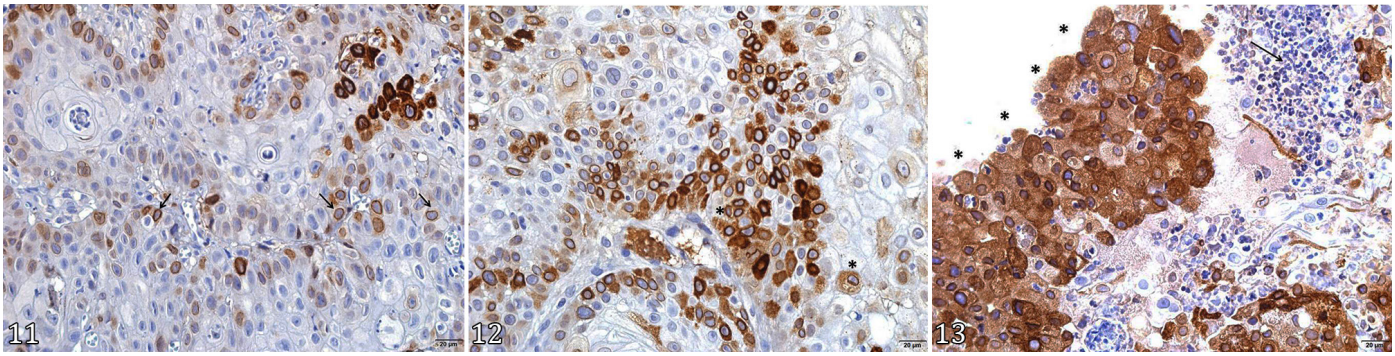

Fig.11-13. Photomicrograph of COX-2 immunostaining of feline cutaneous squamous cell carcinoma (SCC). (**11**) SCC poorly differentiated from Sample 9, with a staining score of 3; note the predominance of perinuclear staining (arrows). IHC, obj.40x. (**12**) SCC poorly differentiated from Animal 10, with a staining score of 3 and cytoplasmic granular staining (asterisks). IHC, obj.40x. (**13**) SCC poorly differentiated from Sample 4, showing immunoexpression by neoplastic keratinocytes mainly on the eroded surface of the tumor (asterisks). Attention was given to the intense chronic-active inflammatory infiltrate (arrow). IHC, obj.40x. FCAV-Unesp, Jaboticabal/SP, 2018.

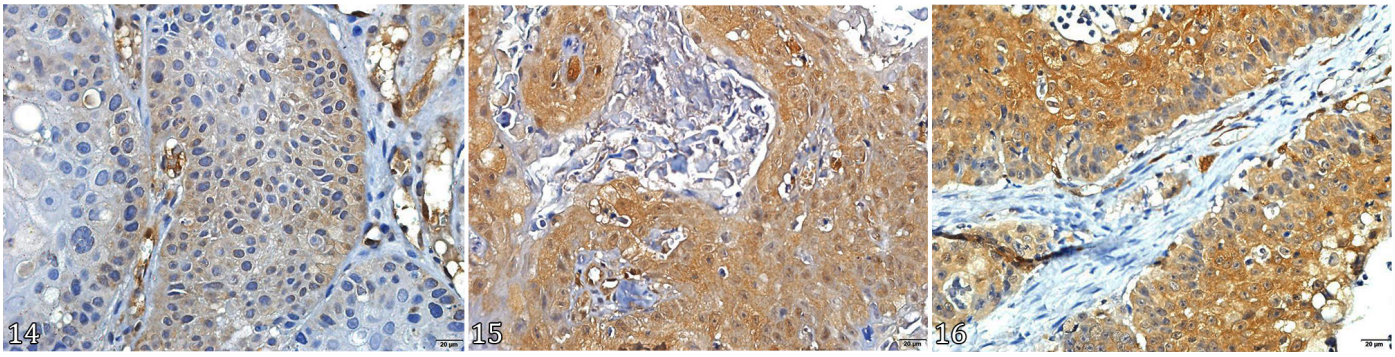

Fig.14-16. Photomicrographs of caspase-3 immunostaining of feline cutaneous squamous cell carcinoma (SCC). (**14**) SCC poorly differentiated from Sample 10, with a distribution percentage of 33.3% and caspase-3 labeling score of 2; note the weak labeling intensity. IHC, obj.40x. (**15**) SCC poorly differentiated from Sample 9, with a distribution percentage of 60.8% and a score of 6, observing the moderate intensity of anti-caspase-3 staining. IHC, obj.40x. (**16**) SCC poorly differentiated from Sample 4, with a distribution percentage of 62.87% and a score of 9, showing strong immunostaining intensity. IHC, obj.40x. FCAV-Unesp, Jaboticabal/SP, 2018.

Table 4. Immunoexpression of Ki-67, COX-2 and caspase-3, tumor volumes on D0 and D21 and type of response to ECT of the cutaneous SCCs of the cats included in the study. FCAV-Unesp, 2018

Samples	Ki-67 Score	COX-2 Score	Caspase-3 score	Volume (D0)	Volume (D21)	Response
1	2	3	2	Pinna: 1.64 Nasal: 0.11	Pinna: 0.52 Nasal: L.A.	Pinna: PR Nasal: CR
2	2	4	6	9.42	5.23	PR
3	1	1	6	Nose: 1.96 Right eye:1.49 Left eye: 2.05	NL.	CR
4	2	6	9	3.45	2.62	SD
5	1	2	0	0.94	0.042	PR
6	0	3	4	0.89	NL	CR
7	1	2	4	2.2	NL	CR
8	2	3	4	0.06	NL	CR
9	2	3	6	0.64	NL	CR
10	3	3	2	0.31	0.27	SD
11	3	3	6	0.52	NL	CR
12	3	0	4	Nasal: 0.42 Left eye:1.36 Right eye:0.19	NL	CR
13	2	1	6	2.07	3.45	PD

SCC = squamous cell carcinoma, FCAV-Unesp = "Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista 'Júlio de Mesquita Filho'", CR = complete remission, PR = partial remission, SD = stable disease, PD = progressive disease, N.L. = no lesion; Tumor volume is expressed in cm^3; ECT = electrochemotherapy, D0 = before ECT, D21 = 21 days after ECT.

Fig.17-24. Photographic images of cutaneous squamous cell carcinoma (SCC) before performing ECT (D0) and 21 days after ECT (D21). (**17**) Lesion of Sample 8 with a volume of 0.06cm^3 on D0. (**18**) Same sample in complete remission on D21. (**19**) Lesion of Sample 10 measuring 0.31cm^3 in volume on D0. (**20**) The same sample with a lesion measuring 0.27cm^3 on D21, which represents a reduction of 12.9% and constitutes a stable disease. (**21**) Lesions from Sample 12 on D0, measuring 0.42cm^3 in the nasal plane, 1.36cm^3 in the left eyelid and 0.19cm^3 in the right eyelid. (**22**) Same sample on D21 showing complete remission of lesions. (**23**) Lesion from sample 13 on D0, measuring 2.07cm^3. (**24**) Same sample on D21, with a lesion measuring 3.45cm^3, representing an increase of 66.6% and configuring a progressive disease.

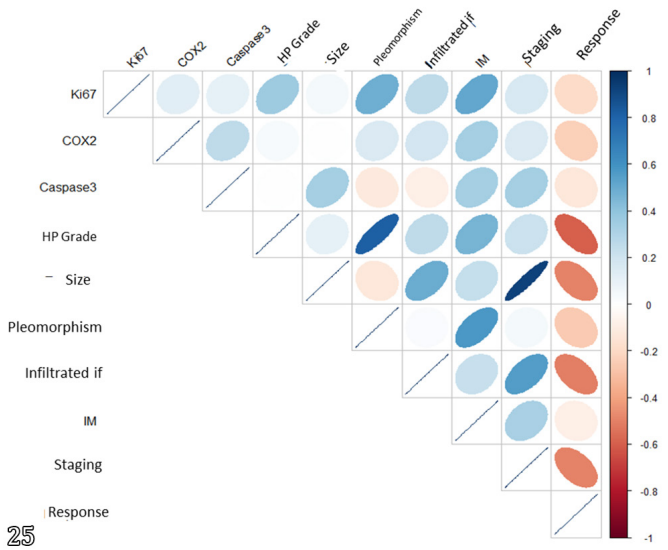

Fig.25. Spearman correlation matrix for the studied variables. FCAV-Unesp, Jaboticabal/SP, 2018.

The assessment of the proliferative index through Ki-67 expression is predictive of the behavior of several canine and human neoplasms (Zuccari et al. 2004, De Nardi 2007). In the present study, 77% of the tumors showed a relatively high percentage of positivity for Ki-67, with 38% to 60.2% immunopositive cells. This evidence supports the accelerated growth pattern of cutaneous SCCs. However, there was no significant correlation between the Ki-67 immunoexpression score and histopathological grade or tumor stage. These findings were identical to those of Melzer et al. (2006), who studied Ki-67 reactivity in the nasal and periocular SCC of cats treated with electron beam radiotherapy. Assessment of the proliferation score prior to therapy can be helpful in deciding the appropriate treatment protocol (Melzer et al. 2006). Ki-67 immunopositivity can be used as a predictive factor for tumor susceptibility to a given treatment. Melzer et al. (2006) found a positive correlation between Ki-67 and response to electron beam radiotherapy.

Cats with fewer Ki-67-positive cells had a shorter mean disease-free survival time than cats with higher Ki-67 reactivity. Similar results were observed by Raybaud-Diogène et al. (1997) in human patients with head and neck SCC treated with radiotherapy. Therefore, SCC with greater proliferative activity responds better to radiotherapy. Proliferative activity through Ki-67 may also be associated with tumor susceptibility to chemotherapy, as most chemotherapy drugs act on dividing cells. Thus, neoplasms with a high proliferative index are more susceptible to these drugs (De Nardi 2007). However, in this study, no correlation was observed between the Ki-67 index and the type of response to ECT. This finding can be justified because the ECT mechanism of action is not correlated with the presence of dividing cells since all cells in the electroporation field, regardless of the cell proliferation rate, are affected by the action of ECT.

The cutaneous SCCs of the cats in this study showed important COX-2 staining, as Nijsten et al. (2004) and Bardagí et al. (2012) observed. In view of this marked COX-2 expression, additional studies are needed on the use of COX-2 inhibitors in the management and prevention of cutaneous SCCs in cats. The immunoexpression of COX-2 in this study was identical to that observed by Bardagí et al. (2012), characterized by cytoplasmic staining, mainly in the perinuclear zone, with different intensities, in addition to displaying a granular pattern, as well as immunopositive cells in areas of erosion or superficial ulceration.

There was a moderate positive association between histopathological grade and MI and a strong significant correlation between histopathological grade and cellular pleomorphism. In addition, there was a significant correlation between pleomorphism and MI. These findings indicate that more undifferentiated SCCs present a higher proliferation rate and a higher rate of cellular pleomorphism, as described by Gross et al. (2007). A strong negative correlation was also found between the histopathological grade and the response to ECT. This demonstrates that the histopathological grade is considered a negative prognostic factor for cutaneous SCC in cats, as described by Hauck (2013). In addition, it is considered a negative predictive factor for the response to ECT.

Tumor size showed a strong significant correlation with tumor staging, as determined by the WHO (Owen 1980), and a negative correlation with the response and tumor staging. It can be observed that animals that did not respond to ECT were those with greater tumor sizes. This is in agreement with Bexfield et al. (2008) and Mali et al. (2013), who observed that in tumors larger than 3cm in diameter, ECT is less effective. This is due to the inadequate concentration of the chemotherapeutic agent in the target cells when electrical pulses are applied due to the heterogeneous distribution of blood flow in larger tumors, as well as the heterogeneous coverage of the electric field throughout the tumor volume (Mir et al. 2006, Testori et al. 2010).

Thus, the clinicopathological variables of histopathological grade, tumor size and staging, degree of cellular pleomorphism and degree of inflammatory infiltrate were considered negative prognostic factors for cutaneous SCC in cats since they correlated with greater tumor aggressiveness. Such variables were also considered negative predictive factors for the response to ECT due to their greater expressions implying less efficacy of ECT.

Table 5. Relationship between the median immunostaining for Ki-67, COX-2 and caspase-3 and the histopathological grade of the cutaneous SCCs of the evaluated felines. FCAV-Unesp, 2018

Sample	Histopathological grade	Ki-67 score	COX-2 score	caspase-3 score
1	Well-differentiate	2	3	2
2	Moderately differentiated	2	4	6
3	Well-differentiate	1	1	6
4	Poorly differentiated	2	6	9
5	Poorly differentiated	1	2	0
6	Well-differentiate	0	3	4
7	Well-differentiate	1	2	4
8	Well-differentiate	2	3	4
9	Poorly differentiated	2	3	6
10	Poorly differentiated	3	3	2
11	Well-differentiate	3	3	6
12	Poorly differentiated	3	0	4
13	Moderately differentiated	2	1	6

SCC = squamous cell carcinoma, FCAV-Unesp = "Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista 'Júlio de Mesquita Filho'".

The results of the present study corroborate previously reported data on the role of EQT in the treatment of feline cutaneous SCC. The overall response rate achieved in this study was 76.9% (61.5% complete remission and 15.4% partial remission). Similar results were found by Spugnini et al. (2009), Tozon et al. (2014) and Silveira et al. (2016).

Some lesions showed partial remission on the 21st day after ECT and subsequently achieved complete remission, and sample 10 had stable disease on D21 and then achieved complete response. Therefore, it is believed that D21 is an early moment to assess the response in larger lesions. According to Spugnini et al. (2015), a minimum duration of 2 weeks is required for a post-ECT response assessment. The need to repeat the protocol is related to the type and volume of the neoplasm (Serša et al. 2006, Silveira et al. 2010). For smaller tumors, a healing time between 4 and 8 weeks is considered, while larger tumors can take up to 10 weeks to heal (Mir et al. 2006, Mali et al. 2013).

Adverse effects resulting from ECT in this study were local and minimal, being easily symptomatically manageable, as observed by Spugnini et al. (2009) and Tozon et al. (2014). According to Testori et al. (2010) and Spugnini et al. (2012), minimal pain and erythema in the treated and adjacent areas are the most commonly reported side effects. The results show that ECT is a safe, effective technique with antitumor efficacy comparable to conventional tumor control techniques, such as surgery and cryosurgery (Thompson 2007). The technique has several advantages, such as the absence of bleomycin-related toxicity, speed and practicality in carrying out the protocol, absent or minimal trans- and post-therapeutic complications, relatively low cost (Silveira et al. 2016) and aesthetically acceptable results. The main limitation is the low efficacy to treat invasive and large tumors.

However, comparative interpretation between the different studies must be made cautiously since different electroporators were used in the different works. Another limitation of this study was the total number of felines included and the small size of the collected fragments. Thus, more homogeneous studies are needed regarding ECT protocols, the type of histopathological grade of the tumors, and their macroscopic characteristics, aiming at standardizing the lesions treated with ECT for a more reliable evaluation of the results. Long-term evaluation of patients treated with EQT for disease-free time and survival time is also important to determine the duration of remission achieved.

CONCLUSION

The results obtained in this research indicate that the determination of cell proliferation and apoptosis rates in feline cutaneous squamous cell carcinoma (SCCs) through immunohistochemical analysis and associated with clinical and pathological tumor characteristics are unrelated to the behavior of SCCs submitted to ECT and the clinical evolution of the patient.

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