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research article

Effects of electrochemotherapy with cisplatin and peritumoral IL-12 gene electrotransfer on canine mast cell tumors: a histopathologic and immunohistochemical study

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Background. The study was aimed to characterize tumor response after combined treatment employing electrochemotherapy with IL-12 gene electrotransfer in dogs with spontaneous mast cell tumors (MCT).

Materials and methods. Eleven dogs with eleven MCTs were included in the study. Histological changes were investigated in biopsy specimens collected before the treatment (T_0), and 4 (T_1) and 8 weeks (T_2) later. Cellular infiltrates were characterized immunohistochemically by using anti CD3, CD20, Foxp3 (Treg), CD68 and anti MHC-class II antibodies. Proliferation and anti-apoptotic activity of neoplastic cells were assessed using anti Ki-67 and Bcl-2 antibodies. Angiogenetic processes were investigated immunohistochemically by using anti Factor VIII and anti CD31 antibodies and micro vessel density quantification.

Results. Histopathological examination of samples at T_0 confirmed the diagnosis and the presence of scanty infiltrates consisted mainly of T-lymphocytes and macrophages. At T_1 and T_2 neoplastic cells were drastically reduced in 7/11 cases, small clusters of neoplastic cells were detected in 3/11 cases and 1/11 cases neoplastic cells were still evident. Proliferation activity of neoplastic cells was significantly reduced at T_1 and T_2 and expression of anti-apoptotic protein at T_1. Microvessel density was drastically reduced in all samples after treatment. The number of T-lymphocytes increased at T_1, although not significant, while Treg were significant higher at T_1 and macrophages at T_2.

Conclusions. The combined electrochemotherapy and IL-12 gene electrotransfer effectively induced a cellular response against neoplastic cells characterized mainly by the recruitment of T-lymphocytes and macrophages and a fibrotic proliferation with reduction of microvessels.

Key words: electrochemotherapy; histopathology; immune cells; interleukin-12; mast cell tumor; microvessel density

Introduction

Electrochemotherapy is an ablative technique for the treatment of solid tumors of different histotypes in human and veterinary oncology, with approximately 80% objective response of the treated tumors.[1,2] It is based on electroporation as drug delivery method to tumors for the chemotherapeutic drugs bleomycin or cisplatin to improve the antitumoral efficacy.[1]

Another biomedical application of electroporation is plasmid DNA delivery to tumors (gene elec-

trotransfer) for gene therapy. Preclinical and clinical studies demonstrated effectiveness of different therapeutic plasmid DNA electrotransfer to tumors as effective and safe method for local as well as loco-regional control of the cutaneous tumors.[3] The most studies explored gene electrotransfer of plasmid DNA coding for IL-12 cytokine.[4-6] Its effectiveness was demonstrated also in treatment of spontaneous tumors in dogs.[7-10]

A new treatment approach is combining local tumor electrochemotherapy with gene therapy with plasmid coding for IL-12 peritumorally to skin.[11] Some preclinical data indicate that this approach provides in situ vaccination by electrochemotherapy that is boosted by immunogene IL-12 peritumoral gene electrotransfer.[3]

Our previous report has provided evidence for this approach in MCT with excellent local tumor control and long lasting progression free survival of the treated dogs.[11] To provide further evidence on the mechanisms of action, this study evaluates the histopatological features of those tumors in more detail to characterize whether this approach provides antitumor response also due to boosting the immune response. Therefore, we characterized tumor response including the cellular infiltrates at different time points post-electrochemotherapy with cisplatin combined with peritumoral IL-12 electrotransfer in dogs, spontaneously affected by MCT and compared these results with those observed in biopsies collected before the treatment. Proliferation and anti-apoptotic activity of neoplastic cells as well as the changes in microvessel density were also investigated, at the same timing.

Materials and methods

Animals and tumors

Between January and December 2010 eleven subjects, 4 males and 7 females of different breeds ranging from 5 to 9 year-old (mean 6.5 years ± 1.3 years) complied with inclusion criteria for the clinical survey (histologically confirmed MCT in different anatomical locations, good general health conditions with normal routine hematologic and biochemical profile without cardiac dysfunctions), were included in the study. The animals included in the study were those that owners refused any other type of standard treatment/surgery with wide excision of nodules.

Before the treatment a staging in all patients was performed according to modified WHO staging criteria with physical examination, examination of thoracic radiographs, abdominal ultrasonography and basic bloodwork, consisting of a complete blood count with differential white cell count. Biochemical parameters (urea, creatinine, serum alkaline phosphatase and alanine aminotransferase) were determined using an automated chemical analyzer.

The study was approved by the Ethical Committee of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (approval No. 323-451/2004-9). Prior to the inclusion a written consent for participation in the clinical study for each animal was obtained from their owners.

Electrochemotherapy and gene transfer

Electrochemotherapy and IL-12 gene electrotransfer were performed as previously described.[11] Briefly, electrochemotherapy with intratumoral injection of cisplatin (*cis*-diamminedichloroplatinum II, Cisplatyl; Aventis, Paris, France), at a concentration of 2 mg/ml in a dose of ~1 mg/cm^3 was performed just before electric pulses were delivered (8 pulses each of 100 μs duration and amplitude to electrode distance ratio of 1300 V/cm and frequency of repetition 5 kHz with electric pulses generator Cliniporator™ (IGEA s.r.l., Carpi, Italy)). Two parallel stainless steel plate electrodes with 6 mm distance between them or 4 needle row electrodes with 4 mm distance according to the tumor size was used. Immediately after the electrochemotherapy 2 mg of the IL-12 plasmid was injected intradermally in equidistant locations around tumor nodule in two locations.[10] One high voltage pulse (amplitude to electrode distance ratio 1200 V/cm, duration 100 μs) and one low voltage pulse (amplitude to electrode distance ratio 140 V/cm, duration 400 μs)[12] were delivered immediately after plasmid injection, using the same electric pulse generator and electrodes as mentioned above.

Tissue sampling and processing

All the subjects were submitted to biopsy before (T_0) the combined therapy and at 4 (T_1) and 8 weeks (T_2) post-treatment. Biopsies were fixed into 10% neutral buffered formalin and then processed routinely for paraffin embedding. Five-micrometers serial sections from all specimens were stained for haematoxylin and eosin (HE), Gomori's modified trichrome stain and also mounted on treated glass slides (Superfrost Plus; Menzel-Glaser, Germany) for immunohistochemistry. Mast cell tumors were classified according to the Kiupel *et al*. (2011) classification.[13]

TABLE 1. Antibodies used in the study

Antibody	Specificity	Type	Species	Source	Dilution	Pretreatment
Anti-human CD3	Pan-T lymphocytes	Policonal	Rabbit	(A0552) Dako UK Ltd. Ely UK	1:50	Citrate buffer pH6
Anti-human CD20	Pan-B lymphocytes	Policonal	Rabbit	(RB-9013-PO) Thermo Scientific, Chesire, UK	1:400	None
Anti-human Foxp3	T-reg lymphocytes	Monoclonal	Mouse	(7979) Affymetrix eBioscience, san Diego, CA USA	1:100	Triss-EDTA pH9
Anti-human CD68	Macrophages	Monoclonal	Mouse	(PG-M1) Thermo Scientific, Chesire, UK	1:100	Proteinase K
Anti-human Ki-67	Proliferating cells	Monoclonal	Mouse	(MIB1; M7240) Dako UK Ltd. Ely UK	1:100	Citrate buffer pH6
Anti-human Bcl-2	Anti-apoptotic protein	Monoclonal	Mouse	(610538) BD Biosciences, Wyckoff, NJ, USA5	1:100	Citrate buffer pH6
Anti-human Von Willebrand Factor -	Endothelial cells	Policlonal	Rabbit	(A0082) Dako UK Ltd. Ely UK	1:300	Citrate buffer pH6
Anti-human CD31	Endothelial cells	Monoclonal	Mouse	(JC70A) Dako UK Ltd. Ely UK	1:100	Citrate buffer pH6

Immunohistochemistry

Sections were dewaxed in xylene and rehydrated through graded alcohols prior to quenching endogenous peroxidase activity with 3% H_2O_2 in distilled water for 20 minutes. Heat induced epitope retrieval with citrate buffer pH 6 was performed. Immunohistochemical labelling was performed manually with the Sequenza slide rack and cover-plate system (Shandon, Runcorn, UK). Non-specific antigen binding was blocked by incubation with UltraVision Protein Block (TA-125-PBQ; Thermo Scientific, Cheshire, UK). A panel of primary antibodies was applied to serial sections and incubated overnight at 4°C (Table 1). Antibody binding was detected by the Biotinylated Goat Polyvalent Secondary (TP-125-BN; Thermo Scientific, Cheshire, UK), Streptavidin Peroxidase (TS-125-HR; Thermo Scientific, Cheshire, UK) and DAB chromogen (SK-4105; ImmPact DAB, Vector, Burlingame, CA) as indicated by manufacturer's instructions and slides were counterstained with haematoxylin. Substitution of the primary antibody with unrelated matched primary antibody was used to provide a negative control. Serial sections of canine lymph node were used as positive control.

Slides were examined by two pathologists (C.S. and A.P.) without knowledge of the corresponding clinical and pathological data.

Quantification of immunolabeling

Bright field images were acquired at x20 magnification with a Leica Microsystem DFC490 digital camera mounted on Leica DMR microscope (Wetzlar, Germany). Counting were performed using a semiautomatic analysis system (LASV 4.3, Leica). Six 10,000 μm^2 random fields of the central and periphery parts of the biopsies were used for counting the number of infiltrating CD3+, CD20+, Foxp3+ (Treg+), CD68+ and MHC Class II+ cells and Bcl-2+ and Ki-67+ neoplastic cells. Microvessel density was determined with Factor VIII and CD31 immunostained section in six 50,000 μm^2 random fields.

Statistical analysis

Statistical analysis was performed using the statistical package SPSS Advanced Statistics 21.0 (SPSS Inc., Chicago, IL, USA). ANOVA test was used to compare the composition of cell infiltrates and microvessel density at the different times of observation and post hoc analysis was made by Bonferroni Test. Statistical significance was based on a 5% (0.05) significance level.

Results

Histologic features

At T_0, all eleven skin tumors of different volumes (ranged from 0.2 to 16.9 cm^3) were diagnosed as low grade MCTs and were characterized by sheets of polygonal neoplastic cells with abundant cytoplasm containing variable number of metachromatic granules, associated with moderate to massive infiltration of eosinophils, in the superficial and deep dermis, sometimes extending also to deep muscular layer (Figure 1A). Mitotic index was low (1 to 2 mitoses/10 HPF). Scanty multinucleated cells were observed. Rarely necrotic areas

were present. Neoplastic cells were associated with reactive proliferation of connective tissue.

At T_1 in biopsies collected from seven dogs with complete response, the neoplastic tissue was substituted by a fibrotic tissue composed by horizontally oriented wavy collagen fibers associated with inflammatory infiltrates mainly constituted by lymphocytes and macrophages (Figure 1B). In three cases (partial response), single or small clusters of neoplastic cells were still evident scattered among the fibrotic connective tissue, while in one dog neoplastic mast cells were still evident with features similar to T_0.

At T_2 biopsies collected from the seven dogs which did not present tumor cells at T_1 were still free of neoplastic cells and consisted of a dense fibrous tissue still infiltrated by mononuclear cells. In other three cases (one with stable disease and two with partial response), thin aggregates of neoplastic mast cells between connective tissue bundles, were still evident (Figure 1C), while in one case with partial response, neoplastic cells were numerous as at T_0 and T_1.

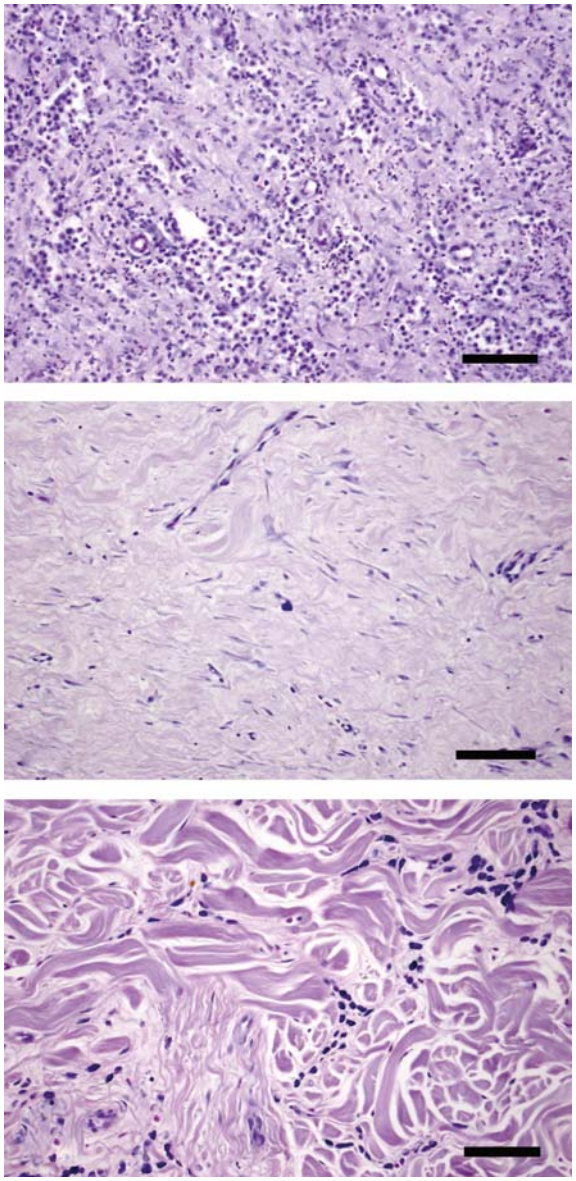

FIGURE 1. Histology of tissue samples collected before the combined therapy (T_0) and at 4 (T_1) and 8 weeks (T_2) post-treatment. **(A)** At T_0 sheets of neoplastic cells with abundant cytoplasm containing variable number of metachromatic granules were present in the superficial and deep dermis; **(B)** At T_1 The neoplastic tissue was substituted by a fibrotic tissue associated with scanty inflammatory infiltrates mainly constituted by mononuclear cells; **(C)** At T_2 in dogs with partial response between connective tissue bundles were evident thin aggregates of neoplastic mast cells. Haematoxylin Eosin; bar = 100 mm.

Immunohistochemistry

Immunohistochemical analysis of biopsies collected before the treatment and at different times of post-treatment revealed, in dermal infiltrates the presence of CD3+ T lymphocytes (Figure 2A, B), CD20+ B lymphocytes, Treg Lymphocytes (Figure 2C, D), and macrophages (Figure 2E, F), in different percentages, with T lymphocytes and macrophages representing the predominant cell populations. Results of immunohistochemical evaluation of inflammatory infiltrates at T_0 and at T_1 and T_2 intervals are presented in the Figure 3. Overall, the T lymphocyte number increased at T_1, even this difference was not statistically significant and was reduced at T_2. Specifically, in biopsies collected from subjects with complete remission, the number of CD3+ lymphocytes was significantly higher than from dogs with stable or progressive disease both at both T_1 (8.1 ± 8.2 CD3+cells/ 10,000 mm^2 *vs* 3.4 ± 1.9 CD3+cells/ 10,000 mm^2; p = 0.008) both at T_2 (6.6 ± 4.4 CD3+cells/ 10,000 mm^2 *vs* 3.2 ± 1.9 CD3+cells/ 10,000 mm^2; p = 0.001). Macrophages significantly increased at T_2 (P = 0.006) and Treg lymphocytes significantly increased at T_1 (p = 0.0001), but no differences were observed at T_2. No differences were observed in the presence of CD20 lymphocytes at T_1 and T_2.

Immunohistochemical studies allowed to determine also the presence of proliferative and anti-apoptotic activities of neoplastic cells (Figure 4A, B; and C, D, respectively) as well as the micro-vessel density (Figure 4E, F) at T_0, T_1 and T_2. Results were summarized in the Figure 5.

Proliferation activity of neoplastic cells was statistically reduced at T_1 (p = 0.0001) and T_2 (p = 0.0001), while the number of neoplastic cells ex-

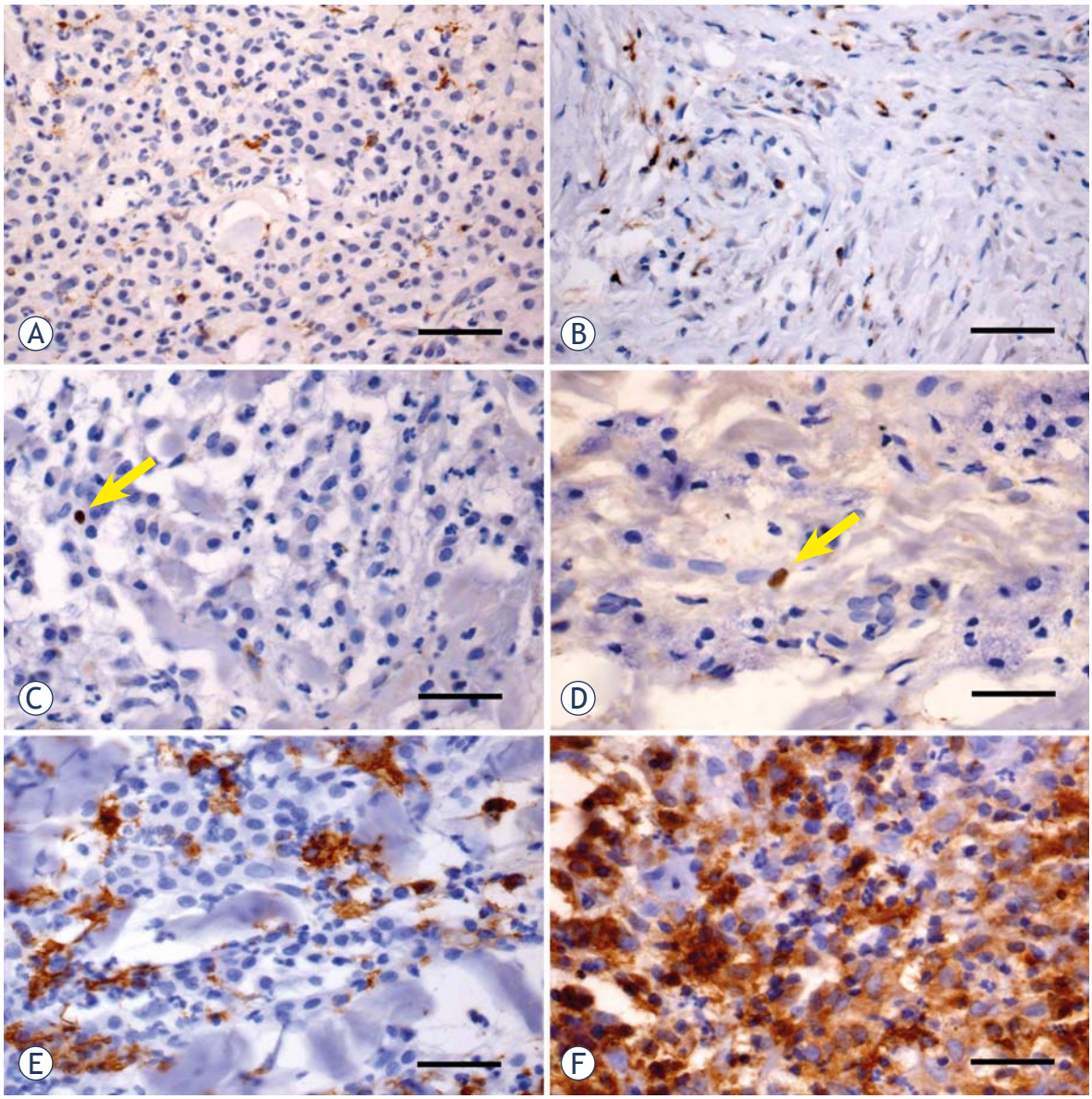

FIGURE 2. Immunoistochemical staining of tissue samples collected at T_0 **(A, C** and **E)** and , T_1 **(B, D** and **E)**. CD3+ lymphocytes infiltrating the neoplastic tissue at T_0 **(A)** and the fibrotic tissue at T_1 **(B)**. Scanty Foxp3+ Treg lymphocytes at the periphery of neoplastic tissue at T_0 **(C)** and in a tissue sample collected at T_1 **(D)**. CD68+ macrophages in the neoplastic tissue at T_0 **(E)** and in the fibrotic tissue at T_1 **(F)**. Immunohistochemical staining using DAB chromogen and haematoxylin counterstain. Bar = 100 mm.

pressing the Bcl-2 anti-apoptotic protein was increased at T_1 ($p = 0.0001$), while was reduced 4 weeks later. Proliferation activity of neoplastic cells in the biopsies collected from subjects with complete response was significantly lower than in the biopsies collected from dogs with stable or progressive disease both at T_1 (1.5 ± 2.1 Ki-67+cells/ 10,000 mm^2 *vs* 2.5 ± 0.7 Ki-67+cells/ 10,000 mm^2; $p = 0.012$) both at T_2 (0.4 ± 0.6 Ki-67+cells/ 10,000 mm^2 *vs* 2.2 ± 1.0 Ki-67+cells/ 10,000 mm^2; $p = 0.0001$), as well as the expression of anti-apoptotic Bcl-2 protein both at both T_1 (1.9 ± 2.0 Bcl-2+cells/ 10,000 mm^2 *vs* 3.2 ± 0.9 Bcl-2+cells/ 10,000 mm^2; $p = 0.004$) both at T_2 (1.4 ± 1.5 Ki-67+cells/ 10,000 mm^2 *vs* 2.8 ± 1.2 Ki-67+cells/ 10,000 mm^2; $p = 0.0001$).

Microvessel density, determined using both anti-Factor VIII and anti-CD31 antibodies, was drastically reduced at T_1 ($p = 0.000$) and T_2 ($p = 0.0001$), when compared with the samples collected before the treatment.

Discussion

Histopathological evaluation of tissue biopsies after electrochemotherapy with cisplatin combined with peritumoral IL-12 gene electrotransfer demonstrates that in the tumors with complete clinical response, pronouncedly reduced number and proliferation rate of the tumor cells was obtained with significantly enhanced immune and anti-vascular response, which confirm our previously reported clinical results.[11]

At the best of our knowledge this is the first clinical study that describes the histopathological and cellular changes induced in vivo by electrochemotherapy and peritumoral IL-12 electrotransfer in dogs bearing MCT, performed with the consent of the owners. The number of biopsies was reduced and made at distance from treatment with the aim to verify its effectiveness.

Already at four weeks post-treatment, in dogs with a complete responses histopathologic examination revealed a marked reduction of neoplastic cells, confirming the anticancer efficacy of the combined electrochemotherapy and gene therapy. This reduction was even more evident four weeks later. On the contrary, in dogs with a partial response the reduction of neoplastic cell was lower at four weeks after treatment and this reduction was absent in dog with stable disease.

The reduced presence of neoplastic cells detected in the tissue samples from dogs with complete response was also associated with a reduced proliferative activity of remnant neoplastic mast cells, characterized by a significant reduction of positivity to Ki-67 antibody and by a significant reduced expression of the anti-apoptotic Bcl-2 protein. The major mechanism of cisplatin tumor destruction consists of the formation of intrastrand and interstrand DNA adducts leading to DNA fragmentation that can ultimately lead to an apoptotic mediate cell death as demonstrated in vitro and in vivo in murine models[14] and the decreased expression of anti-apoptotic factor as the Bcl-2 protein promotes higher death rate of the cancer cells.[15]

The time interval of 4 weeks of the first post-treatment biopsy did not allow evaluation of the tumor necrosis, since at this time point dense fibrotic connective tissue with progressive reduction of microvessel density was already present. In experimental animal models has been demonstrated that electroporation induces a higher internalization of cisplatin molecules with a rapid necrosis of neoplastic cells[16] and vascular disrupting action by causing a rapid shutdown of tumour blood flow leading to reduced tumour oxygenation, increased tumour hypoxia and tumour necrosis.[17]

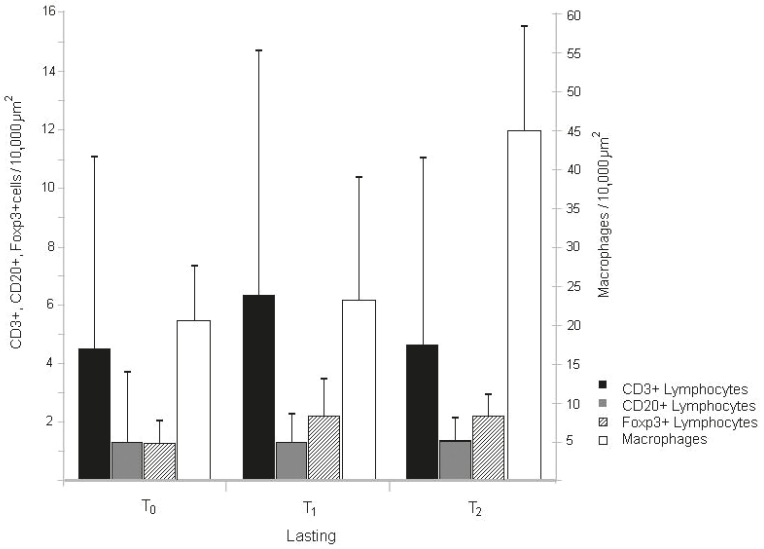

FIGURE 3. Histogram of number of immune cells in 10,000 µm² of tissue samples collected al T_0, T_1 and T_2. Slight increase of CD3+ lymphocytes at T_1, while macrophages significantly increased at T_2 and Treg lymphocytes at T_1.

In this study, electrochemotherapy was associated with IL-12 gene electrotransfer with proven good local and locoregional antitumor effects.[11] The potential for inducing an antitumoral immune response to treat neoplastic disorders is well known, but the application of this technology has not yet been meaningfully transferred to the clinic. Local administration of recombinant cytokines directly into the tumor has proven to be much safer than systemic delivery.[13] IL-12 is a natural occurring cytokine that showed potential success for treating cancer by inducing a specific anti-tumoral immune response.[18] Gene therapy with IL-12 pDNA showed to be effective to treat multiple tumor histotype in rodent models and induce an anti-tumoral immune response capable of affecting neoplastic tissues.[14-16] Likewise, IL-12 gene therapy in canine species can induce tumor regression in spontaneous neoplasms of different histotypes.[17,19,20]

Another therapeutic approach that is currently extensively explored is combination of the ablative techniques with different immunomodulatory approaches, either with immune checkpoint inhibitors, Treg depletion or immunostimulation.[3] In this context ablative techniques are considered as in situ vaccination with further boosting of the immune responsiveness of the organism. For this purpose, we postulated that electrochemotherapy

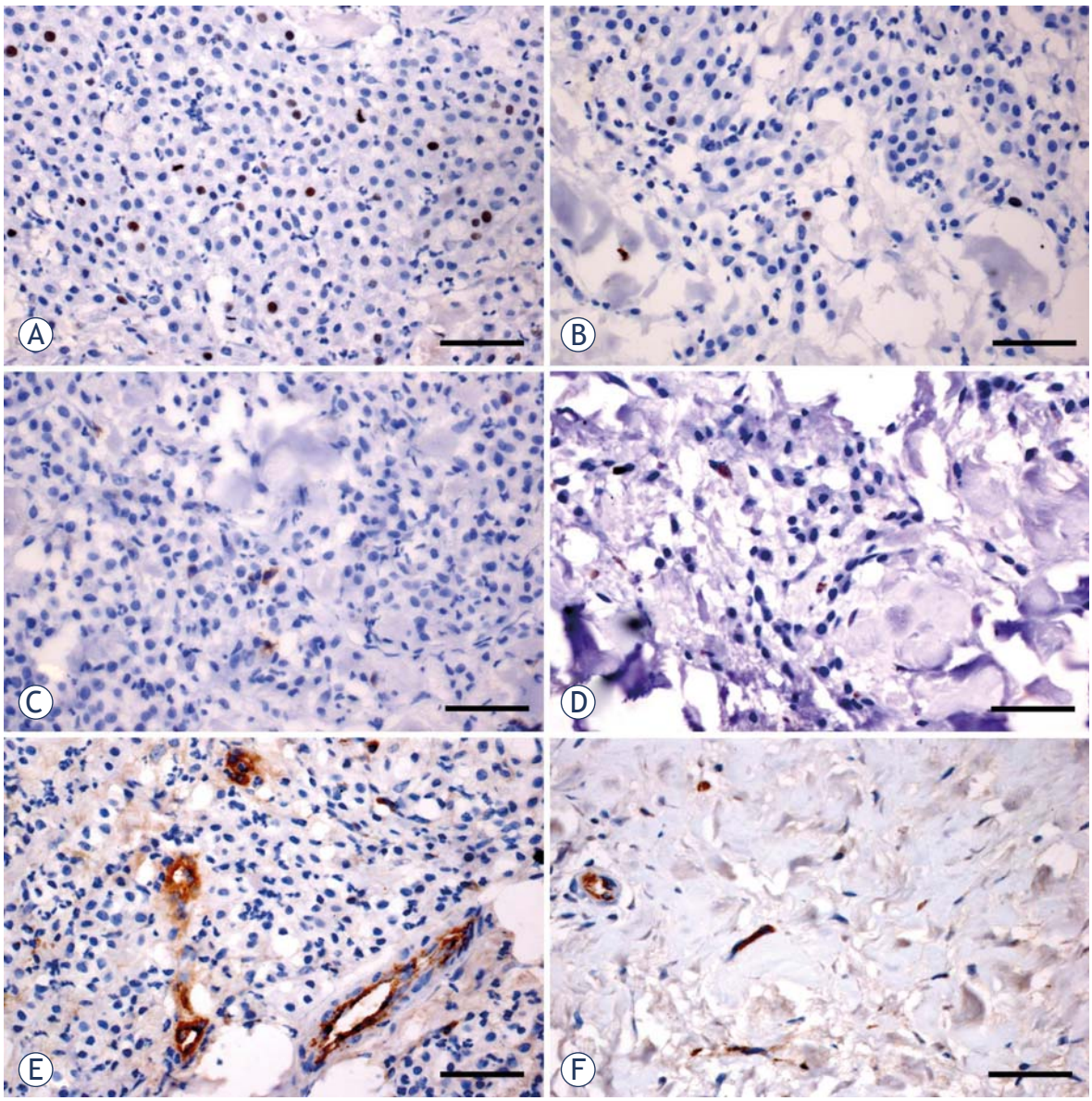

FIGURE 4. Immunoistochemical staining of tissue samples collected at T_0 **(A, C** and **E)** and , T_1 **(B, D** and **E)**. Ki-67+ neoplastic cells at T_0 **(A)** and at T_1 in a dog with partial response **(B)**. Bcl-2+ neoplastic cells at T_0 **(C)** and at T_1 in a dog with partial response **(D)**. Microvessels stained using an anti-CD31 primary antibody at T_0 **(E)** and in the fibrotic tissue at T_1 in a dog with a complete response **(F)**. Immunohistochemical staining using DAB chromogen and haematoxylin counterstain. Bar = 100 mm.

could be in situ vaccination, which can be boosted with peritumoral IL-12 gene electrotransfer, given peritumorally.[3] Our clinical study on MCT supports this hypothesis, providing evidence that this treatment combination has excellent local tumor control and also the long lasting disease free interval (between 3 and 4 years). Furthermore the present study immunohistologically supports the value of peritumoral IL-12 gene electrotransfer in boosting immune response of the organism, providing evidence of the presence of immune cells (T-lymphocytes and macrophages) in the tumors treated with electrochemotherapy and peritumoral IL-12 gene electrotransfer compared to tumors before therapy. There is also another study on sarcoids in horses, using the same combined treatment in which increased number of CD4 and CD8 lymphocytes sub-population confirmed induction of local immune response in treated tumors.[21]

Even though in our study the number of examined biopsies was limited, at 4 weeks post-treatment our immunohistochemical results unequivo-

cally demonstrated an increase of T-lymphocytes in cellular infiltrates, higher in subjects with a complete response. T cells are the essential components of the adaptive immune response and they interact closely with antigen presenting cells to release various cytokines that characterize the specific immune response also to neoplastic cells. The T-lymphocyte increase detected after four weeks post treatment was not evident 4 weeks later, indicating that probably the anti-tumoral immune response induced by IL-12 is only transient as previously hypothesized or that due to the complete tumor regression, they could not be observed in higher numbers in the biopsies.[15]

The presence of B cells in examined subjects was not affected by IL-12 gene electrotransfer 4 as well as 8 weeks after the treatment. On the contrary, macrophages increased 8 weeks post-treatment, which was not related with the response to the therapy. The macrophages, as any innate immune cells, act when tissue homeostasis is perturbed, releasing soluble mediators such as cytokines, chemokines, matrix remodeling proteases and reactive oxygen species and contribute to the maintenance of the inflammation and this late increase of these cells detected in treated subjects should be related with the tissue damage induced by the therapy.

Tregs play an important role in down regulating immune responses and an imbalance between numbers of Tregs and CD4+ and CD8+ T-lymphocytes contributes to the outcomes in cancer and infectious diseases. Many studies have demonstrated the *in vivo* role of these cells in the suppression of the immune response to the cancer in human[22,23] and veterinary oncology.[24,25] While Treg are immunosuppressive at sites of inflammation, the increase detected in our study should be explained by previous studies demonstrating that the adjuvant activity of IL-12 is short-lived due to regulatory Treg re-infintration. In fact, quantitative analysis of Treg kinetics in IL-12 treated tumors revealed a transient loss of these cells followed by a rapid fold expansion of tumor Treg between days 3 and 10 post treatment.[26] So the increase of Treg observed in our study should be related with the timing of tissue sampling after the treatment or due to the presence of inflammation.

In conclusion, our study confirmed the synergic power of electrochemotherapy and IL-12 gene electrotransfer in canine species at immunohistopathological level. It led to number of complete responses and extends survival through the induction of interferon gamma, inhibiting angiogenesis and increasing cytotoxic activity.[3]

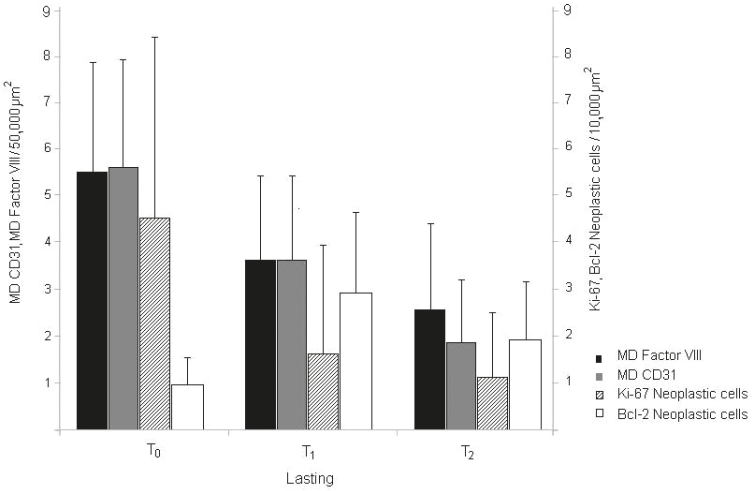

FIGURE 5. Histogram of microvessel density determineg using both primary antibodies against Factor VIII and CD31positive-cells in 50,000 µm² of tissue samples and number of Ki-67+ and Bcl-2+ neoplastic cells in 10,000 µm² of tissue samples collected al T_0, T_1 and T_2. Microvessel density was drastically reduced at T_1 and T_2. as well as the proliferation activity of neoplastic cells, while Bcl-2 expression was increased at T_1.

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