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PEQUEÑOS ANIMALES

Nº 2

- Immunophenotyping of Peripheral Blood, Lymph Node, and Bone Marrow T Lymphocytes During Canine Leishmaniosis and the Impact of Antileishmanial Chemotherapy

  Inmunofenotipado de la sangre periférica, los ganglios linfáticos y los linfocitos T de la médula ósea durante la leishmaniosis canina y el impacto de la quimioterapia antileishmanial

- Veterinary Guidelines for Electrochemotherapy of Superficial Tumors

  Directrices veterinarias para la electroquimioterapia de tumores superficiales

SUMARIO

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EDITA:
IMPULSO VET
impulsovet@impulsovet.es

Immunophenotyping of Peripheral Blood, Lymph Node, and Bone Marrow T Lymphocytes During Canine Leishmaniosis and the Impact of Antileishmanial Chemotherapy

Inmunofenotipado de la sangre periférica, los ganglios linfáticos y los linfocitos T de la médula ósea durante la leishmaniosis canina y el impacto de la quimioterapia antileishmanial

Palabras clave: terapia antileishmanial, médula ósea, leishmaniosis canina, células T efectoras, citometría de flujo, ganglio linfático, células mononucleares de sangre periférica, células T (Treg) reguladoras

Keywords: *antileishmanial therapy, bone marrow, canine leishmaniosis, effector T cells, flow cytometry, lymph node, peripheral blood mononuclear cells, regulatory T (Treg) cells*

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Marcos Ferreira Santos[1], Graça Alexandre-Pires1, Maria A. Pereira[2], Lídia Gomes1, Armanda V. Rodrigues[2], Alexandra Basso[1], Ana Reisinho[1], José Meireles[1], Gabriela M. Santos-Gomes[2] and Isabel Pereira da Fonseca[1]*

1. CIISA-Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária, Universidade de Lisboa, Lisbon, Portugal
2. GHTM-Global Health and Tropical Medicine (GHTM), Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova De Lisbon (UNL), Lisbon, Portugal

Tabla de contenido:

Los perros son un reservorio importante de Leishmania infantum, agente etiológico de la leishmaniosis canina (CanL), una enfermedad visceral zoonótica de preocupación mundial. Los protocolos terapéuticos basados en medicamentos antileishmaniales se utilizan comúnmente para tratar a los perros enfermos y mejorar su condición clínica.

Dogs are a major reservoir of *Leishmania infantum*, etiological agent of canine leishmaniosis (CanL) a zoonotic visceral disease of worldwide concern. Therapeutic protocols based on antileishmanial drugs are commonly used to treat sick dogs and improve their clinical condition. To better understand the impact of *Leishmania* infection and antileishmanial drugs on the dog's immune response, this study investigates the profile of $CD4^+$ and $CD8^+$ T cell subsets in peripheral blood, lymph node, and bone marrow of sick dogs and after two different CanL treatments. Two CanL groups of six dogs each were treated with either miltefosine or meglumine antimoniate combined with allopurinol. Another group of 10 clinically healthy dogs was used as control. Upon diagnosis and during the following 3 months of treatment, peripheral blood, popliteal lymph node, and bone marrow mononuclear cells were collected, labeled for surface markers CD45, CD3, CD4, CD8, CD25, and intracellular nuclear factor FoxP3, and T lymphocyte subpopulations were immunophenotyped by flow cytometry. CanL dogs presented an overall increased frequency of $CD8^+$ and $CD4^+CD8^+$ double-positive T cells in all tissues and a decreased frequency of $CD4^+$ T cells in the blood. Furthermore, there was a higher frequency of $CD8^+$ T cells expressing $CD25^+FoxP3^+$ in the blood and bone marrow. During treatment, these subsets recovered to levels similar to those of healthy dogs. Nevertheless, antileishmanial therapy caused an increase of $CD4^+CD25^{+-}FoxP3^+$ T cells in all tissues, associated with the decrease of $CD8^+CD25^-FoxP3^-$ T cell percentages. These findings may support previous studies that indicate that *L. infantum* manipulates the dog's immune system to avoid the development of a protective response, ensuring the parasite's survival and the conditions that allow the completion of *Leishmania* life cycle. Both treatments used appear to have an effect on the dog's immune response, proving to be effective in promoting the normalization of T cell subsets.

Abbreviations: ALT, *ALT, alanine aminotransferase; AST, aspartate aminotransferase; BID, bis in die; BUN, blood urea nitrogen; CanL, canine leishmaniosis; CD, cluster of differentiation; CG, control group; CVBD, canine vector-borne disease; DNA, deoxyribonucleic acid; FMO, fluorescence minus one; FoxP3, forkhead box protein 3; IFN-γ, interferon gamma; IL-2, interleukin 2; IL-10, interleukin 10; Megl+Al, meglumine antimoniate with allopurinol; Milt+Al, miltefosine with allopurinol; SID, semel in die; TGF-β, transforming growth factor beta; Th1, type-1 T-helper; Th2, type-2 T-helper; TNF-α, tumor necrosis factor alpha; Treg cells, regulatory T cells; UPC, urine protein-to-creatinine ratio.*

Tabla de contenido:

Introduction

Leishmaniosis is considered a neglected tropical disease [1] that affects humans and domestic and sylvatic animals. Parasites of the genus *Leishmania* are obligatory intracellular protozoa and the etiological agent of this parasitic disease [2]. The main host cell for *Leishmania* parasites is the macrophage, which the parasite is able to manipulate and prevent activation by various mechanisms and, thus, avoid their intracellular death and perpetuate the infection [3–5]. Canine leishmaniosis (CanL), endemic in about 50 countries and two major regions, South America and the Mediterranean basin, is caused by *Leishmania infantum* [6]. Dogs affected by this disease can present a wide variety of specific and unspecific clinical signs [7,8]. CanL conventional treatments improve the clinical condition of dogs and reduce the parasite burden [9]. Although when therapy is discontinued, relapses are common [10–12], indicating that treatment does not promote parasite clearance in all cases. Thus, it is important to improve the efficacy of the treatment protocols applied to CanL to promote the clinical cure of the dog, ensure parasite clearance, and prevent further transmission. According to the most recent guidelines [8], the recommended CanL treatment protocols combine allopurinol with either meglumine antimoniate or miltefosine. Meglumine antimoniate is a pentavalent antimonial considered a multifactorial drug whose effects are still unclear. However, some authors have referred the promotion of *Leishmania* DNA damage by oxidative stress and influence on macrophage microbicidal activity [13–15]. Pentavalent antimonials, which belong to the same family of meglumine antimoniate, such as sodium antimony gluconate, have been shown to interfere with the host's immune system by activating macrophages to release interleukin 12 (IL-12), leading to the subsequent production of interferon-γ (IFN-γ) by other immune cells, that induce the phosphorylation of extracellular signal-regulated kinase 1 (ERK-1) and ERK-2, driving the production of reactive oxygen species (ROS) [16]. Moreover, they also appear to induce the expression of class I molecules of the major histocompatibility complex (MHC), stimulating $CD8^+$ T cells that lead to apoptosis of infected cells [17,18]. Although these drugs have proved antileishmanial activity *in vitro* and *in vivo*, pentavalent antimonials have failed to treat visceral leishmaniosis in human patients who are also infected with HIV or receiving immunosuppressive therapy [17], indicating that a complete cure is dependent on T cell-mediated responses [19,20]. Miltefosine is an alkylphosphocholine compound able to induce apoptosis by mechanisms still not entirely clear, although the specific disturbance of the lipid content on the parasite's membrane and the modulation of macrophage activity are the most consensual modes of action [18,21–24]. Several studies have reported the immunomodulatory properties of miltefosine, with *in vitro* studies showing the induction of the release of tumor necrosis factor α (TNF-α) and nitric oxide (NO) by peritoneal macrophages of BALB/c mice [25] and enhancement of IFN-γ receptors, thus restoring responsiveness to this cytokine in macrophages infected by *L. donovani* and promoting an IL-12-dependent Th1 response [26]. Also, in healthy human peripheral blood cells, it was found that miltefosine was able to increase the production of IFN-γ, acting as a co-stimulator of the IL-2-mediated T cell activation process, together with increased expression of CD25, showing the possible immunomodulatory activity of miltefosine [27]. Allopurinol, a purine analog of adenosine nucleotide, blocks RNA synthesis, inhibiting *Leishmania* growth [28,29]. To date, meglumine antimoniate or miltefosine in combination with allopurinol are both considered first-line treatments in Europe [7,8]. Recently, in Brazil, miltefosine therapy was approved for CanL treatment [30]. Taking into account the emergence of a greater number of reports on drug resistance, whether it be in humans or

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dogs [13,17,21,31], it is crucial to deepen the understanding of the mode of action of the most used antileishmanial therapies.

In dogs, disease outcome is mainly determined by the cell-mediated immune response, with T cells playing a key role in cytokine release, which interacts with infected macrophages, influencing macrophage activation and subsequent killing of internalized parasites. According to the cytokine environment, naive $CD4^+$ T lymphocytes can differentiate into a protective subset (Th1) or a Th2 cell subset, which favors the progress of infection [32]. A protective Th1 immune response is characterized by a high production of pro-inflammatory cytokines as is the case of IFN-γ, TNF-α, and IL-2. These cytokines stimulate the cytotoxic activity of $CD8^+$ T cells and activate macrophage respiratory burst, leading to the synthesis of ROS and induce NO production, which can cause major damage to the parasite membrane, leading to the death of the parasite [32–34]. On the other hand, a Th2 response directs the release of anti-inflammatory cytokines and stimulates the humoral immune response, favoring the establishment of infection and disease exacerbation [6,7]. Previous works on symptomatic dogs with CanL have demonstrated that the lack of adequate cell-mediated immune response might be associated with decreased levels of $CD4^+$ T cells and high antibody titers [35–38]. *In vitro* studies of cytotoxic $CD8^+$ T cells from asymptomatic dogs demonstrated a role in resistance to CanL by enhancing IFN-γ production and causing the lysis of infected macrophages [39].

A critical role of immune regulation has been attributed to a subgroup of cells denominated regulatory T (Treg) cells, which seem to be recruited to the sites of *Leishmania* infection, enabling parasite survival and ensuring the transmission cycle [40,41]. Experimental studies of cutaneous leishmaniosis performed in *L. major*-infected mice showed that Treg cells are essential for the development and maintenance of persistent cutaneous disease [40]. The fast increase of $CD4^+CD25^+$ Treg cells at the sites of *L. major* infection suppressed parasite-eliminating immune mechanisms [41]. Accumulation of IL-10-producing Treg cells observed in the bone marrow of patients with *L. donovani* visceral leishmaniosis can cause immunosuppression, prevent the release of pro-inflammatory cytokines, like IFN-γ, avoid macrophage activation, and be associated with unresponsiveness to treatment [42]. Another study showed increased $CD4^+CD25^+$ Treg cells exhibiting high levels of Forkhead box Protein 3 (FoxP3) gene expression along with transforming growth factor β (TGF-β) in spleen and draining lymph nodes of BALB/c mice infected with *L. infantum* [43]. This cell subpopulation contributes to immunosuppression and control of parasite-mediated immunopathology during infection. Treg cell subsets that constitutively express CD25 and synthesize IL-10 and TGF-β drive the suppression of cell-mediated immune responses [44]. These cells are considered potent suppressors of the activation of $CD8^+$ T cells [45]. Nevertheless, another study showed a reduced percentage of $CD3^+CD4^+FoxP3^+$ Treg cells in dogs infected with *L. infantum*, independently of antibody titer [46]. Although $CD8^+$ T suppressor cells have been identified, their mode of action and purpose are not fully understood [47]. Some studies have shown that resting $CD4^+$ lymphocytes are resistant to $CD8^+CD25^+FoxP3^+$ Treg cells, which indicates that the initiation of cell-mediated immune response is not likely to be affected by $CD8^+$ Treg cells. In contrast, $CD8^+$ Treg cells can play a critical role in suppressing ongoing $CD4^+$ T cell responses [48]. Besides, the activity of $CD4^+CD25^+FoxP3^+$ Treg cells appears to be mediated through the release of immune-suppressive cytokines and by cell contact-dependent mechanisms [48]. With regard to leishmaniosis, few studies focus on Treg cells, and less are those that have analyzed the $CD8^+$ Treg cell fraction. Tiwananthagorn et al. [49] reported that in the liver of *L. donovani*-infected mice, $CD4^{+-}$

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FoxP3⁺ Treg cells, but not CD8⁺FoxP3⁺ T cells, are essential for the increased susceptibility to *Leishmania* infection and high IL-10 production.

T cells expressing both CD4 and CD8 molecules have been identified in peripheral blood and secondary lymphoid organs of several species, such as pigs, monkeys, humans, chickens, rats, mice, and dogs [50–56]. These CD4⁺CD8⁺ double-positive (dp) T cells appear to constitute memory CD4⁺ helper T cells that, upon activation, develop the ability to express the CD8α chain and, in cases such as pigs, produce high levels of IFN-γ in response to stimulation with viral antigens [50]. This subpopulation has been identified as being increased in chronic diseases, such as cancer, autoimmune diseases, and viral infections [57–61]. Several studies have also reported the presence of CD25 and FoxP3 in dp T cells of dogs, revealing a possible regulatory activity among this subpopulation [62,63].

Thus, the current study aims to evaluate the kinetics of CD4⁺ and CD8⁺ T cell subsets in tissues that commonly harbor *Leishmania* parasites in both sick and treated dogs. Sick dogs (CanL) were treated by two of the most used protocols for CanL during a 3-month period, and peripheral blood, lymph node, and bone marrow T cells were immunophenotyped.

Materials and Methods

Dog Selection

Twenty-three household dogs living in the endemic area of the Metropolitan Region of Lisbon (Portugal) were diagnosed with CanL at clinical stage I/II, according to the LeishVet Consensus Guidelines [64], and at stage C, following the Canine Leishmaniasis Working Group Guidelines [65]. Twelve of these sick dogs fulfilled the minimum requirements to enter the study (**Figure 1**), which included having at least 1.5 years of age, weighing more than 5 kg, not having been vaccinated for leishmaniosis, being negative for circulating pathogens potentially responsible of canine vector-borne diseases (CVBDs), and have not undergone any treatment in the last 8 months that could interfere with the immune response (such as corticosteroids, antibiotics, or immunomodulators). The present study also included a control group of 10 clinically healthy dogs that were negative for *Leishmania* antibodies and other CVBDs and not vaccinated for leishmaniosis. All dog owners gave written consent after being informed about the objectives of the study and every procedure. The selected animals included 15 males and 7 females of various breeds, with ages ranging between 2 and 9 years and weight between 7.6 and 32.1 kg. Clinical examination and sample collection were done by veterinarians at the Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon.

As previously described by our group [66], dogs diagnosed with CanL that presented biochemical parameters such as increased blood urea nitrogen (BUN), creatinine, and/or alanine aminotransferase (ALT), aspartate aminotransferase (AST), and urine protein-to-creatinine (UPC) ratio between 0.2 and 0.6, which point to the possibility of developing hepatic and renal lesions, were treated with miltefosine [Milteforan®, Virbac S.A., France; 2 mg/kg *per os, semel in die* (SID) for 4 weeks] combined with allopurinol [Zyloric®, Laboratórios Vitória, Portugal; 10 mg/kg, *per os, bis in die* (BID) for at least 6 months] and correspond to Group Milt+Al. Dogs that exhibited changes in serum proteins and UPC ratios between 0.2 and 0.4 were treated with meglumine antimoniate (Glucantime®, Merial Portuguesa, Portugal; 100 mg/kg SID for 4 weeks) combined with allopurinol (10 mg/kg, *per os*, BID for at least 6 months) and were included in Group Megl+Al. To prevent new infections during the study and *Leishmania* transmission, deltamethrin-impregnated collars were applied to all dogs.

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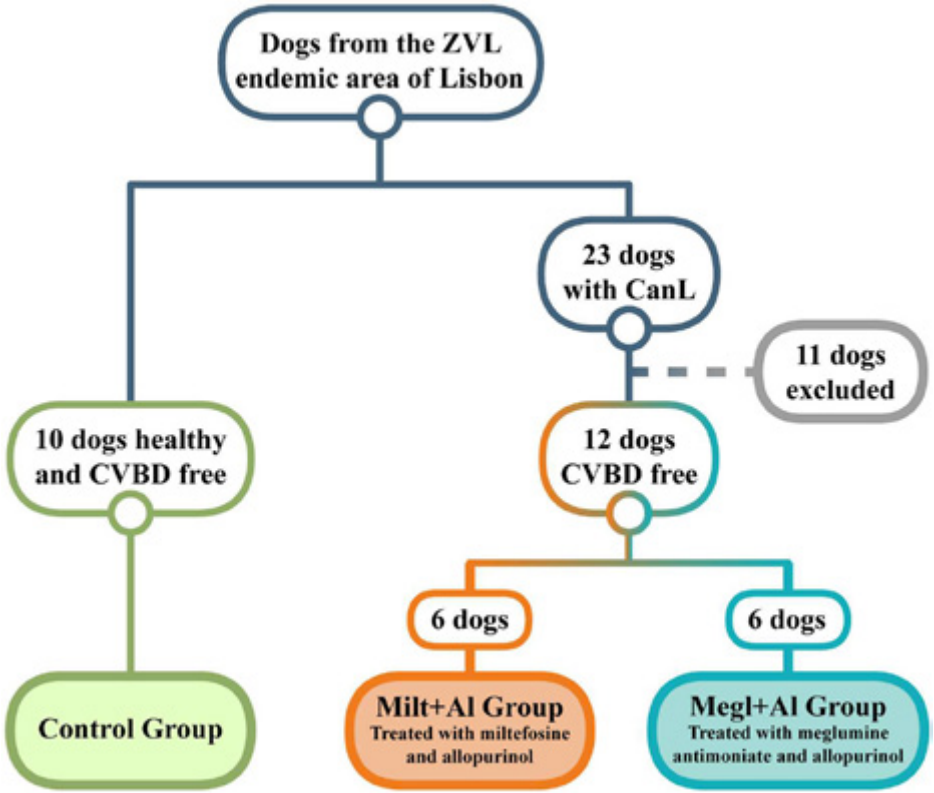

Figure 1. *Dog selection diagram used in the current study. From a population of dogs living in an endemic area of zoonotic visceral leishmaniosis (ZVL), two groups clinically diagnosed with canine leishmaniosis (CanL) were established and treated with either miltefosine in combination with allopurinol (Milt+Al) or meglumine antimoniate in association with allopurinol (Megl+Al). A group of clinically healthy dogs and free of any canine vector-borne disease (CVBD) was also selected as the control group.*

Experimental Design

To investigate the effect of *Leishmania* infection and antileishmanial treatments in helper, cytotoxic, and regulatory T cell subsets, peripheral blood, popliteal lymph node, and bone marrow mononuclear cells were isolated from sick dogs (CanL) before the beginning of treatment (M0) and monthly after treatment (M1, M2, and M3). These cells were immunophenotyped by evaluating the surface expression of CD45, CD3, CD4, CD8, and CD25 and the intracellular expression of FoxP3. To reduce the number of animals used in this study and to ensure any ethical concern for animal discomfort and well-being, the amount of sample collection and its periodicity were reduced to a minimum. Furthermore, peripheral blood, popliteal lymph node, and bone marrow samples were collected from sick dogs before the onset of treatment (M0) to establish the baseline levels of cell populations, avoiding the need of an additional group of untreated sick dogs. Peripheral blood, popliteal lymph node, and bone marrow samples were also collected from clinically healthy dogs [control group (CG)]. The present study followed the directive 86/609/EEC of the Council of the European Union and was approved by the Ethics and Animal Welfare Committee of the Faculty of Veterinary Medicine, University of Lisbon.

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Isolation of Peripheral Blood, Lymph Node, and Bone Marrow Mononuclear Cells

Peripheral blood mononuclear cells were obtained through density gradient centrifugation (Histopaque®-1077 solution, Sigma-Aldrich, Germany). Dog peripheral blood was resuspended in PBS (1:1 v/v), overlaid on half of that total volume in Histopaque®-1077 solution and centrifuged 400 × g for 30 min at 18°C. Peripheral blood mononuclear cells were then harvested at the interface of PBS and Histopaque® and washed twice in cold PBS (300 × g, 10 min, 4°C). Whenever red blood cells were still visible in the pellet, a step of lysis was done by adding 5 ml of RBC Lysis Buffer (eBioscience, USA) for 5 min and stopping the reaction with 10 ml of PBS, followed by a centrifugation at 300 × g (4°C) for 10 min. The pellet was then resuspended in Flow Cytometry Staining Buffer (FCSB) (eBioscience), and the total volume was adjusted for 2×10^7 cells ml^{-1}. Lymph node and bone marrow aspirates were centrifuged at 400 × g (4°C) for 5 and 15 min, respectively, and resuspended in FCSB with the total volume also adjusted for 2×10^7 cells ml^{-1}. These samples were then kept on ice until antibody labeling.

Flow Cytometry

To characterize regulatory and effector T cell subpopulations, a multicolor panel was designed for flow cytometry analysis, and each fluorochrome-conjugated antibody was titrated for optimal staining (**Table 1**). Cell suspensions (50 µl) were incubated with the following monoclonal antibodies (30 min at 4°C in the dark): rat anti-dog CD45 (clone YKIX716.13, eBioscience Inc.), mouse anti-dog CD3 (clone CA17.2A12, AbD Serotec, UK), anti-dog CD4 (clone YKIX302.9, eBioscience Inc.), rat anti-dog CD8 (clone YCATE55.9, AbD Serotec), and mouse anti-dog CD25 (clone P4A10, eBioscience Inc.) (**Table 2**). Then, cells were washed twice with 1 ml of FCSB and centrifuged at 400 × g (4°C) for 5 min. Afterward, 1 ml of FoxP3/Transcription Factor Fixation/Permeabilization Working Solution (eBioscience Inc.) was added, and cells were incubated overnight at 4°C in the dark. Next, 500 µl of 1× Permeabilization Buffer (eBioscience Inc.) was added, and cells were centrifuged at 400 × g (4°C) for 5 min, followed by two washes at 400 × g (4°C) for 5 min with 1 ml of 1× Permeabilization Buffer and a last washing step with 500 µl of FCSB. Cells were resuspended in a total of 100 µl of FCSB and incubated for 15 min at 4°C in the dark. Intracellular staining with anti-mouse/rat FoxP3 (clone FJK-16s, eBioscience Inc.) monoclonal antibody was done by incubating for at least 30 min (4°C) in the dark, followed by two washes with 1× Permeabilization Buffer at 400 × g (4°C) for 5 min. For flow cytometry acquisition (three-laser equipped CyAn ADP apparatus, Beckman Coulter, using the Summit v4.3, Dako Colorado Inc. software), cells were resuspended in a final volume of 300 µl of FCSB. For each sample, a minimum of 20,000 gated events were acquired, and data analysis was performed using FlowJo version 10.0.7 (Tree Star, CA). To define the best gating strategy to be applied (**Figure 2**), compensation was done with unstained, single-stained, and "fluorescence minus one" (FMO) samples (**Table 2**).

A recent study [67] showed relevant proof that the doublet discrimination usually made in flow cytometry analysis, with the reasoning that they constitute experimental artifacts, may hide cell-to-cell contact, in particular, T cell–monocyte association that is not disrupted during sample processing. Thus, in the current study, a simple approach was used to compare the frequency of doublets in healthy, sick, and treated dogs following the gating strategy shown in **Figure 3A**.

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Instrument: Beckman Coulter Cyan ADP						
Laser lines	405 nm	488 nm			642 nm	
Emission filters	450/50	530/40	575/25	680/30	665/20	750LP
Fluorochrome	eFluor® 450	FITC	PE	PerCP/Cy5.5	APC	Alexa Fluor® 700
Biomarker	CD45	CD3	CD25	FoxP3	CD4	CD8
Brightness						
Antibody	rat anti-dog	mouse anti-dog	mouse anti-dog	anti-mouse/rat	rat anti-dog	rat anti-dog
Clone	YKIX716.13	CA17.2A12	P4A10	FJK-16s	YKIX302.9	YCATE55.9
Company	eBiosciences	AbD Serotec	eBioscience	eBioscience	eBioscience	AbD Serotec
Volume	5 µl per test (1:20)	8 µl per test (1:12.5)	5 µl per test (1:20)	5 µl per test (1:20)	5 µl per test (1:20)	10 µl per test (1:50)

The green-shaded squares indicate the level of brightness for each corresponding fluorochrome, from dim (1 square) to the brightest (5 squares).

TABLE 1. *Flow cytometer setup, fluorochrome panel, and labeling.*

Sample type	Marker						Blood	Lymph node	Bone marrow
	CD45	CD3	CD4	CD8	CD25	FoxP3			
Unstained							X	X	X
Single-stained							X		
							X		
							X		
							X		
							X		
							X	X	X
FMO-CD25							X	X	X
FMO-FoxP3							X	X	X
All							X	X	X

The green-shaded slots indicate the antibody label used in each sample type, while the crosses indicate which samples were analyzed in each tissue.

TABLE 2. *Fluorochrome compensation panel graph by sample type and tissue.*

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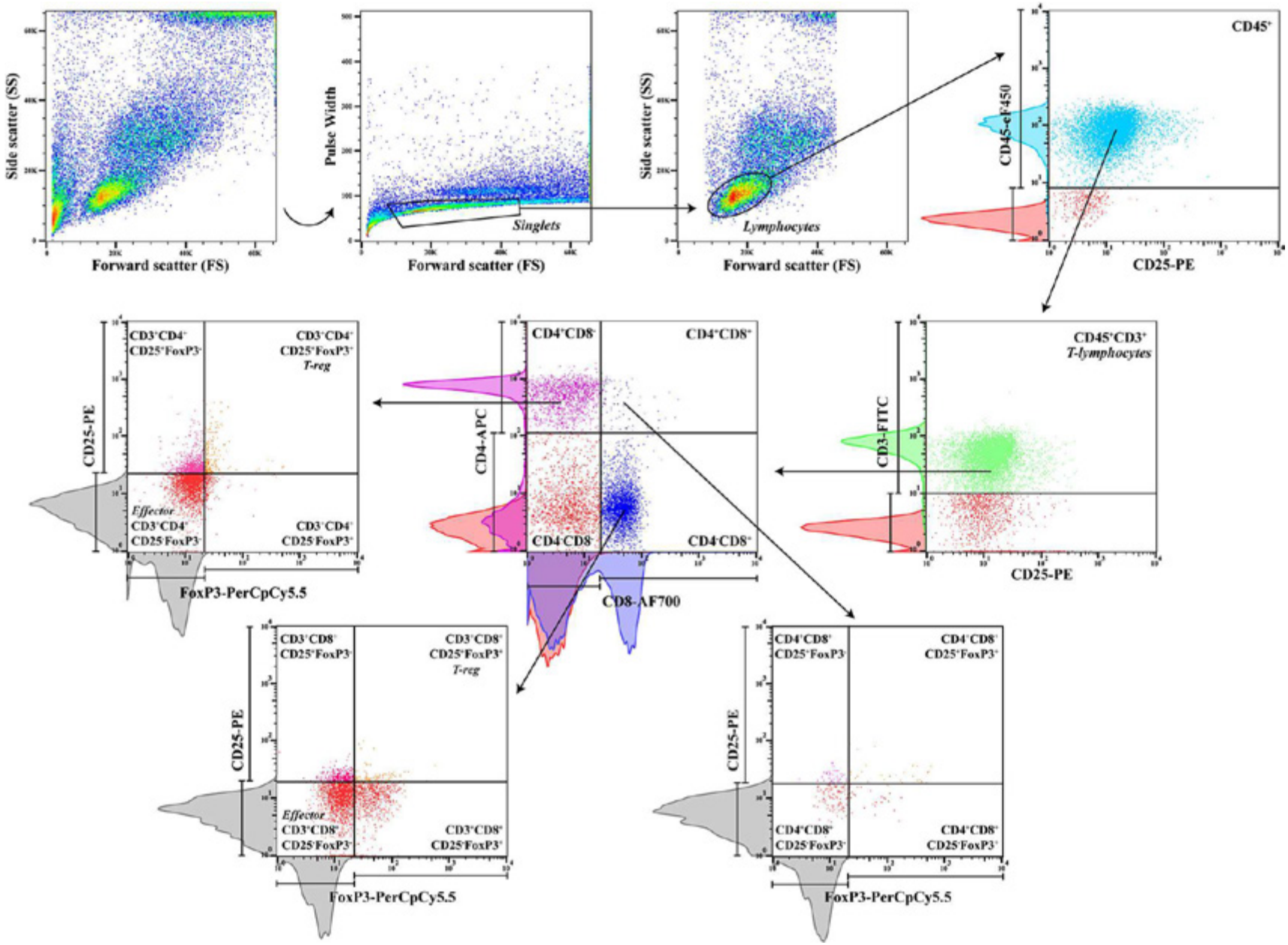

Figure 2. *Gating strategy. Peripheral blood sequential gating strategy for a panel of six antibodies to identify the different cell subpopulations after doublet exclusion. CD45, a pan-leukocyte marker, and CD3, a T-lymphocyte specific marker, were used to define the T-lymphocyte population, with posterior separation of CD4+ and CD8+ cells, CD4+CD8+ double-positive T cells, and subsequent regulatory CD25+FoxP3+ and effector CD25−FoxP3− cells. Red histograms from unstained control samples and colored histograms from single-stained control samples were used to define the sequential gating, along with gray histograms from fluorescence minus one (FMO) controls to gate for rare cells (CD25+FoxP3+).*

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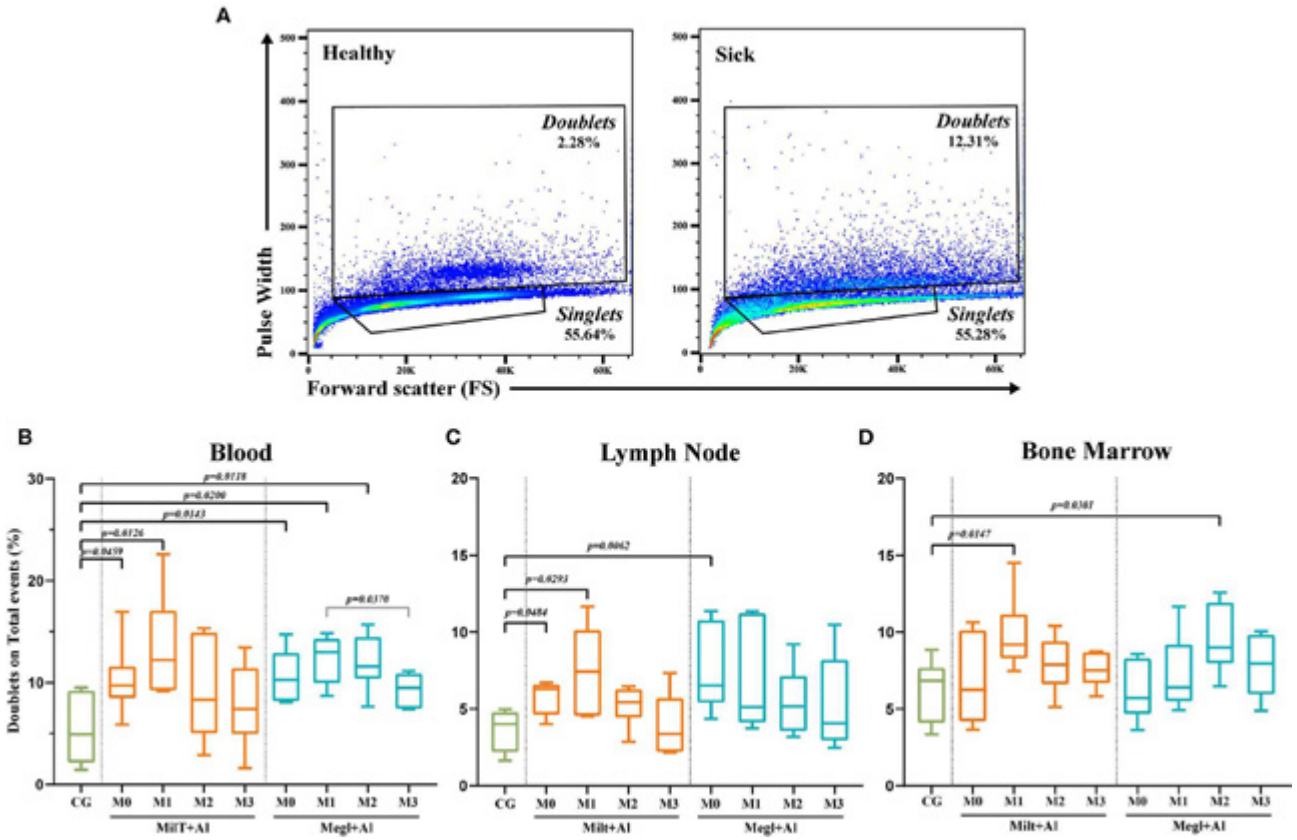

Figure 3. *Doublet analysis. (**A**) Gating strategy example in the blood of a healthy [control group (CG)] and a sick dog (M0). Percentage of doublets gated on total events for blood (**B**), lymph node (**C**), and bone marrow (**D**) before and after the beginning of treatment. Results of 22 dogs are represented by box and whisker plots and median, minimum, and maximum values. The non-parametric Kruskal–Wallis test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatment groups and the control group (CG). The repeated measures ANOVA test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. p-values are indicated in every statistically significant comparison.*

Statistical Analysis

Statistical analysis between control, infected, and treated groups was performed using GraphPad Prism software package (version 8.0.1, GraphPad Software Inc.). The Kolmogorov–Smirnoff test was used to assess data normality. The non-parametric Kruskal–Wallis Test (one-way ANOVA on ranks) with Dunn's *post hoc* test was used to evaluate differences in cell subset levels between sick, treated, and control groups. Lastly, the repeated measures ANOVA test with Tukey's *post hoc* test was used to compare dogs between the several months M0, M1, M2, and M3.

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Results

Canine Leishmaniosis Promotes a High Frequency of Cell Doublets That Reach Healthy Values During Treatment

A significant increase of events in the doublets gate in both blood (*p*Milt+Al = 0.0459; *p*Megl+Al = 0.0143) (**Figure 3B**) and lymph node (*p*Milt+Al = 0.0484; *p*Megl+Al = 0.0062) (**Figure 3C**) was observed in sick dogs (M0) when compared with the control group. One month after Milt+Al treatment (M1), blood (p = 0.0126), lymph node (p = 0.0293), and bone marrow (p = 0.0147) presented a significantly high frequency of doublets. Although, during treatment, doublets return to frequencies close to those of the control group. In dogs treated with Megl+Al, peripheral blood exhibited significantly high percentages of doublets in the first (*p*M1 = 0.02) and second (*p*M2 = 0.0118) months of treatment. On the other hand, the bone marrow presented only a transient increase of doublets 2 months (*p*M2 = 0.0301) after the beginning of the treatment (**Figure 3D**).

Canine Leishmaniosis Chemotherapy Causes an Imbalance of T Lymphocyte Population

Peripheral blood (**Figure 4A**) and lymph node (**Figure 4B**) of dogs with active leishmaniosis (M0) presented T lymphocyte ($CD45^+CD3^+$) levels similar to clinically healthy dogs. However, the subsequent administration of either treatment resulted in lymphocyte frequency reduction. Dogs under Megl+Al therapy showed a significant reduction of the percentage of blood T cell population ($CD45^+CD3^+$ cells) after 2 (*p*M2 = 0.0239) and 3 (*p*M3 = 0.0046) months of treatment. However, in the lymph node, a significant frequency reduction of the T cell population was observed at 1 (*p*M1 = 0.0319) and 2 (*p*M2 = 0.0328) months with this therapy. Furthermore, bone marrow T cells (**Figure 4C**) frequency significantly increased after the first month of treatment with Megl+Al (*p*M1 = 0.0399), reaching values similar to clinically healthy dogs by the sec-

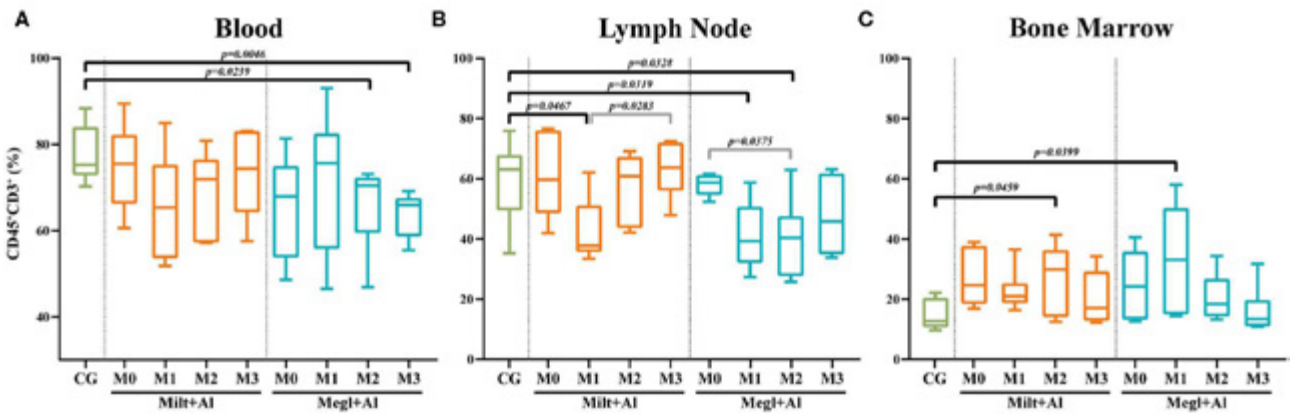

Figure 4. *Frequency of lymphocytes (CD45+CD3+) in the blood (**A**), lymph node (**B**), and bone marrow (**C**) of healthy [control group (CG)], sick (M0), and treated dogs (M1, M2, and M3). Results of 22 dogs are represented by box and whisker plots and median, minimum, and maximum values. The non-parametric Kruskal–Wallis test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatment groups and the CG. The repeated measures ANOVA test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. p-values are indicated in every statistically significant comparison.*

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ond month (M2). One month after the beginning of treatment with Milt+Al, a transient reduction of lymph node T cells (*p*M1 = 0.0467) was observed. The bone marrow, in turn, showed a transient higher frequency of T cells (*p*M2 = 0.0459) 2 months after treatment, recovering to levels identical to those of control dogs in the third month (M3).

Anti-leishmanial Therapy Favors the Predominance of CD4⁺ T Cells Over CD8⁺ T Cells

According to several authors, the CD4⁺/CD8⁺ T cell ratio acquired by flow cytometry analysis can be considered a simple and fast way to assess cell-mediated immune response [65,68]. When compared with healthy dogs, blood (*p*M0 = 0.0177) (**Figure 5A**), and lymph node (*p*M0 = 0.0246) (**Figure 5B**) cells of sick dogs presented a significant decrease of the CD4/CD8 ratio to values close to 1, pointing to similar frequencies of CD8⁺ and CD4⁺ T cells. During treatment, this ratio progressed toward values closer to 2, indicating the predomination of CD4⁺ T cells. On the other hand, the bone marrow CD4⁺/CD8⁺ T cell ratio (**Figure 5C**) of sick dogs was similar to that of healthy dogs, with ratios ranging between 0.5 and 1. These values point toward a variation between a slight predomination of CD8⁺ T cells and an identical frequency of both T cell subsets.

Canine Leishmaniosis Increases CD4⁺CD8⁺ Double-Positive T Cell Frequency in Peripheral Blood, Lymph Node, and Bone Marrow

Sick dogs (M0) showed increased frequencies of CD4⁺CD8⁺ dp T cells in the blood (**Figure 6A**) (*p*Milt+Al = 0.0182; *p*Megl+Al = 0.0015), lymph node (**Figure 6B**) (*p*Milt+Al = 0.0234; *p*Megl+Al = 0.0318), and bone marrow (**Figure 6C**) (*p*Milt+Al = 0.005; *p*Megl+Al = 0.006) when compared to healthy dogs. The administration of either treatment protocol resulted in a maintenance of these high frequencies of CD4⁺CD8⁺ dp T cells in all tissues during the first month of treatment (M1), progressively normalizing by the following month (M2),

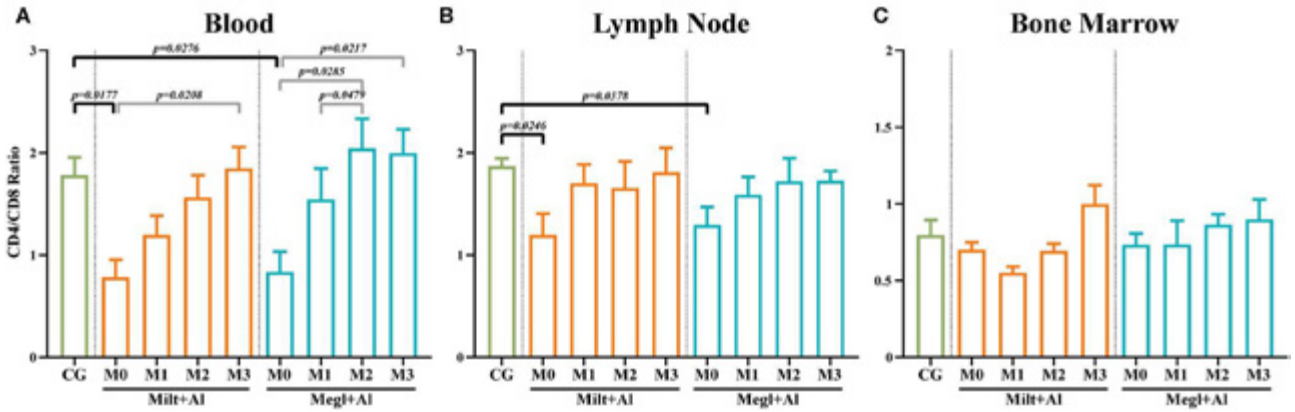

Figure 5. *CD4/CD8 ratio in the blood (**A**), lymph node (**B**), and bone marrow (**C**) of healthy [control group (CG)], sick (M0), and treated dogs (M1, M2, and M3). Results of 22 dogs are represented by mean values ± SEM. The non-parametric Kruskal–Wallis test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatment groups and the CG. The repeated measures ANOVA test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. p-values are indicated in every statistically significant comparison.*

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with the exception of lymph node of dogs treated with the Megl+Al protocol that recovered 1 month after treatment.

CD4⁺CD8⁺ Double-Positive T Cells Expressing Regulatory Phenotype Decrease in Peripheral Blood of Sick Dogs and Increase in the Lymph Node and Bone Marrow After Treatment

Lymph node (**Figure 6E**) and bone marrow (**Figure 6F**) of sick dogs showed a significant frequency reduction of dp T cells expressing CD25 molecules (lymph node: *p*Milt+Al = 0.0024; *p*Megl+Al = 0.0319/bone marrow: *p*Milt+Al = 0.0018; *p*Megl+Al = 0.0293), which recovered to values similar to clinically healthy dogs during treatment. However, in peripheral blood, treatment caused a significant decrease of this T cell subset (**Figure 6D**).

In turn, the percentage of CD25⁺FoxP3⁺ dp T cells in the blood of sick dogs (**Figure 6G**) was higher than that in healthy dogs (*p*Milt+Al = 0.0484; *p*Megl+Al = 0.0095), while being similar to the control group in the lymph node (**Figure 6H**) and bone marrow (**Figure 6I**). Treated dogs presented a normalization of the frequencies in the blood after 1 month of treatment, while showing a progressive increase in this subpopulation, reaching higher frequencies than the control group, in the lymph node (*p*Milt+Al = 0.0072; *p*Megl+Al = 0.0061) and bone marrow (*p*Milt+Al = 0.0310; *p*Megl+Al = 0.0411) in the third month.

Leishmania Infection Results in the Increase of Blood CD8⁺ T Cell Frequencies With CD25⁺FoxP3⁺ Phenotype

Blood of sick dogs (M0) exhibited a significant decrease in the frequency of the CD4⁺ T cell subset (*p*Milt+Al = 0.0253; *p*Megl+Al = 0.0467) (**Figure 7A**) along with a high frequency of the CD8⁺ T cell subset (*p*Milt+Al = 0.0018; *p*Megl+Al = 0.0052) (**Figure 7B**). Both treatments were able to recover normality for the CD4⁺ and CD8⁺ T cell fractions. However, dogs under the Megl+Al protocol recovered to values similar to those of clinically healthy dogs during the first month of treatment (M1), faster than the group treated with Milt+Al that only recovered after the second month (M2).

The frequency of blood T cells with CD4⁺CD25⁺ phenotype showed some fluctuation, mainly during Megl+Al treatment (**Figure 7C**), although with no statistical differences when compared with clinically healthy dogs. However, a significant increase in the frequency of the CD8⁺CD25⁺ T cell subset (*p*Milt+Al = 0.0071; *p*Megl+Al = 0.0246) was observed in sick dogs (M0) when compared with that of the control group (**Figure 7D**). This cell subset returned to normal values immediately after the beginning of both treatments (M1).

CD4⁺CD25⁺FoxP3⁺ (*p*Milt+Al = 0.0411; *p*Megl+Al = 0.0310) and CD8⁺CD25⁺⁻FoxP3⁺ (*p*Milt+Al = 0.0118; *p*Megl+Al = 0.0052) T cell subsets of sick dogs (M0) presented higher frequencies than those of the control group (**Figures 7E,F**). After administration of both treatments, an increase in the frequency of the CD4⁺CD25⁺FoxP3⁺ T cell subset was observed (*p*Milt+Al(M1) = 0.0092; *p*Megl+Al(M2) = 0.0029), with the values returning to healthy levels at M2 and M3, for the Milt+Al and Megl+Al groups, respectively. Likewise, the CD8⁺CD25⁺⁻FoxP3⁺ T cell subset recovered to values comparable to those of control dogs after 3 months for both treatment protocols.

Effector T cell subsets of sick dogs (M0) presented different patterns. CD4⁺CD25⁻FoxP3⁻ T cells were significantly lower than those of the control group (*p*Milt+Al = 0.0086; *p*Megl+Al = 0.0073). However, dogs recovered to healthy values 1 month after the beginning of treatment with Megl+Al (M1) and after 2 months of Milt+Al therapy (M2) (**Figure 7G**). On the other hand, CD8⁺CD25⁻FoxP3⁻ T cells of sick dogs were similar to those of healthy dogs, but subsequent treatments led to a significant reduction in cell frequency

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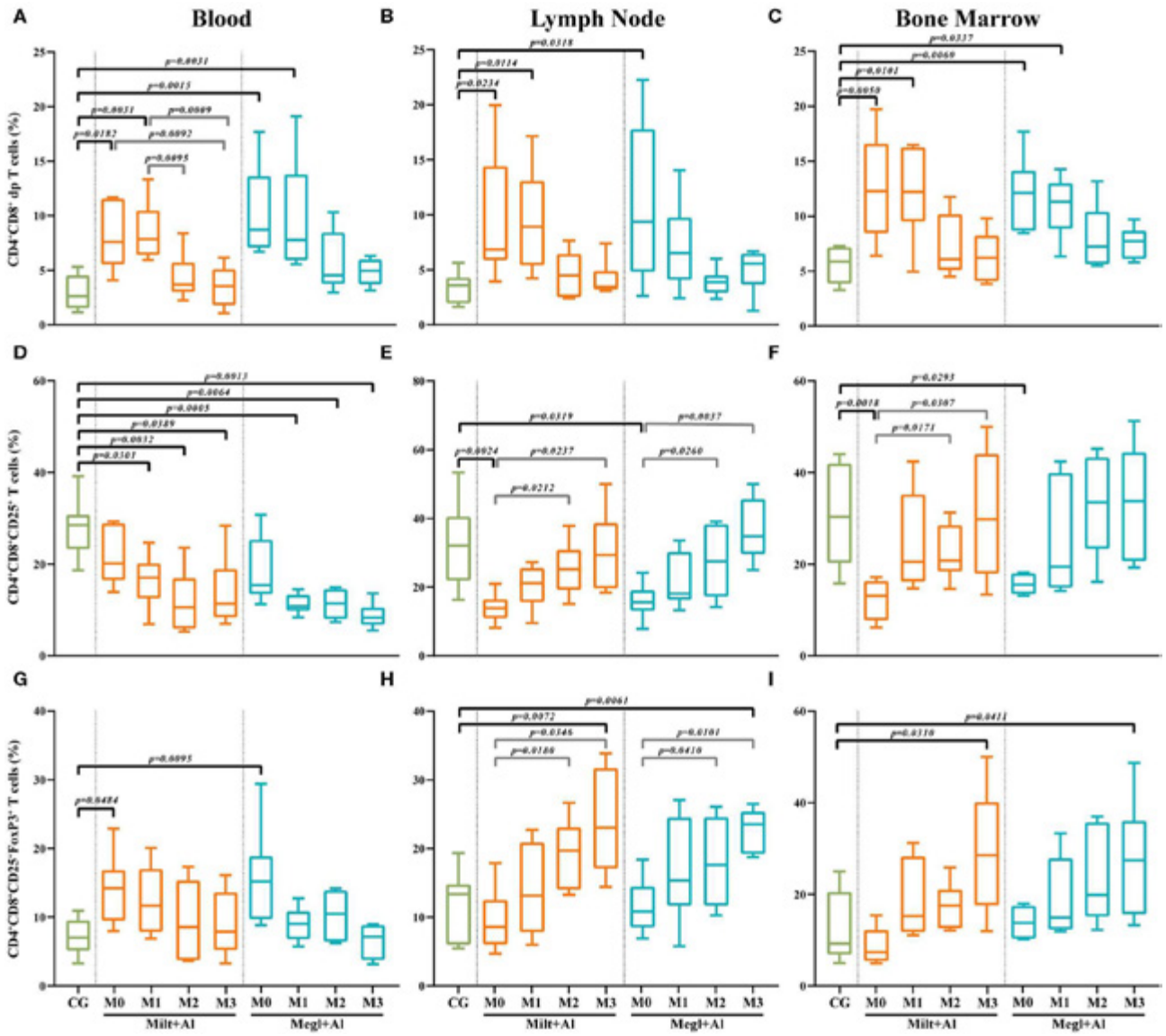

Figure 6. *Frequency of CD4+CD8+ double-positive (dp) T cells. The frequency of dp T cells (**A–C**) expressing CD25 (**D–F**) and CD25 and FoxP3 (**G–I**) was evaluated in the peripheral blood (**A,D,G**), lymph node (**B,E,H**), and bone marrow (**C,F,I**) of healthy [control group (CG)], sick (M0), and treated dogs (M1, M2, and M3). Results of 22 dogs are represented by box and whisker plots and median, minimum, and maximum values. The non-parametric Kruskal–Wallis test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatment groups and the CG. The repeated measures ANOVA test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. p-values are indicated in every statistically significant comparison.*

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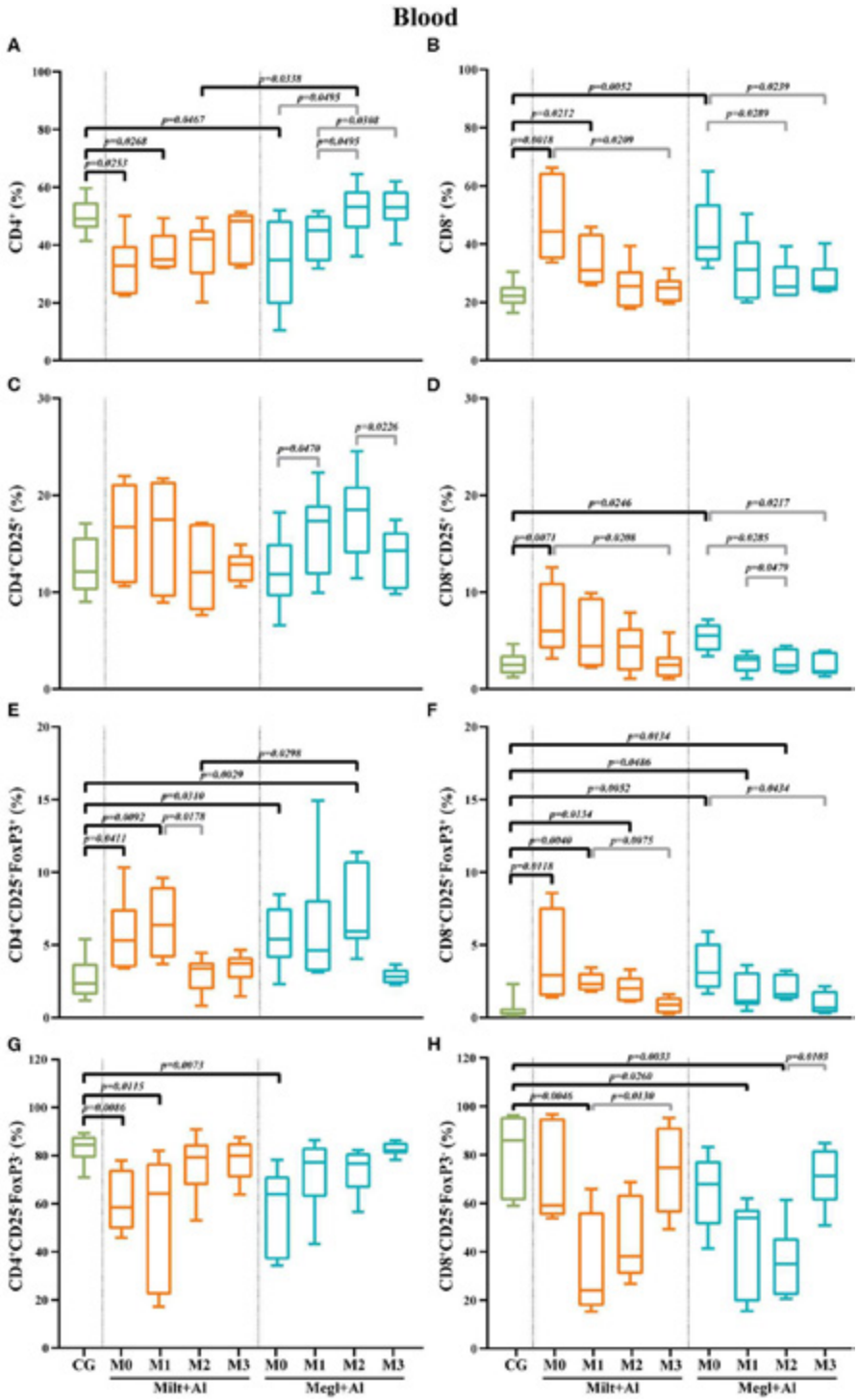

Figure 7. *Frequency of CD4+ (**A**), CD8+ (**B**), regulatory (CD25+FoxP3+) (**C–F**), and effector (CD4+CD25−FoxP3−/CD8+CD25−FoxP3−) (**G,H**) T lymphocytes in the blood of healthy [control group (CG)], sick (M0), and treated dogs (M1, M2, and M3). Results of 22 dogs are represented by box and whisker plots and median, minimum, and maximum values. The non-parametric Kruskal–Wallis test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatment groups and the CG. The repeated measures ANOVA test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. p-values are indicated in every statistically significant comparison.*

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(pMilt+Al(M1) = 0.0046; pMegl+Al(M1) = 0.026), with the Megl+Al group recovering to normal frequencies by the third month (M3) and the Milt+Al group after the second month (M2) (**Figure 7H**).

Canine Leishmaniosis Promotes the Increase of Lymph Node CD8⁺ T Cell Frequencies, and Treatment Leads to an Imbalance of Effector and Regulatory T Cell Subsets

In the lymph node of sick dogs, the frequency of CD4⁺ T cells was similar to that of healthy dogs (**Figure 8A**), but the CD8⁺ T cell fraction presented a higher percentage (pMilt+Al = 0.0052; pMegl+Al = 0.0120) (**Figure 8B**). Furthermore, treatment administration caused a reduction of the CD8⁺ T cell frequencies to values similar to control dogs. Three months after the onset of treatment with Megl+Al, the CD4⁺ T cell fraction was significantly diminished (p = 0.0389) when compared with clinically healthy dogs.

In sick dogs, the level of CD4⁺ (**Figure 8C**) and CD8⁺ (**Figure 8D**) T cells with CD25⁺ phenotype was similar to healthy dogs. However, both treatment protocols led to a transient increase of the CD4⁺CD25⁺ T cell subset frequencies after 1 month of Milt+Al treatment (p = 0.0463) and 2 months of Megl+Al (p = 0.0471). The CD8⁺CD25⁺ T cell subpopulation of dogs under the Milt+Al protocol showed a significant increase 2 (p = 0.0200) and 3 (p = 0.0071) months after the beginning of treatment (**Figure 8D**).

Likewise, sick dogs showed similar frequencies of CD4⁺CD25⁺FoxP3⁺ and CD8⁺CD25⁺FoxP3⁺ T cells compared to healthy dogs. Moreover, after treatment, these dogs exhibited a significant increase in the frequency of the CD4⁺CD25⁺FoxP3⁺ T cell subset (**Figure 8E**). In dogs treated with Milt+Al, a peak of the frequency of CD4⁺ Treg cells was observed 2 months (pM2 = 0.0182) after the beginning of treatment. One and 2 months after administration, Megl+Al also promoted a CD4⁺ Treg frequency increase (pM1 = 0.0172; pM2 = 0.0098) that subsequently reverted to normal values. Moreover, Milt+Al caused a significant increase in the frequency of CD8⁺CD25⁺FoxP3⁺ T cells (pM1 = 0.0027; pM2 = 0.0071; pM3 = 0.0145), while the Megl+Al protocol only resulted in a transient increase of this subpopulation 1 month after treatment (pM1 = 0.0399) (**Figure 8F**).

Effector T cell subsets in the lymph node of sick dogs were similar to those of healthy dogs. After treatment administration, CD4⁺CD25⁻FoxP3⁻ T cell frequencies showed a progressive reduction during the first and second month with both the Milt+Al (pM1 = 0.0301; pM2 = 0.0434) and the Megl+Al protocol (pM1 = 0.0225; pM2 = 0.0212) (**Figure 8G**). CD8⁺CD25⁻FoxP3⁻ T cell frequencies also presented a significant reduction after drug administration (pMilt+Al = 0.0134; pMegl+Al = 0.0021), with the Milt+Al-treated dogs recovering cell frequency levels by the second month (M2) and the Megl+Al-treated dogs by the third month (M3) (**Figure 8H**).

Leishmania Infection Causes the Increase of Bone Marrow CD8⁺ T Cell Frequencies With CD25⁺FoxP3⁺ Phenotype

In the bone marrow of sick dogs, the frequency of CD4⁺ T cells (**Figure 9A**) was similar to clinically healthy dogs. The administration of Milt+Al did not cause significant alterations in the CD4⁺ T cell fraction, while dogs under the Megl+Al protocol exhibited a transient frequency increase (p = 0.0134) 2 months after the onset of treatment. Meanwhile, a prominent increase of the frequency of CD8⁺ T cells was observed in sick dogs (pMilt+Al = 0.0293; pMegl+Al = 0.0495) (**Figure 9B**). This high frequency of CD8⁺ T cells in the bone marrow persisted during both treatments (Milt+Al: pM1 = 0.0367; pM2 = 0.0310) (Megl+Al: pM1 = 0.0463; pM2 = 0.0411), returning to values similar to control dogs by the third month (M3).

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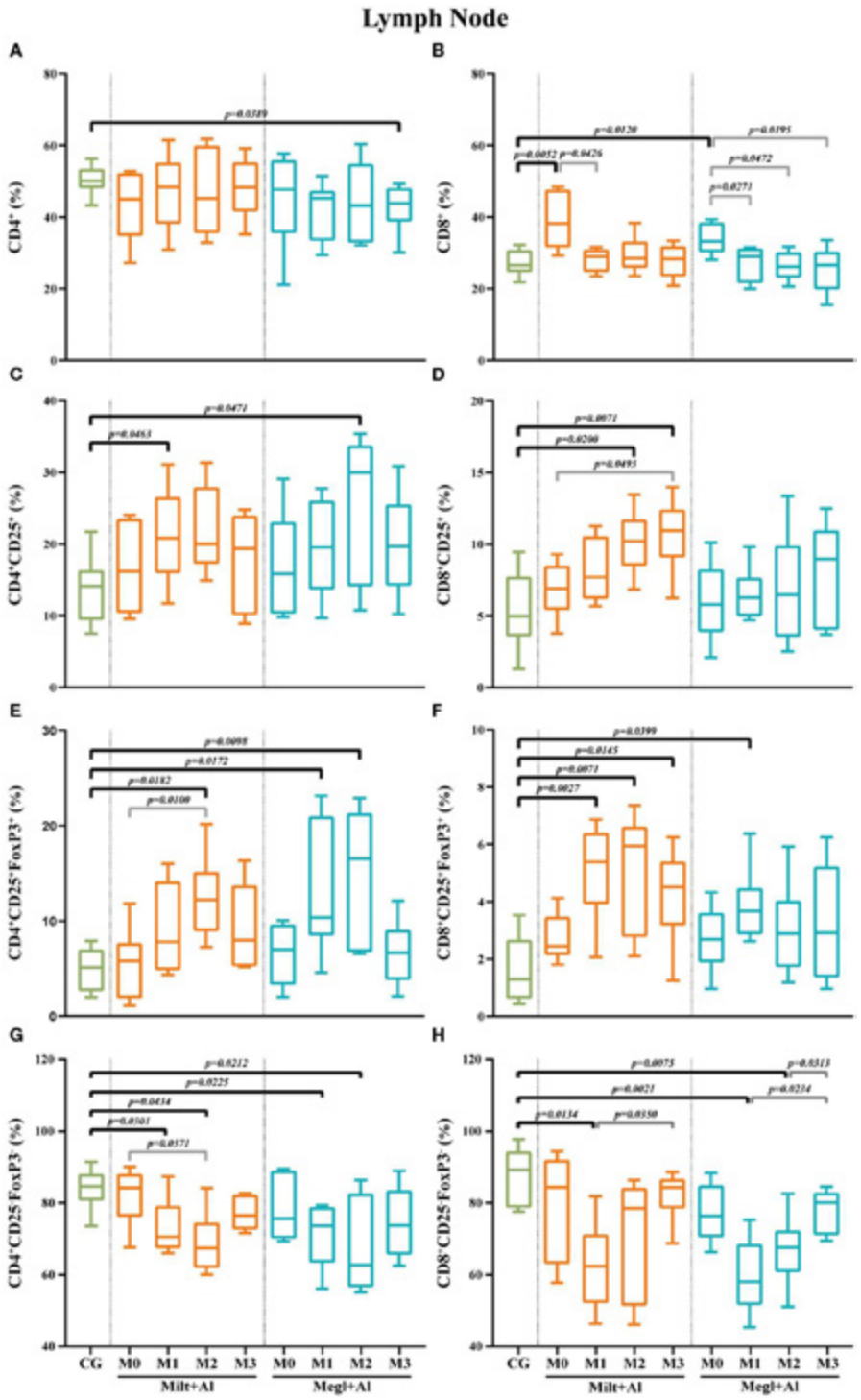

Figure 8. *Frequency of CD4+ (**A**), CD8+ (**B**), regulatory (CD25+FoxP3+) (**C–F**), and effector (CD4+CD25−FoxP3−/CD8+CD25−FoxP3−) (**G,H**) T lymphocytes in the lymph node of healthy [control group (CG)], sick (M0), and treated dogs (M1, M2, and M3). Results of 22 dogs are represented by box and whisker plots and median, minimum, and maximum values. The non-parametric Kruskal–Wallis test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatment groups and the CG. The repeated measures ANOVA test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. p-values are indicated in every statistically significant comparison.*

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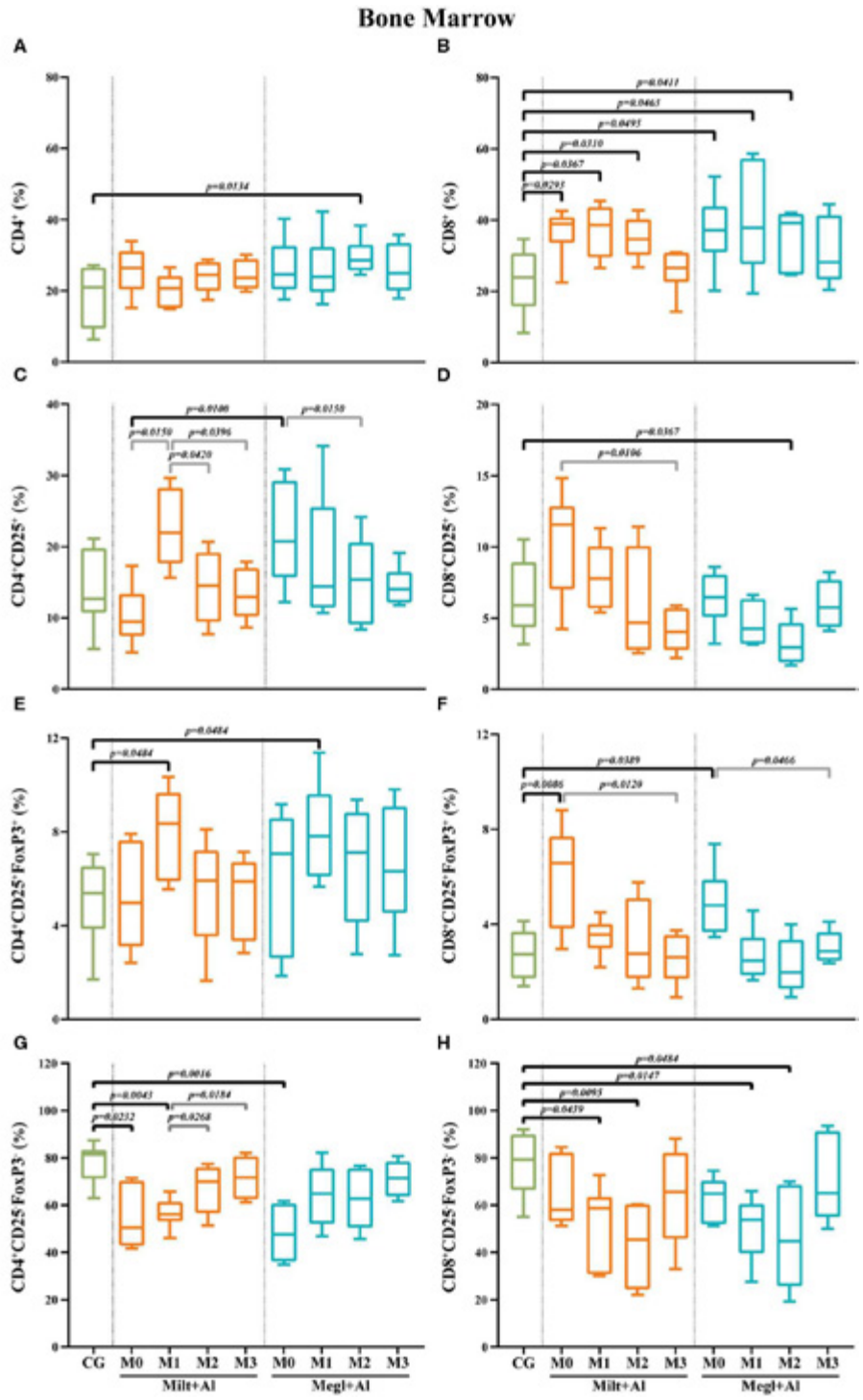

Figure 9. *Frequency of CD4+ (**A**), CD8+ (**B**), regulatory (CD25+FoxP3+) (**C–F**), and effector (CD4+CD25−FoxP3−/CD8+CD25−FoxP3−) (**G,H**) T lymphocytes in the bone marrow of healthy [control group (CG)], sick (M0), and treated dogs (M1, M2, and M3). Results of 22 dogs are represented by box and whisker plots and median, minimum, and maximum values. The non-parametric Kruskal–Wallis test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatment groups and the CG. The repeated measures ANOVA test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. p-values are indicated in every statistically significant comparison.*

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Regarding the $CD4^+CD25^+$ T cell subpopulation (**Figure 9C**), no considerable differences were observed in the bone marrow of sick dogs when compared with that of clinically healthy dogs. Moreover, dogs treated with Megl+Al evidenced a transient decrease of the frequency of $CD8^+CD25^+$ T cells by month 2 (*p*M2 = 0.0367) that quickly recovered (**Figure 9D**).

In the bone marrow of sick dogs, the frequency of Treg cells ($CD4^+CD25^+FoxP3^+$) was similar to that of control dogs (**Figure 9E**). Nevertheless, an increase of the frequency of this cell subset was observed 1 month (*p*Milt+Al = 0.0484; *p*Megl+Al = 0.0484) after either treatment, followed by normalization. Similar to peripheral blood, the $CD8^+CD25^{+-}FoxP3^+$ T cell subset frequencies (**Figure 9F**) of sick dogs was significantly higher (*p*Milt+Al = 0.0086; *p*Megl+Al = 0.0389). Both treatments led to a reduction of cell frequencies to values similar to those of the control group.

$CD4^+CD25^-FoxP3^-$ T cell frequencies of sick dogs were significantly lower in comparison with those of healthy dogs (*p*Milt+Al = 0.0232; *p*Megl+Al = 0.0016) (**Figure 9G**). However, the Megl+Al group recovered to values close to those of healthy dogs 1 month earlier than the Milt+Al group. The frequencies of $CD8^+CD25^-FoxP3^-$ T cells of sick dogs, on the other hand, were similar to those of healthy dogs, with the administration of either treatment leading to a significant decrease 1 (*p*Milt+Al = 0.0439; *p*Megl+Al = 0.0147) and 2 months (*p*Milt+Al = 0.0095; *p*Megl+Al = 0.0484) after the beginning of treatment (**Figure 9H**).

Discussion

CanL treatment has an inherent connection with the ability of the dog's immune system to develop a competent cellular immune response against *L. infantum*. Thus, comprehending the cellular immune response and the dynamics of T cell subsets in dogs naturally infected with *Leishmania*, especially in organs that usually harbor these parasites, is of utmost relevance not only for the treatment and management of CanL but also as guidelines for the development of prophylactic and therapeutic tools. A better knowledge of the effect of antileishmanial therapy on the cellular immune response of dogs can facilitate the development of strategies to reduce the transmission of the parasite and, consequently, lead to a decrease in the incidence of zoonotic visceral leishmaniosis. Therefore, in the current study, T cell subpopulations of dogs naturally infected with *L. infantum* were phenotypically characterized before treatment and during the influence of antileishmanial drugs.

In the current study, it was found that sick dogs have increased doublet frequencies in peripheral blood and lymph node, decreasing to values similar to clinically healthy dogs after treatment. As was proposed by Burel *et al.* [67], these changes in the doublet levels associated with CanL and during the first months of treatment may reflect a possible cell-to-cell interaction between T lymphocytes and antigen-presenting cells. It is also possible that the doublets could increase as a result of interaction of Treg:lymphocyte, as Treg cells, which seem to be increased in CanL, appear to exert immune suppression by mechanisms dependent on cell contact [69]. In the present work, it was not possible to delve deeper into these interactions since this was a secondary objective of the study. In this sense, not enough events were collected in the doublet region to obtain meaningful information on further subpopulations. This way, further detailed studies are needed to corroborate this hypothesis, with the correlation between CanL and the level of doublets being able to be used as a possible marker of disease to monitor treatment success and predict potential relapses [67].

Several authors have correlated symptomatic dogs with decreased levels of $CD4^+$ T cells and CD4/CD8 ratios in peripheral blood [35,70], along with high antibody titers. Other authors verified that

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higher infectivity to sand flies by naturally infected dogs was associated with lower proportions of CD4⁺ T cells in the blood [37]. Furthermore, it has also been shown that the administration to dogs infected with *Leishmania* of antileishmanial drugs, such as amphotericin B and meglumine antimoniate, promoted the increase of the percentage and the absolute cell count of CD4⁺ T cells in the blood, respectively [36,71]. On the other hand, other treatment protocols, such as allopurinol in monotherapy, although able to improve the number of circulating CD4⁺ T cells in the blood, were not able to restore values to those within the normal range [68]. Thus, the findings obtained in the current study are in line with previous reports. Sick dogs presented low CD4/CD8 ratios in peripheral blood and lymph node, recovering to values equal to the healthy group after the administration of both treatments. Following our results, and according to several authors [36–38,68,71], the CD4/CD8 ratio can be a useful indicator of the immunological condition of sick dogs and a possible tool with prognostic value. Some authors also describe a decline of the percentage of CD3⁺ lymphocytes in the peripheral blood of CanL symptomatic dogs, as a direct consequence of the reduction in the frequency of CD4⁺ T cells [36,56]. Other authors, on the contrary, have reported a significant increase of CD3⁺ T cells in sick dogs, especially in dogs severely affected [72]. Nevertheless, the administration of antileishmanial therapy in both situations restored CD3⁺ lymphocytes within normal values [36,56,72]. Moreover, the results of the present study point to a dual effect of antileishmanial therapy on bone marrow and lymph node. Both treatments led to a reduction in the frequency of lymph node T cells (CD45⁺CD3⁺) along with an increase in the bone marrow. Interestingly, only meglumine antimoniate in association with allopurinol resulted in a decrease of the frequency of blood T cells.

Protective immunity against CanL is usually considered to be dependent on a Th1 immune response [6]. The predominance of IFN-γ-producing CD4⁺ T cells is crucial for macrophage activation in order to kill internalized *Leishmania* through the production of NO and ROS [73,74]. A reduction of the CD4⁺ T cell population is usually associated with the inability to control the infection, allowing the survival and replication of *Leishmania* parasites in macrophages, which can subsequently lead to increased infectibility to sand flies [37]. Murine studies have shown that *Leishmania* parasites negatively interfere with the ability of IFN-γ to induce the expression of MHC-II mRNA, leading to parasitized macrophages with a low expression of MHC class II molecules [75]. Thus, due to their reduced capacity as antigen-presenting cells, these macrophages are therefore unable to provide co-stimulatory signals to CD4⁺ T cells [76,77], which, in turn, are not stimulated, do not proliferate, and do not produce IFN-γ. Although the complete role of CD8⁺ T cells in CanL is still debated, there are studies of leishmaniosis in humans and mice showing a functional duality. CD8⁺ T cells can either play a protective role by releasing IFN-γ, or they can be pathogenic to the host, causing excessive inflammation at the site of infection [73] as a result of cytotoxic activity, which can exacerbate disease progression [78]. Following the results of previous reports [35,36,79], the sick dogs included in the current study also showed an increased frequency of CD8⁺ T cells in the blood, lymph node, and bone marrow, along with significantly decreased levels of CD4⁺ T cells in the blood. These findings suggest that CD8⁺ T cells are at the forefront of the fight against *Leishmania* infection, especially in tissues that commonly harbor *Leishmania* parasites. Nonetheless, antileishmanial therapy led to the recovery of the T cell population in all tissues. And whether due to the direct action of the antileishmanial drugs or the availability of free antigens as a consequence of *Leishmania*'s death caused by therapy, a shift of T cell population occurs, leading to a rapid reduction in the frequency of CD8⁺ T cells in the blood and lymph node.

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Regulatory T cells are generally considered to be a subset of $CD4^+$ T cells, which express the non-constitutive IL-2R-α chain (CD25) and the transcriptional factor FoxP3 [80,81]. The main function of these cells is to suppress excessive or misguided immune responses and prevent autoimmune diseases [74,82]. Few are the Treg studies done in CanL, which account for the lack of overall information on these subpopulations [83]. In dogs experimentally infected with *L. infantum*, FoxP3 RNA was increased in the skin and liver, but in the lymph node, the authors verified a decrease associated with disease progression [84]. Figueiredo et al. [85] referred that CanL enhanced FoxP3 expression in the jejunum and colon. However, the skin of *L. chagasi* (syn. *L. infantum*)-infected dogs revealed lower levels of FoxP3 expression [86]. Another study found no correlation between TGF-β or IL-10 producing $CD4^+$ Treg cells in the blood and spleen and the parasitic load of naturally infected dogs [87].

In the present study, sick dogs showed increased frequencies of blood $CD4^+$ Treg cell associated with decreased percentages of $CD4^+$ ($CD25^-FoxP3^-$) effector T cells, signaling a lack of adequate cellular immune response, which can prolong the presence of the parasite, facilitating parasite transmission. Antileishmanial therapy allowed the normalization of blood $CD4^+$ Treg and effector T cell subsets, especially in dogs under the meglumine plus allopurinol protocol, restoring the action of $CD4^+$ effector T cells.

Curiously, and following the obtained results, CanL does not seem to cause significant changes in $CD4^+$ Treg cells and $CD4^+$ effector T cell subsets of lymph nodes. Similarly, in a study with mice infected with *L. infantum*, a high frequency of $CD4^+CD25^+$ T cells expressing FoxP3 was found in the lymph nodes in the first weeks of infection, followed by a decrease in the subsequent chronic phase of the disease [43], supporting the observed results in the present study. In addition, the administration of CanL drugs caused a transient disturbance in Treg cells and effector T cell subsets. By directing the reduction in the frequency of effector T cells associated with the increase of the Treg cell subset, therapy appears to promote the development of a suppressive immune response located in the dog's lymph node. Despite this, 3 months after the start of treatment, the values normalize. Therefore, it is possible that miltefosine and meglumine antimoniate, which were administered to sick dogs only during the first 4 weeks of treatment, are primarily responsible for the development of a suppressive immune response that can limit inflammation.

In patients with visceral leishmaniosis caused by *L. donovani*, the bone marrow revealed an increase of Treg cells ($CD4^+CD25^+FoxP3^+$) that outnumbered effector T cells ($CD4^+CD25^+FoxP3^-$) [42]. These Treg cells were shown to be a source of IL-10 and persisted in patients even after successful chemotherapy with sodium antimony gluconate. In the current study, both treatments induced a quick increase in the frequency of the $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^-FoxP3^-$ T cell subsets in the bone marrow of dogs, but for a short period of time, normalizing by the second month of observation. In this case, the findings support the hypothesis that the increase in the frequency of $CD4^+$ Treg cells can be a possible consequence of miltefosine and meglumine antimonial drugs.

In CanL, as in other diseases in which the immune system is deeply involved, the presence and action of $CD8^+$ Treg cells are still a matter of discussion. In a study of human visceral leishmaniosis, the authors proposed that IL-10 produced by $CD8^+$ T cells could lead to a downregulation of cytokine production, in particular pro-inflammatory cytokines like TNF-α and IFN-γ, blocking this way the anti-leishmanial macrophage activity [88]. Subsequent studies have shown the presence of a subset of $CD8^+$ Treg cells that can inhibit the $CD4^+$ T cell-mediated immune response by inducing apoptosis of activated $CD4^+$ T cells [89]. This way,

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the increased frequency of the $CD8^+CD25^+FoxP3^+$ T cell subset in the blood and bone marrow of sick dogs shown in the current study could represent a complementary mechanism of immune regulation that may favor parasite survival [78]. Treatment of CanL with miltefosine or meglumine antimoniate in combination with allopurinol directs blood $CD8^+$ Treg cells to progressively return to normal values. These antileishmanial drugs seem to cause a shift in blood and bone marrow lymphocytes by reducing the increased frequency of the $CD8^+CD25^+FoxP3^+$ T cell subset and reduce effector $CD8^+$ ($CD25^-FoxP3^-$) T cells to restrain the local inflammatory immune response and cytotoxicity in order to lessen possible tissue damage.

$CD4^+CD8^+$ dp T cells have been identified in dogs with and without CanL [56,90,91]. In the current study, the frequency of $CD4^+CD8^+$ dp T cell subsets was revealed to be increased in peripheral blood, lymph node, and bone marrow of dogs with CanL. Considering the chronic profile of CanL, these findings are in line with previous studies [57–61] that have established a link between increased dp T cells and chronic diseases. Furthermore, dp T cells have also been associated with increased production of IFN-γ in pigs [50], similar to previous results found in dogs with CanL [66]. Moreover, the presence of $CD4^+CD8^+CD25^+FoxP3^+$ T cell subset in the peripheral blood of sick dogs reveals a possible regulatory activity, as proposed by other authors [62], while the lymph node and bone marrow presented decreased percentages of CD25, reflecting a possible cytotoxic role [63] resulting from the infection with *L. infantum*. In turn, in the present study, the administration of either treatment led to a change in both profiles, with dp T cells in the blood losing the regulatory phenotype, possibly in order to fight the infection, while the lymph node and bone marrow apparently switching to a regulatory profile to nullify a possible excessive cytotoxic damage. In any case, since the role of these $CD4^+CD8^+$ dp T cells is not yet fully understood *in vivo*, further in-depth studies are still needed in these subpopulations in order to elucidate their modes of action.

The immune response to *Leishmania*, in humans, mice, or dogs, seems to be far complex and influenced by several types of immune cells and different immune mediators, establishing an elaborate network. Either way, there seems to be a consensus that *Leishmania* parasites lead to differentiation of specific cell immunophenotypes in different tissues. CanL in this study led to an increased frequency of $CD8^+$ T cells in all tissues, along with increased $CD4^+CD8^+$ dp T cell frequencies, resulting in a predominant pro-inflammatory profile. $CD8^+$ Treg cell frequencies were also significantly increased in the blood and bone marrow, showing a possible action on immune responses mediated by $CD4^+$ T cells, which can lead to parasite tolerance and disease progression. In the present work, the administration of either treatment protocol led to an overall recovery of the T cell subpopulations by the end of observation, reflecting the clinical improvement of the dogs [66]. Nonetheless, it should be noted that both protocols resulted in an increase of $CD4^+$ Treg cell frequencies in all tissues, possibly in order to significantly reduce the frequency of $CD8^+CD25^-FoxP3^-$ T cells present and to control the local inflammatory immune responses. Lastly, with respect to the effectiveness of either treatment, despite not being the scope of this work, the recovery of many subpopulations was achieved more quickly with the Megl+Al protocol than with the Milt+Al protocol, which is in agreement with previous results [66].

Monitoring T cell subsets by using specific biomarkers and analyzing the effectiveness of CanL treatments allow a better understanding of the interplay between the parasite and the dog's immune response, which should improve patient management, lead to the development of more efficient and less toxic chemotherapies, and encourage the use of prophylactic measures that favor the reduction of zoonotic visceral leishmaniasis.

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Data Availability Statement

The datasets generated for this study are available on request to the corresponding author.

Ethics Statement

The animal study was reviewed and approved by Ethics and Animal Welfare Committee of the Faculty of Veterinary Medicine, University of Lisbon. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author Contributions

GS-G, IP, and MS conceived and designed the study. AB, AR, IP, JM, MP, and MS collected samples. IP, LG, and MS processed samples and did subsequent microscopic, serological, and molecular tests. GA-P and MS conducted the experiments. GA-P, GS-G, IP, and MS analyzed the data. GS-G and MS conducted statistical analysis. GS-G, IP, and MS drafted the manuscript. AVR, GA-P, GS-G, and IP made in-depth reviews of the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BID, bis in die; BUN, blood urea nitrogen; CanL, canine leishmaniosis; CD, cluster of differentiation; CG, control group; CVBD, canine vector-borne disease; DNA, deoxyribonucleic acid; FMO, fluorescence minus one; FoxP3, forkhead box protein 3; IFN-γ, interferon gamma; IL-2, interleukin 2; IL-10, interleukin 10; Megl+Al, meglumine antimoniate with allopurinol; Milt+Al, miltefosine with allopurinol; SID, semel in die; TGF-β, transforming growth factor beta; Th1, type-1 T-helper; Th2, type-2 T-helper; TNF-α, tumor necrosis factor alpha; Treg cells, regulatory T cells; UPC, urine protein-to-creatinine ratio.

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Reviewed by: Sonja Härtle, Ludwig Maximilian University of Munich, Germany
Hans-Joachim Schuberth, University of Veterinary Medicine Hannover, Germany

*Correspondence: Isabel Pereira da Fonseca, ifonseca@fmv.ulisboa.pt

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Veterinary Guidelines for Electrochemotherapy of Superficial Tumors

Directrices veterinarias para la electroquimioterapia de tumores superficiales

Palabras clave: electroporación, cáncer, oncología, mascotas, perros, gatos, caballos, procedimientos

Keywords: *electroporation, cancer, oncology, pets, dogs, cats, horses, procedures*

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Matías Tellado[1], Lluis M. Mir[2] and Felipe Maglietti[3]*

1. VetOncologia, Buenos Aires, Argentina
2. Université Paris-Saclay, CNRS, Gustave Roussy, Metabolic and Systemic Aspects of Oncogenesis (METSY), Île-de-France, France
3. Instituto Universitario del Hospital Italiano-CONICET, Buenos Aires, Argentina

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Tabla de contenido:

La electroquimioterapia (ECT) consiste en la aplicación de pulsos eléctricos para aumentar la ingesta de medicamentos quimioterapéuticos (bleomicina, cisplatino o calcio) en las células tumorales. Se ha convertido en una opción de tratamiento muy valiosa en oncología veterinaria. Es una modalidad de tratamiento eficaz y segura, que no solo es beneficiosa como tratamiento paliativo, sino también para un enfoque curativo.

Electrochemotherapy (ECT) consists in the application of electric pulses to increase chemotherapeutic drug intake (bleomycin, cisplatin, or calcium) into the tumor cells. It has become a very valuable treatment option in veterinary oncology. It is an effective and safe treatment modality, which is not only beneficial as a palliative treatment, but also for a curative approach. Performing the treatment adequately will ensure the best results possible, in the minimum number of sessions, and reduce complications. Usually, only one session is enough to achieve excellent results, but the treatment can be repeated. Several sessions can be necessary in the case of incompletely treated or very extended lesions, as well as in the occurrence of new lesions. ECT is effective for superficial or oral tumors of any histology that are accessible to the electrodes. Intravenous bleomycin is the preferred drug and route of administration, leaving other ways of administration and drugs for selected cases. The guidelines presented here are destined to veterinarians who want to develop their understanding of the basis of ECT and wish to perform it adequately and effectively. In this paper, we also discuss common problems and how to solve them, and we include practical tips to improve the treatment results based on common questions and mistakes of beginner users.

Introduction

Electrochemotherapy: Concept, Field of Application, and Advantages

Electrochemotherapy (ECT) is a well-established treatment modality that has been performed in veterinary medicine since 1997 in Europe [1] and since 2008 in Latin America. Its use in human medicine began with a clinical trial published in 1991 [2,3] and became a standard therapy in 2006 when the standard operating procedures for ECT in human patients were published, and the appropriate equipment was made available [4].

In veterinary medicine, ECT as a standard of care is now available in many countries across the globe. The use of ECT had a steep growth after veterinary electroporators were produced and training courses became available, for example in Slovenia, Brazil and Argentina. The impact solely in Latin America is revealed by the fact that annual meetings in Brazil and Argentina gather users from around the world to share their knowledge and experience. Despite a relatively low number of publications until now, more than 20,000 patients had been treated in more than

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120 centers just in Latin America (information gathered from the last users meeting held in Brazil in 2019).

In its beginnings, ECT was used for the palliative treatment of cutaneous and subcutaneous tumors of any histology. But nowadays, it can be used as a first-line treatment, alone or in combination with other therapies. It has been used to treat tumors in different anatomical regions, aided by the electrodes specially designed for veterinary and human medicine [5–11].

ECT consists in the transient and reversible permeabilization of cells through the application of an electric field. This increases the cellular uptake of certain molecules, augmenting their cytotoxic and anticancer efficacy by up to 1,000-fold [12]. Bleomycin, cisplatin, or calcium are the drugs proven to be effective, with a clear advantage for bleomycin among them [13,14]. The reasons behind this advantage are that bleomycin selectively kills replicating cells, preserving healthy non-replicating tissues, induces an immune system response, and it can be administered intravenously, providing an adequate dose availability in the treatment area [13,15].

As the drug is introduced into the cell by a physical phenomenon, penetration is not cell-type dependent. Tumors of any histology can be treated with very good results, with an objective response rate of around 70–100%. This high response rate can be observed in dogs, cats, horses, and of course in human patients as well [11,16,17]. In particular, in malignant melanoma, the objective response rate in early stages is around 90% [9], in squamous cell carcinomas is around 80% [18], in sarcoids is around 97% [11], and in mast cell tumors of less than 2 cm^3 is around 100% [19].

The application of the electroporation pulses also induces a *vascular-lock* phenomenon that produces the instantaneous interruption of the blood flow in the treated area. This provides additional benefits to the treatment, i.e., entrapment of the drug inside the tumor, tumor starvation, and immediate hemostasis in the treated area [20,21].

The immune response plays a crucial role in the efficacy of the treatment, as it was demonstrated in immunocompromised mice that showed a remarkably lower response to the treatment [13,22,23]. Also, in veterinary medicine, the role of the immune system is very important as it increases treatment effectiveness, and can even give rise to abscopal effects in some particular cases [24].

All in all, a very good response can be obtained in one or two sessions for most cases, depending on the size and location of the tumor. It is also important to recall that the therapy can be combined with other treatment modalities increasing their effectiveness, for example in combination with debulking surgery, chemo, or radiotherapy [25]. Finally, ECT can be applied when no other treatment options are available, still with good results, making ECT a very attractive tool for the veterinary oncologist [26].

In precision medicine, treatments are selected and personalized regarding patients' characteristics, genetics, and environmental factors, for increasing their effectiveness and reducing side effects [27]. Precision medicine relies on the biological and chemical particularities of the patient and its disease on the one hand, and on the biological and chemical effects of the treatment on the other hand. ECT is an original contribution to precision medicine, as it is a physical approach. It is important to note that all the classical physical approaches up to now (including radiotherapy, brachytherapy, cryotherapy, hyperthermia, high frequency focused ultrasound, and even surgery as a <<mechanical>> approach) are ablative methods, meaning that they do not selectively discriminate between cancerous and normal cells. On the contrary ECT is not an ablative approach. As will be discussed in the next sections, the exquisite combination of electropermeabilization and a non-permeant cytotoxic drug like bleomycin results in a very

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precise and selective destruction of the tumor cells. Thus, in the preservation of the normal cells and tissues structures, dramatically reducing the side effects of the classical chemotherapy and of the ablative procedures. As such, ECT is an efficient, easy to apply, and safe new method for precision medicine. In particular, ECT precision allows to efficiently and securely treat the margins and surroundings of the tumor mass because the few tumor cells present in this volume will be eliminated while the normal cells in the same volume will remain viable. This precision in killing replicating cells, i.e., tumoral cells, explains why ECT spares healthy tissues and preserves organ function, which in certain patients is the most important aspect for choosing a treatment, particularly for preserving life quality. This selectivity confers the treatment reduced side effects, which are mild and self-limited, and even provides very good cosmetic results in the treatment of cutaneous and subcutaneous tumors [28]. It is also noteworthy that, even in the case of tumors that have genetic characteristics that confer resistance to multiple drugs, and even to radiotherapy, ECT still plays an important role due to its capability to overcome these barriers. ECT forces the drug into the cells, achieving excellent results where other treatments had failed. Finally, not only the electric pulses per se are an immunological adjuvant as demonstrated in [23], but moreover, ECT causes immunogenic cell death [29], an important aspect of the success in cancer precision medicine.

Many times in the veterinary setting, ECT is performed following standard procedures made for human patients, and that reduces its effectiveness. ECT in vet medicine should be performed considering the unique characteristics of the patients and taking into account the differences among the species that are treated. To achieve its maximum potential adequate treatment planning is crucial. The proper selection of the electrode will depend on the anatomy of the patient, tumor location, invasion depth, and size of it. Also, it is very important choosing the right drug, and the adequate way of administration.

In this work, we report the Veterinary Guidelines for Electrochemotherapy of superficial tumors. Based on published data in peer-reviewed journals and enriched with a careful compilation of the users' questions, and common difficulties. We report a list of potential deviations that can reduce treatment efficacy, and we provide ways to attenuate their consequences. The prevention of these deviations in the practice of the ECT is as important as the accurate application of these guidelines.

Materials, Equipment, and Patients

Patients

The main goal is to provide cancer patients with the best treatment available and maximize their chances of success.

In selected patients, ECT alone can be a very good treatment option which can provide excellent results. However, in veterinary medicine, it is common to receive patients in very advanced stages of the disease, with large tumors where ECT alone may not be effective or will require too many sessions. In these cases, combining ECT with other treatment modalities is very effective as will be described later. ECT can thus be used alone or as a neoadjuvant, adjuvant, or concomitant treatment with surgery, chemotherapy, or radiotherapy [25,30]. Adequate treatment selection, based on the patient's oncological situation, is crucial for obtaining the best results.

The owner should understand the expectations of the selected treatment, and the alternatives, and after that, sign an informed consent.

Tabla de contenido:

Indications of ECT

1. Cutaneous or subcutaneous tumors (primary or metastatic) of any histology, which cannot be satisfactorily treated with their respective first-line treatments [26,31].
2. Primary or metastatic tumors affecting the quality of life due to bleeding, ulceration, or pain [26].
3. Oral or Nasal tumors, as a single treatment, or in combination with surgery [8,9,32].
4. Incompletely resected tumors (including surgical scars, skin flaps, and other surgical repairs), or for extending safety margins during surgery [33].
5. Elective ECT treatment when other first-line therapies are possible. A thorough explanation of other treatment options had to be made, and they had to be rejected by the owner [31,34].
6. Reducing cancer burden in primary or metastatic tumors that are accessible to the electrodes (superficial or by surgical approach) [26,35].
   a. In patients under systemic therapy, for the treatment of the lesions that do not show good response.
   b. In large tumors before surgery, to improve relapse-free survival.
   c. In patients without treatment options, to improve quality of life in a palliative intent.

Pre-treatment Tests and Considerations

Mandatory pre-treatment tests are listed below.

1. Pregnant or lactating animals; special care should be taken with unspayed females as bleomycin and cisplatin can harm the developing fetus.
2. Blood tests to determine kidney and liver function should be within normal parameters. Hematological and coagulation parameters should be normal and comparable to those required for a simple surgical procedure.
3. Anesthetic risk including cardiological examination should be evaluated.
4. Histopathological diagnosis of the tumor.
5. Complete oncological staging including three-view chest radiographs, abdominal ultrasonography, CT scan, or MRI if needed, to evaluate the presence of metastasis or other comorbidities.
6. According to each specific case, pain management may be required before ECT and may be adjusted thereafter.
7. Assess the risk in patients who previously received bleomycin; the maximum cumulative dose for dogs is 200,000 IU/m2, while for cats it is not yet established [36]. Patients who reached this dose should be treated with cisplatin (except cats) or calcium administered intratumorally. If this is not possible, consider another treatment option.
8. History of allergy or hypersensitivity to bleomycin or cisplatin.

In dogs and cats, special attention should be paid to the following cases, as the treatment can be difficult to perform, and the chances of success are reduced.

1. Tumors in, or close to the larynx. Before initiating the anesthetic procedure, make sure that the lesion does not impede the intubation. Consider that after the treatment, the lesion will swell, and may obstruct the airway. If the lesion is large and blocks a considerable part of the airway, debulk it and treat the tumoral bed. Temporary or permanent tracheostomy may be an option for reducing the risk of airway obstruction after the treatment and during the recovery.
2. Tumors in the tongue. The tongue has terminal irrigation, and a very broad application of the electric pulses might induce necrosis of its rostral part, due to the vascular-lock phenomenon. If the tumor is located caudally, it may obstruct the airway after the treatment. In both cases, consider surgical debulking followed

Tabla de contenido:

by ECT in the tumoral bed. A feeding tube may be needed in the early recovery days.

3. Tumors that compromise the cribriform plate, the retro-orbital region, paranasal sinuses, or other unreachable structures. Tumors located in the caudal region of the nasal duct may invade the ethmoidal cells, which cannot be treated successfully using standard electrodes as they are very difficult to reach. This will ultimately lead to treatment failure. Consider other treatment modalities like surgery plus radiotherapy.

Special considerations for treating horses.

1. They must be treated under general anesthesia, and they must have the appropriate pre-surgical examinations for the procedure. Tetanus vaccine or hyperimmune serum can be administered at the discretion of the veterinarian.
2. The use of the IV route is costly considering the high volumes of drug required, and there are not enough safety studies in this species. Also, the use of intravenous cytostatics should be avoided in animals that may be destined for human consumption.
3. The most frequently treated neoplasias are sarcoids, cutaneous malignant melanoma, and squamous cell carcinoma, all of them with good results [19,37]. Other histologies can be treated whenever their approach is feasible using the available electrodes.
4. The treatment of ulcerated lesions must be adapted to the living conditions of the animal and may require the administration of antibiotics and/or repellants.

Assessing Size and Number of Tumors

To determine the best treatment strategy, count and measure all the lesions. It is recommended to take pictures including a ruler, to document response to the treatment. They should be taken at the same angle and perspective every time. Also, other imaging procedures may be used to document the effect of the treatment.

For calculating the tumoral volume use the following formula,

$$Tumoral\ volume\ [cm^3] = a \cdot b \cdot c \cdot \frac{\pi}{6}$$

Where a, b, and c are the length, width, and thickness of the tumor, respectively. For thin tumors (or when lesion thickness cannot be measured) replace the c by the b, as can be seen in the following formula,

$$Tumoral\ volume\ [cm^3] = a \cdot b^2 \cdot \frac{\pi}{6}$$

The tumoral burden should be put in context to the size of the animal treated, however, the size of the tumor by itself has an impact on the treatment outcome.

At this point, it has to be determined if the ECT will be performed as a palliative or curative intent, and the number of sessions needed for that end.

ECT as a Curative Intent

Tumors of up to 3 cm^3 can be treated easily with good results, whatever their shape. It can be used as an alternative to surgery when it is difficult, to avoid postoperative complications, or when the tumor is close to important structures [38–40].

In the case of extensive superficial tumors, of <1 cm thickness, even if they are larger than 3 cm^3, ECT is a good option when first-line treatment modalities have failed or if they are not feasible [38,41–43].

Special care should be taken with the thickness of the tumor, as the length of the needle of the electrode could limit the possibility of treating the tumoral bed. Please, note that only the conductive part of the needles should be considered (some devices use electrodes that despite the needle being long, some

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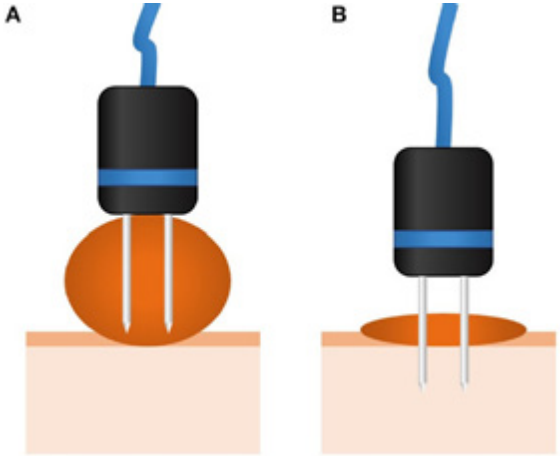

Figure 1. *Scheme showing the treatment of a tumor thicker than the needle's length. In (A), as can be seen, in a thick tumor the tumoral bed is not treated, leading to a relapse. In these cases, surgical debulking is recommended to achieve the situation in (B). Otherwise, the same situation in (B) can be achieved by multiple ECT sessions, which should be already planned when the patient is evaluated for treatment.*

part of it is isolated). Tumor thickness should be less than the length of the needle's conductive part, which usually varies from 1 to 4 cm, depending on the manufacturer. If the tumor is thicker than the conductive needle's length, debulking followed by ECT of the tumoral bed and margins is recommended. If debulking is not performed, a second session of ECT must be scheduled to complete the treatment, and performed after the response to the initial session is achieved. These additional rounds of ECT are part of the treatment planning, and should be established during the patient's initial evaluation [44]. See **Figure 1**.

ECT as a Palliative Intent

ECT is a very useful treatment strategy, and in this context, superficially extensive or large tumors can be treated to improve the quality of life, provided there are no curative alternatives [45].

Owner Information and Informed Consent

Owners should be informed about all treatment options, the benefits and drawbacks of ECT, and its possible side effects. Owners should also be informed about the expected outcome of the treatment. Once these issues are understood, a proper informed consent should be signed by the owner, which should include: (i) the risks associated with the drug used for the procedure, i.e., lung fibrosis when using bleomycin in a patient with a high accumulated dose, and risk of alopecia and/or changes in the pigmentation of the treated skin (see **Figure 2**), (ii) the risk of pain after the procedure, for which an adequate management plan has to be provided, (iii) the risk of formation of a fistula, extensive necrosis, or serious post-treatment anatomical defects when the tumor compromises the whole thickness of the tissue, that may require reconstructive surgery, as well as (iv) the possibility of needing more treatment sessions in certain cases.

Electroporators and Pulse Parameters

The standard accepted pulse parameters for ECT are 8 monopolar square-wave pulses of 100 µs, delivered at a repetition frequency of 1–5,000 Hz, with a voltage-to-distance ratio of 1,000–1,300 V/cm, depending on the electrodes used. The repetition frequency does not affect the response. When frequencies higher than 100 Hz are used, only one muscle contraction is seen improving tolerance, and comfort for the patient [46,47].

Monopolar square-wave pulses are the preferred type of pulses for ECT. However, many configurations of bipolar pulses have also displayed good results [34,48]. Further research is needed to determine if there are benefits of using bipolar pulses in gene electrotransfer or ECT [24,49].

Many electroporators are available for performing ECT in veterinary medicine. Some of them are for laboratory use, and others are specifically designed for their use in veterinary clinics.

Automatic devices are preferred since configuration errors are prevented. The electroporator should be able to maintain the voltage (without drops) in each one of the 8 pulses of the train. That is possible if the generator can deliver an adequate maximum output current. Ac-

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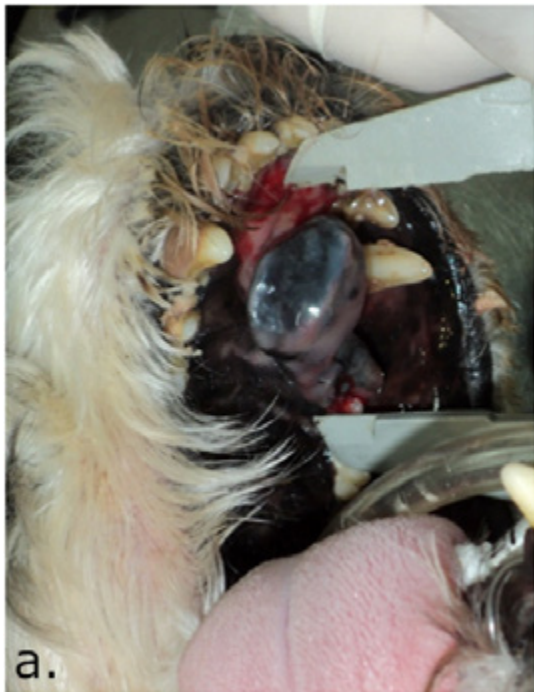

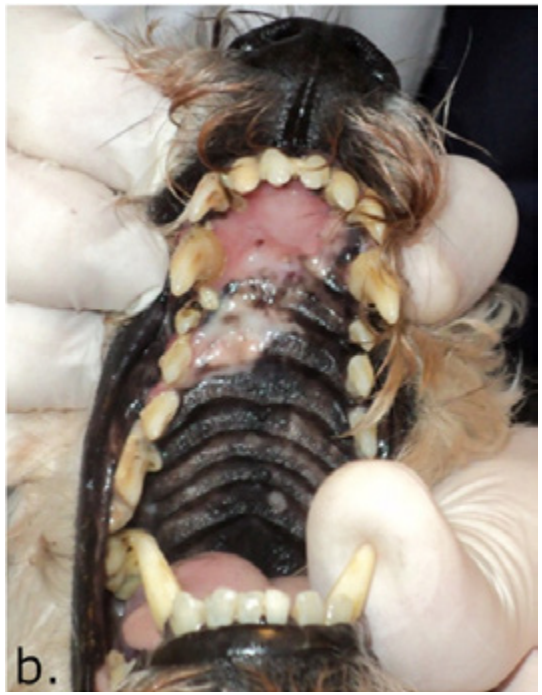

Figure 2. *Picture of the palate of a dog treated with ECT using IV bleomycin where induced hypopigmentation can be seen. In (**a**), the dog with a melanoma in the palate before the treatment. In (**b**), 4 months after ECT, a complete response was obtained and a hypopigmented area is seen where the tumor was located. Note that as the bone was not compromised, the palate integrity was preserved. In some cases, hypopigmentation may partially revert with time.*

cording to our experience, current outputs of 35 A (during the 100 μs of the pulse duration) are enough for treating all kinds of tumors.

It is very important to always use the electrodes provided by the manufacturer of the device. The basis for choosing the best electrode for a patient is discussed in the following section.

If a configurable electroporator is used, it should be properly set. To this end, verify the distance between the needles of the electrode that will be used and multiply this distance in cm by 1,000 to obtain the voltage to be used. For instance, if you are using needle electrodes separated 0.4 cm from each other, you have to set the device output voltage at 400 V (1,000*0.4 = 400). In the case of plates applied on the skin, you should multiply the distance by 1,300. Thus, in the same example, you have to set the device output voltage to 520 V (1,300*0.4 = 520). In the case of plates applied directly onto the tumor, use 1,000 V/cm, like for the needles. Then set the pulse length to 100 μs, and the pulse interval between 100 μs and 1 s (for a repetition frequency between 5,000 and 1 Hz). Set the device to deliver 8 pulses [50].

Drugs Used

For performing ECT only 3 drugs have been validated; bleomycin administered intravenously or intratumorally, cisplatin intratumorally, or calcium intratumorally. The decision basis for the use of each of them is presented in the following section.

Tabla de contenido:

Methods: Treatment

Anesthesia

The treatment should be performed under general anesthesia, even when using plate electrodes. The sensations and muscle contractions induced by the pulse delivery can be painful and stress the patient, eliciting a pain-induced aggression [51].

The anesthetic procedure required for ECT is similar to the one required by surgery in the same region. It should be chosen according to the expertise and familiarity of the professional who is going to perform it.

As an example, for dogs and cats, a typical regime would be as follows: (i) premedication with intramuscular administration of xylazine 0.5 mg/kg and tramadol 2 mg/kg; (ii) induction made with intravenous administration of propofol 3 mg/kg; (iii) maintenance performed with inhaled isoflurane 2–3 % and intravenous fentanyl 2 µg/kg. Regional anesthesia may be added, according to the criterion of the anesthesiologist, to improve patients' comfort and reduce the dose of the anesthetic drugs. For instance, infraorbitary nerve block can be performed when treating the nose. Anti-inflammatory drugs can be used during or after the procedure and maintained the following days as it will be seen later.

In horses, general anesthesia is always recommended. A typical regime would be combined regional-general anesthesia, to reduce the depth of the anesthetic plane required [52]. The induction should be carried out in a safe and carefully chosen environment. The patient should be under the proper analgesic plan during the whole procedure.

In small animals, post-anesthetic observation until the patient is fully awake is very important, especially when the nose or the mouth is treated.

Drug Administration

Begin by weighing the patient. Then, measure the lesions to calculate the tumoral volumes. After the measurements, proceed to trim the fur of the area to be treated to have a good visualization of the tumor and its margins. Use an iodine solution for cleaning and disinfecting the whole area to be treated (tumor and margins). It is highly recommended to use sterile electrodes or to sterilize them following recommendations from the manufacturer, even though the risk of infection after ECT is very low [53].

For the dilution and administration of the antineoplastic drugs, it is mandatory to wear gloves and a laboratory coat, to use face and respiratory protection. If possible, it is recommended to work in a class II laminar air flow cabinet [54].

Intravenous Route

We recommend using intravenous bleomycin for all cases, regardless of the size of the lesion (only bleomycin can be used through the intravenous route, as it is explained later). This recommendation is based on several reasons: (i) an adequate distribution and concentration of the drug are almost always achieved in the tumor and its margins when the timing of administration is observed, (ii) avoids leaving areas of the tumor with insufficient drug concentration due to errors in the intratumoral administration technique, (iii) drug administration is safer, as spilling and leaking that can occur during intratumoral administration are avoided, and (iv) by itself is an immune system activator, contributing to the local immune response induced by the treatment [23].

Note that intravenous bleomycin administration is not recommended for horses due to the huge volume of drug needed.

The potency of bleomycin is measured in units of antimicrobial activity. In many countries, bleomycin is either dosed in mg or International Units (IU), whereas Units (USP) is the term used in the USA. The equivalence would be 1 USP unit = 1 mg (by potency) = 1,000 International Units (IU) [55].

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Use the weight of the patient to estimate the body surface area (BSA) with the following formula.

$$Body\ Surface\ Area\ [m^2] = k \cdot weight[kg]^{2/3}$$

Where k is 0.101 for dogs and 0.1 for cats [56].

The Technique of Administration for the Intravenous Route

The drug is administered at a dose of 15,000 IU/m^2 BSA in bolus (in 30–45 s), and the maximum dose is capped at 30,000 IU (corresponding to 2 m^2 BSA) [50]. The application of the electric pulses can begin 5–8 min later, after the drug has diffused into the tumoral tissue.

After administration, approximately half of the Bleomycin dose administered is eliminated by renal excretion, so a reduced dose can be used in patients with decreased renal function [57,58]. Remember that the maximum cumulative dose for dogs is 200,000 IU/m^2, while for cats it is not yet established [36], to avoid bleomycin's induced lung fibrosis, its major side effect. The drug is metabolized in tissues by the bleomycin hydrolase enzyme, which is in very low concentration in the skin and lungs, explaining the sensitivity of these tissues to bleomycin toxicity [58].

The great difference in sizes and body weights among veterinary patients needs to be considered when estimating the treatment window. This time can be affected by many factors, among them are the tissue blood flow rates [59] which are influenced by the heart rate. As is known, heart rate is related to body weight [60], so we can arbitrarily consider a treatment window closer to 5–25 min for cats and small dogs, and 8–40 min for the rest. Similarly in human medicine, patients younger than 65 years old have a treatment window of 5–15 min, vs. an 8–40 min treatment window for older patients, this difference is related to the kidney function [61]. Further study of bleomycin pharmacokinetics is needed to properly define the treatment window in patients of different species and body weights.

The optimal efficacy for applying the pulses is obtained up to 40 min after the administration of the drug. However, this time can be extended in elder patients as well as in patients with impaired renal function. In any case, it is recommended to continue the application of electric pulses to the remaining lesions even after that time, as there is still an effect on the lesions. It is advisable to mark these lesions, to recognize them during the follow-up [57,62].

Intratumoral Route

The intratumoral administration is acceptable for small tumors of up to 2 cm^3 [63]. In large tumors, this route can be challenging, in particular in horses and cats. On the contrary, the intravenous route will provide an adequate distribution of the drug in all cases, and for that reason it should be preferred whenever possible.

Bleomycin, cisplatin, and calcium can be administered intratumorally. The delivery of the electric pulses should begin immediately after its administration.

Bleomycin

The recommended concentration of bleomycin for intratumoral administration is 1,000 IU/ml, and the dose is 250 IU/cm^3 of tumor [50].

The tumor must be completely infiltrated with a total dose lower than the one that would be used in the intravenous route. If this is not possible, use the intravenous route of administration under the conditions described in the previous section.

In unusual species, like turtles, snakes, or birds, among others, the intratumoral route is preferred due to the lack of information on the dosing and effects of systemic bleomycin in these animals.

Cisplatin

When using cisplatin, the recommended concentration is 1 mg/ml, and the recommended injection dose is to fill the tumor volume with the drug [50]. In case cisplatin is not available at the recommended or superior concentra-

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tion, we suggest using bleomycin. The use of a lower concentration is possible but requires a close follow-up, as the patient may need retreatment [64].

Cisplatin is a good option for treating horses [11,19]. On the contrary, this drug is not recommended in cats [65].

Carboplatin can be used if cisplatin is not available, however, its effectiveness has only been demonstrated *in vitro* [66].

Calcium

When using calcium chloride, the recommended concentration is 9 mg/ml. Inject a volume of calcium chloride solution equal to half of the volume of the tumor [67,68]. Electroporation with calcium can provide good results and can be used when bleomycin or cisplatin are not available, as the third option. It should also be restricted to small tumors [67].

The Technique of Administration for the Intratumoral Route

Insert the needle at a single point in the center of the tumor, and radially administer the drug to avoid spilling. When injecting the drug, pay attention not to remove the needle too quickly to avoid spilling or leaking the medication. As only small tumors should be treated with this technique, safety margins are covered by drug diffusion from the lesion providing an adequate concentration for successful treatment of the margins. Healthy tissue, if infiltrated by any of the previous drugs can necrotize, and thus direct injection of the surrounding healthy tissue should be avoided [69].

The electric pulses should be applied immediately after the administration of the drug, as it washes out quickly [50]. If there is more than one lesion to treat, it is recommended to administer the drug and deliver the pulses to them, one by one. It is important to point out that if the drug is administered to all tumors in the first place, and the pulse delivery is performed after that, the last ones to be pulsed may not have an adequate drug concentration.

In horses, where intravenous bleomycin is not possible, large tumors can be treated in this way; inject half of the lesion and pulse it immediately after. Then, inject the other half and pulse it. By doing this, a large lesion can be successfully treated.

Electrode Selection

Electrode Types and Their Advantages

In general most devices come with two types of electrodes; the needles electrode, and the plate electrodes (see **Figure 3**).

Always use electrodes provided by the manufacturer of the device and observe the maximum number of uses intended for them. Exceeding the maximum number of intended uses may significantly reduce the effectiveness of the treatment. If available, disposable electrodes are recommended.

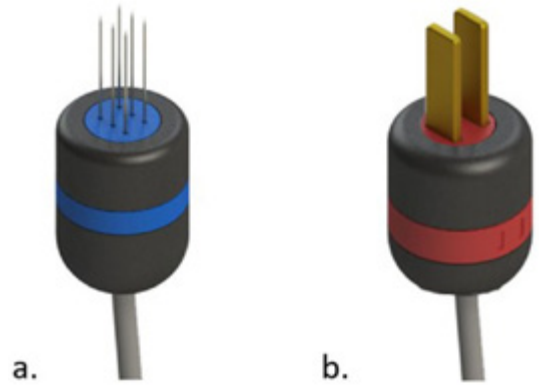

Figure 3. *Common types of electrodes for electroporation. In (**a**) needles electrode. In (**b**) plate electrodes.*

Needles Electrodes

This type of electrode is recommended for the treatment of the great majority of tumors.

In veterinary patients, the skin is thicker than in humans [70], being a very resistive layer that can interfere with the homogeneity and the intensity of the electric field at the tumor level. Moreover, skin thickness and thus skin electrical impedance is very variable, depending on many factors, i.e., the species, the race, the age, and the part of the body, among others. By using needles, this thick layer is surpassed, and the elec-

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tric field can be applied with an adequate distribution. Therefore, needle electrodes are always preferred.

The needles are usually distributed in two rows of three or four, separated 0.4–0.5 cm from each other. There are other patterns, like the hexagonal, that behave in the same way.

The whole tumor surface should be covered to adequately treat the tumor. In particular, mast cell tumors should be treated spirally from the periphery to the center (see **Figure 4**). As the electric field drops off very quickly outside of the electrodes, a minimum superposition in the application is needed to avoid leaving untreated areas. If the tumor is thicker than the length of the needles, surgical debulking may be needed. Also, it can be treated in multiple sessions, which should be at least 4 weeks apart, to avoid overtreatment of the area, provided there is no growth of the tumor. If the growth of tumoral tissues is seen, the following treatment session should be performed as soon as possible. A particular case is when the tumor is only partially treated in the first session. In this case, the remaining untreated tissues can be treated the following day without concern.

For the decision basis on when to retreat a previously treated tumor, please see section Follow-up and Retreatment.

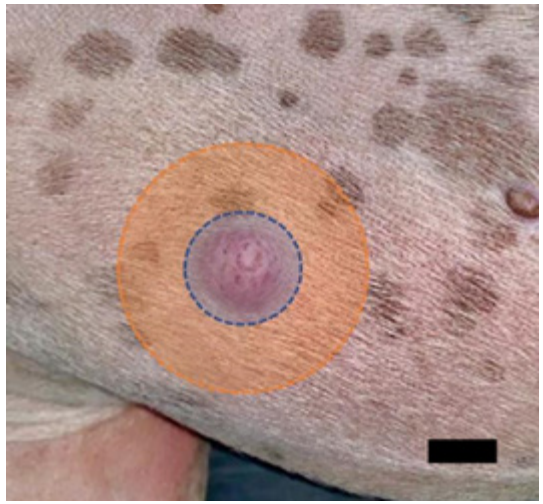

Figure 4. *Principle of treatment for mast cell tumors. First, the periphery should be treated (orange area), and then the tumor (blue area). Scale-bar 1 cm.*

Parallel Plates Electrodes

Parallel plates electrodes are very useful to treat superficial lesions with a few millimeters invasion. The depth of treatment of these kinds of electrodes varies according to the distance between the plates [71]. According to the simulations presented in **Figure 5**, in electrodes with plates separated 4 mm from each other the maximum treatment depth is <3 millimeters. Lesions that invade deeper than this, should be treated with needle electrodes. It is important to remember, that for treating superficial lesions through the skin the voltage-to-distance ratio should be 1,300 V/cm [72]. But, for the treatment of the tumoral bed after debulking surgery, 1,000 V/cm is enough. As the skin is removed, the electric field can reach deeper parts of the tissues [71].

Plate electrodes are very useful for treating the eyelids, and ears by putting them in-between the plates.

Other designs work similarly to plate electrodes, such as contact electrodes and L-shaped electrodes. These kinds of electrodes are used for the treatment of superficial tumors only, and tissues cannot be treated by placing them in between the conductive parts, as is possible with plate electrodes. Particularly in some devices, an L-shaped electrode is configured to deliver 4 pulses, and for that reason, orthogonal rotation between two pulse trains is required.

Always monitor the contact between the tissue and the electrodes [73]. If it is not adequate, conductive gel should be used to improve the electric field distribution and thus the result of the treatment. The gel should have a conductivity similar to the tissue treated. For general purposes ultrasonography gel is adequate. Remember that an excessive amount of gel is better than an insufficient one to improve contact between the tumor and the electrode [74]. Avoid using petroleum jelly as it can impede the flow of the electrical current reducing treatment efficacy. Also, avoid excessive overlapping of two adjacent applications, particularly in healthy tis-

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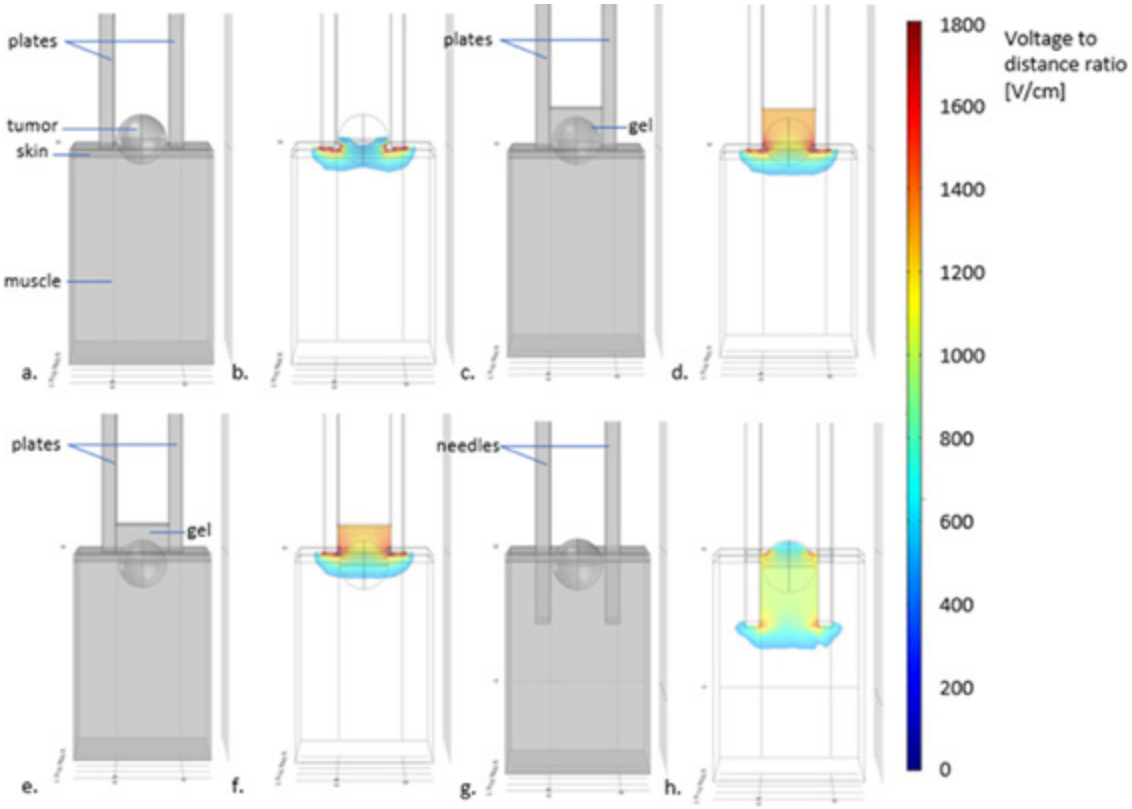

Figure 5. *Scheme displaying the depth of treatment with different electrodes and visual recommendations for the use of plate or needles electrodes. In (a) plates electrodes used for treating a superficial tumor, without using conductive gel. In (b) the simulation of the electric field distribution (using COMSOL Multiphysics 4.3—in color the electric field intensities above the threshold for tissue reversible electroporation) reveals that the superficial parts of the tumor may not be adequately treated. In (c) the scheme shows the addition of gel between the plates. In (d), the simulation shows that now, the distribution of the electric field allows treating the tumor completely. In (e), the case of a tumor with an invasion depth >3 mm treated with non-penetrating plate electrodes. As it can be seen in (f), even with the use of gel, the deepest parts of the tumor may not be adequately treated. Indeed, the field intensity drops below the electroporation threshold at a distance lesser than the separation of the plates. For these cases, needle electrodes should be used, as depicted in (g) where the same tumor (with an invasion depth >3 mm) is treated with needle electrodes without conductive gel. The electric field simulation in (h) shows that the whole tumor is now completely treated.*

(R) evolution

PEQUEÑOS ANIMALES

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Figure 6. *ECG monitoring during an ECT procedure. An artifact can be seen during the delivery of the pulses (white arrow).*

sue, as it may provoke undesired tissue damage or excessive necrosis (see also section Actionable Recommendations).

Safety Recommendations

The electroporator used should comply with basic safety standards, including arc detection, short-circuit detection, and voltage drop alarm to ensure an adequate pulse delivery.

Artifacts in ECG monitors may be seen during pulse delivery, due to electrical interference between devices, and they should not be mistaken for arrhythmias or any other cardiac alteration (see **Figure 6**).

Metallic surgical instruments should be kept away from the electrodes, and the area of treatment, avoiding their contact during pulse delivery. This point will be addressed in section Actionable Recommendations.

Antibiotics and Analgesia After the Treatment

Prophylactic antibiotics can be administered orally, or intramuscularly, before or after the procedure.

The use of non-steroidal anti-inflammatory drugs (NSAIDs) is the recommended option for pain control, because, after the treatment, pain can be provoked by the inflammation of the treated tissue. In large lesions, the association of NSAIDs with opioids may be useful. If the treatment is on the nose, tongue, eyelids, or close to the larynx, the use of corticosteroids is preferred over NSAIDs during the first 48 h, due to their greater anti-inflammatory effect. NSAIDs can be used after the suspension of the corticosteroids. However, simultaneous use of both, which is common in human medicine, is counter-indicated in dogs and cats as it increases side effects [75].

Wound Care

No dressing is needed for the wound after the treatment. In the subsequent days after the treatment, the treated area may present oozing that can be cleaned by the owner. Elizabethan collars can be used in cats and dogs to prevent the animal from licking the treated area.

Evaluating the Results

Follow-Up and Retreatment

Follow-up is planned individually depending on patients' needs and is recommended at 15 days, and at 1, 2, 4, and 6 months after the treatment. At each follow-up, the lesion should be measured and photographed to document the response to the treatment.

Once the tumor is treated, it slowly reduces in size with little or no necrosis. The mechanism of action of bleomycin consists of cutting the DNA strands, and the cells die when trying to divide. For this reason, tumoral cells are "marked" to die, but only die after they try to divide [58,62]. As long as the lesion keeps shrinking, no more treatment sessions are needed, because there is no benefit in treating already treated cells. On the contrary, it may induce necrosis of the tissue. The maximum therapeutic effect is seen after 6–8 weeks, but it may take longer. Sometimes, the tumor enters a quiescent state, and after 2 or 3 months starts shrinking again.

Before scheduling a new treatment session, the full response should be await-

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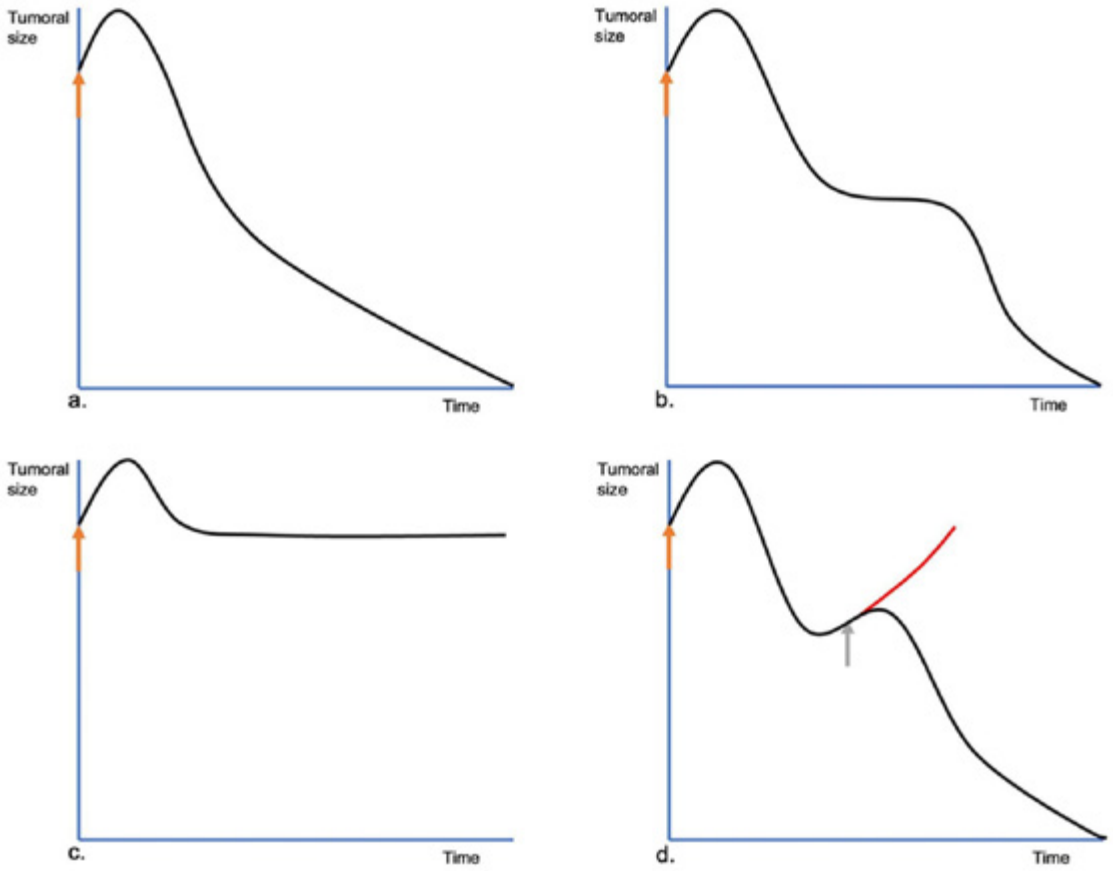

Figure 7. *Different evolutions of the tumors treated by ECT. In (a), the common evolution. At first, immediately after the ECT session (orange arrow), the treated lesion swells, increasing its size, but on the following day, it starts to reduce steadily until its complete remission. In (b), the two-times evolution. The lesion, after the initial swelling, starts shrinking, but it stops. The lesion remains steady for some time and then resumes shrinking until its final response. In (c), the no-change evolution. After the initial swelling, the tumor shrinks to its pretreatment size, without shrinking any more, or shrinking very slowly. In (d), the tumoral escape evolution. After the initial response, the tumor starts growing again. A new ECT session should be performed as soon as possible (gray arrow) to prevent a relapse.*

ed, instead of performing the next session on a fixed time basis. This applies also to the retreatment of a previously treated lesion. Note that in the case of previously untreated tissues or parts of the tumor that were left untreated (in the case of very large lesions, for example in horses) there is no need to delay a new session to treat these untreated tissues. In the case that the treated lesions prove to grow again, the new treatment session should not be delayed.

Even though tumor lysis syndrome is very uncommon, when treating large tumors special measures should be taken to prevent it, in any case, a quick diagnosis and prompt treatment is essential.

In the follow-up of a tumor treated with ECT, four kinds of evolutions can be seen. The *common* evolution is what happens in most cases. The tumor shrinks after an initial swelling and keeps shrinking until the final response is achieved (**Figure 7a**). Sometimes, in the beginning, the lesion behaves like the previous, but shortly after, it stops shrinking. It stays the same size for a variable time, then it resumes shrinking. We call this a *two-times evolution* (**Figure 7b**). This could be attributed to the non-dividing tumoral cells, which enter the cell division cycle and die only at that moment since they have their DNA strands cut by bleomycin

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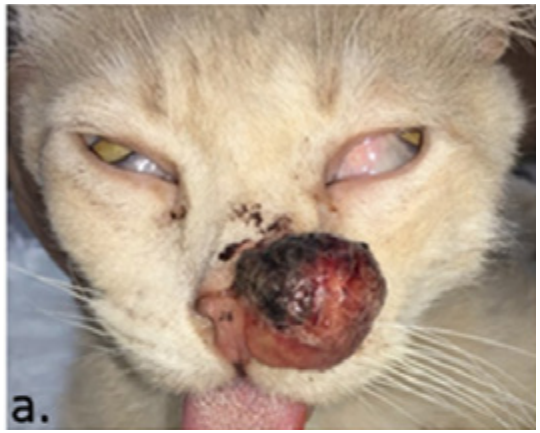

Figure 8. *Feline patient with a squamous cell carcinoma in the nose. In (a) the day of the ECT. In (b) 1 month later, the tumor regrew, and a second ECT session was performed. In (c), a complete response was obtained 1 month after the second ECT. In (d), the patient remains disease-free 4 months later.*

without killing them immediately. Another type of evolution is characterized by lesions that after the treatment do not show an evident shrinking, due to their high population of quiescent cells (see **Figure 7c**). We call this *no change evolution*. The *tumoral escape evolution* is seen when after the initial reduction, the tumor starts growing again (see **Figure 7d**). In this case, a new treatment session should be scheduled with no delay as the tumor has been insufficiently treated (see **Figure 8**). It is important to note that the tumor evolution is different from the response, as the response can be the same in the four cases. Understanding the types of evolution is essential to determine whether to repeat a treatment session or not. In the first three types of evolution, it is recommended to wait and make a close follow-up of the lesion. Especially in the two-times evolution, or in the no-change evolution, as they may end in the tumoral escape evolution. This is particularly important, as the treatment of lesions that are evolving following one of the first three types of evolution, may be unnecessary and can even lead to tissue necrosis, due to overtreatment. If there are doubts about a steady lesion, its recommended to perform a biopsy to avoid confusing a remaining of the tumor with residual scar tissue.

There is no data regarding minimum intervals between retreatments, but at

(R) evolution

PEQUEÑOS ANIMALES

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least 4 weeks is a period recommended when using intravenous bleomycin.

Consider retreatment if

- The lesion was not sufficiently treated in the first session.
- The lesion starts to grow
- New lesions develop.

Combination With Other Therapies

ECT does not preclude other treatments, on the contrary, it may increase their effectiveness. For that reason, many standard treatments such as surgery [10,44,51], chemotherapy [30], immunotherapy [24,76], and radiotherapy [77] may be more effective in combination with ECT [25].

For tumors larger than 3–4 cm^3 ECT can be combined with surgery as a neoadjuvant cytoreductive tool to perform a less extensive surgery, allowing function or organ sparing. ECT can also be used as an adjuvant therapy to clean insufficiently resected margins. And finally, it can be used intraoperatively, to clean the tumoral bed of the resection [10,25,44,51].

Chemotherapy or metronomic chemotherapy can be used adjuvant to ECT. However, considering the beneficial role of ECT as an activator of the immune system, response may be impaired by the immunosuppressive effect of systemic chemotherapy [29]. It must be carefully assessed whether the risks outweigh the benefits for this combination of neoadjuvant, concomitant, or adjuvant chemotherapy with ECT [25].

Radiotherapy can be used in combination with ECT, particularly for tumors that invade an area beyond the reach of the electrodes. The other way around is also possible, ECT can be used after radiotherapy to treat radioresistant relapses [77,78].

Finally a procedure step by step on how to perform the treatment is presented in **Figure 9**.

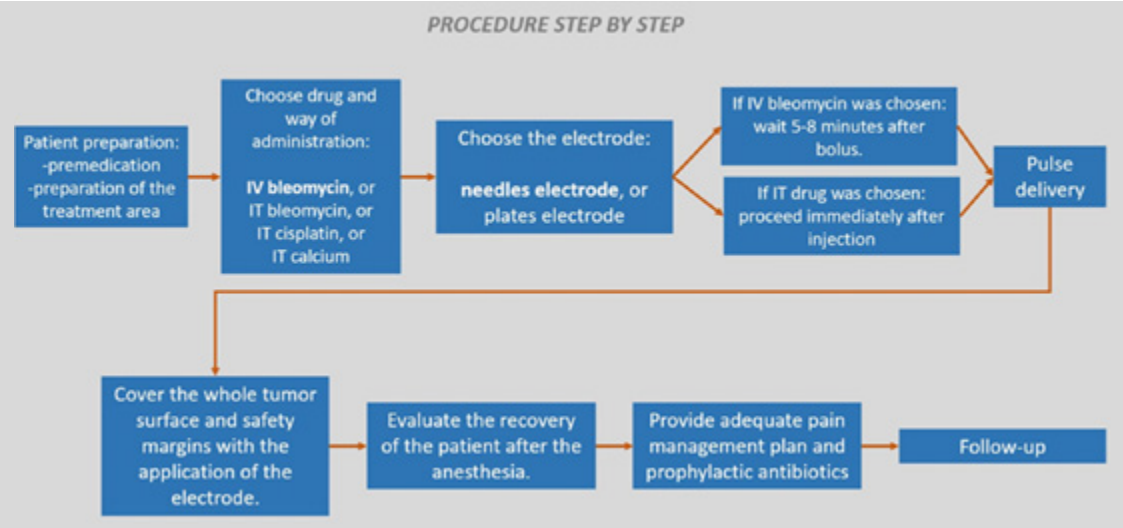

Figure 9. *Electrochemotherapy procedure step by step. In bold, the preferred choices when possible.*

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Actionable Recommendations: Issues Often Observed in the Procedure That Should be Avoided to Improve Results

Related to the Patient

Issue

Sometimes, too much time goes by between the first consultation and the ECT procedure. In very active tumors, this can completely change the status of the patient, and a re-staging may be needed to confirm that ECT is still the best treatment option.

Recommendation

Pre ECT evaluations should be performed a maximum of 4 days before the treatment. If needed, ask for new imaging procedures.

Issue

Like the previous one, if a long time goes by between the first consultation and the ECT, even if the tumor did not grow much, the patient can become seriously deteriorated. This is notorious for tumors that impede appropriate feeding. This situation can increase the anesthetic risk, and the procedure might become impossible.

Recommendation

Make sure that you will provide appropriate clinical support (sufficient hydration, support in feeding, and adequate analgesia) with the aim that the ECT treatment is performed under the best conditions.

In severely deteriorated patients it is possible to treat only half of the lesion. If its evolution is positive in a few days, then treat the second half of the lesion as soon as possible.

Related to the Technique

Issue

The optimal treatment time is up and remains tissue to be treated.

Recommendation

Continue treating all the remaining tissues that should be treated, regardless of the time elapsed. All the lesions treated outside the optimal treatment window should be marked and carefully followed for prompt retreatment in case of regrowth or absence of response. It is advisable to plan the timing of the administration of bleomycin, especially in patients with very extensive lesions, or when the procedure is performed intraoperatively.

When using intratumoral administration of the drug, the injected tumor volume should be treated as quickly as possible. If the tumor is large and the injection lasts more than 2 min [19], deliver the pulses in the injected part and after that, proceed to inject and pulse the other parts.

For more details please refer to section Drug Administration.

Issue

The tumor is thick, and the treatment of the deepest parts cannot be assured.

Recommendation

The electric field drops very quickly outside the region between the needles or plates of the electrodes. For that reason, special considerations must be made when treating the whole tumor volume including its deeper parts. It is very important to cover the whole tumor, especially its bed, to reduce the risk of relapse. When using surface electrodes, the depth of treatment is around 3 mm maximum. For tumors that invade in greater depth, needle electrodes should always be used to guarantee the adequate reach of the electric field to the deeper tissues [79]. If the thickness of the tumor is greater than the needles' length, then proceed with debulking before ECT, as was explained before.

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PEQUEÑOS ANIMALES

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Issue

Inappropriate use of the needle electrodes in rounded or pedunculated lesions.

Mitigation Procedure

It is very important to ensure the treatment of the whole tumor, carefully avoiding overtreatment. It is of utmost importance to cover the base of the lesion with the electric field. In rounded or pedunculated lesions, a common mistake is to insert the needles electrode perpendicularly to the tumor's surface. This approach overtreats the center of the tumor producing irreversible electroporation, and does not cover the tumoral bed. The correct approach is to insert the needles electrode perpendicularly to the surface where the tumor sits. A slight superposition of the application of the electrode is recommended, and large separation or too many superpositions of the electrode's application should be avoided (see **Figure 10**).

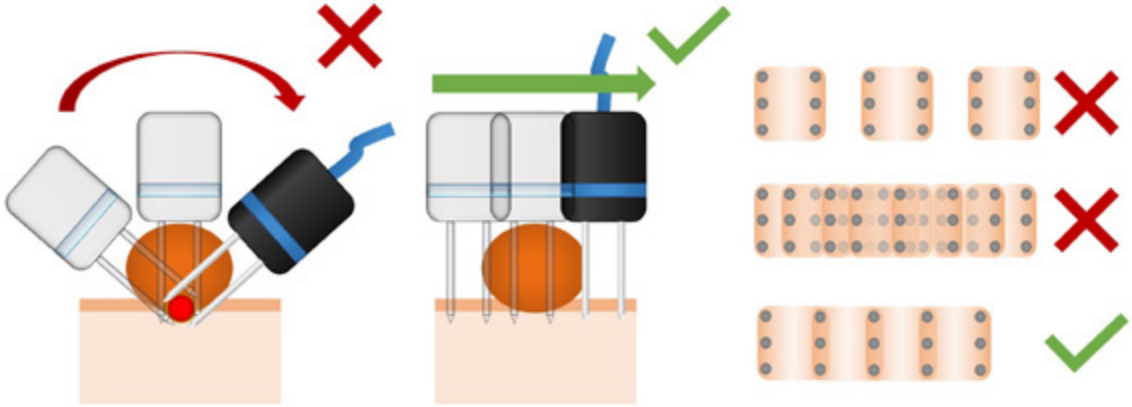

Figure 10. *Dos and don'ts about the technique of electric field delivery using needle electrodes. At left, a common mistake during the treatment of rounded lesions is the application perpendicularly to the surface. In this way, the tumoral bed may not be correctly treated, and the center of the mass may be overtreated. In the middle, the delivery of the pulses perpendicularly to the surface where the tumor lies. This provides adequate treatment of the bed of the lesion. At right, is a scheme of the distance between the applications of a 6-needle electrode. As it can be seen, if they are too separated (up), untreated areas may lead to a relapse. If they are too superposed (middle), overtreatment may produce necrosis and unintended tissue damage. The right distance is a slight superposition of the applications (down).*

Issue

The tumor is surrounding a tooth.

Recommendation

The presence of the tooth between the needles may seriously affect the electric field distribution. The tumor may be treated if it fits between the needles without increasing their separation. But, because the progression of the disease would probably lead to a loosening of the tooth (which will have to be removed later anyway), it is recommended to remove the tooth and treat the area correctly.

Issue

Metallic implants or surgical instruments close to the electrodes can conduct electricity through them and provoke a short circuit and damage the device.

Mitigation Procedure

Particular attention must be brought, to avoid the insertion of a needle in contact or very close to any metallic implant or surgical instrument.

Issue

The presence of blood in the treatment area may increase conductivity and induce an arc or deviate the electric field causing an incomplete/inaccurate treatment of the tumor.

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Recommendation

Adequate hemostasis is essential during the procedure.

Issue

ECT using drugs that are not bleomycin, cisplatin, or calcium.

Recommendation

Use only the proven chemotherapeutic drugs for ECT which are; bleomycin, cisplatin, and calcium, according to the prescriptions recommended in section Drug Administration. There are drugs that, in classical chemotherapy, are effective for specific cancers, but the choice of the appropriate drug for ECT does not depend on the histological type. Most of the commonly used chemotherapy drugs have been tested with little or no benefit by adding the electric pulses [80] because these drugs already penetrate the cells without restrictions, even in the absence of any kind of cell permeabilization.

Related to the Device

Issue

The electroporator is not working properly.

Recommendation

Always deliver a pulse to the air before anesthetizing the patient to ensure the devices' proper functioning. If the problem persists, try powering it off, and back on, checking the correct connection of the electrode and the pedal to the device, and then repeating the test. If the problem persists, contact the manufacturer.

Issue

An electrical power supply breakdown is an unpredicted event that can occur in almost any setting. Most medical-grade devices come with an internal battery that allows them to continue operating. Electroporation devices often do not have batteries and for that reason, they cannot keep working during an electrical power supply breakdown.

Recommendation

A regular uninterrupted power supply (UPS) of 750W can provide enough power to complete 4 or 5 treatment sessions depending on your devices' electrical consumption.

Issue

Many treatment sessions are required to achieve a response and/or the treatment is difficult due to the loss of sharpness of the needles.

Recommendation

In human medicine, disposable electrodes are the only option for the treatment, but in veterinary medicine, it is still possible to use non-disposable electrodes. Needle electrodes may become seriously affected even after a single ECT session. Oxidation of the needles' surface may isolate parts of them, which can greatly affect the electric fields' distribution and thus reduce treatment efficacy. This oxidation may not be seen in plain sight. Use disposable electrodes if possible (see **Figure 11**). If they are not available in your setting, maintenance of the electrodes should be made by sanding the needles before the treatment. This will remove the oxidation and improve electrical conduction.

Figure 11. *Examples of handles with disposable needles. In (**a**) the handle for the disposable shown in (**c**). In (**b**) the handle for the disposable shown in (**d**).*

Keep your electrodes in good condition, make regular evaluations and consider acquiring new ones if they show signs of wear.

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Needles' sharpness can also be affected, and the ulterior use of these needles may produce unintended trauma to the tissue. Already used needles might also tend to excursion inside the tissue, moving away from each other (which can produce an insufficient electric field) or toward each other (which can produce an arc or a short circuit). If needles are bent, correct the bending before inserting them into the tissue.

Issue

The electroporator shows a warning when trying to deliver the pulses.

Recommendation

Some tumors display a very high electrical conductivity, which means that very high electric currents are needed to sustain an adequate electric field. The electroporators can be unable to deliver such high currents. If the device that you are using is warning that the electric field was not achieved correctly, remove the needles half the way out and deliver the pulses. Note that the deepest part of the lesion will not be treated, and a new session has to be scheduled as soon as the superficial part (the treated volume) responds. Refrain from modifying treatment parameters as they can induce necrosis and/or seriously diminish treatment efficiency.

Discussion: The Use of ECT in Veterinary Practice

ECT is a well-established practice in veterinary medicine. It gained status rather quickly because of its high efficiency and negligible side effects, and because it provides adequate treatment when other treatment modalities have failed or are cumbersome.

While ECT is nowadays regularly used in cats, dogs, and equines, patients from a varied spectrum of other species have also been treated successfully, among them: ferrets [81], elephants, fishes, turtles [82,83], hedgehogs [84], snakes [85], birds [86,87], pigs [88], among others.

In human medicine, ECT is a very valuable tool. It started as a palliative treatment, and it began its routine use in 2006, after the ESOPE study [5] and the approval of the medical grade electroporator, the Cliniporator (Igea, Carpi, Italy). Its main indications are cutaneous and subcutaneous tumors of any histology that are not candidates for other treatment modalities. Recently, it can be performed as the first approach upon patient's request, or for the treatment of internal, deep seated tumors [50]. The United States and the rest of the world followed with the development of other generators. In Latin America, its use began in Argentina in 2020 after the approval of another medical grade electroporator, the OncoPore (BIOTEX SRL, Buenos Aires, Argentina). Besides the indications established in the Updated Standard Operating Procedures for ECT published by Gehl et al., intense research is undergoing to extend ECT applications to other organs, such as the liver [12], the brain [89], the pancreas [12], and the bones [12]. An endoscopic electrode, the EndoVe, was developed for the treatment of colorectal cancer [90] and the esophagus [91]. ECT is nowadays a valuable asset for the oncologist. It can be used alone or in combination with other therapies, and provides a new treatment option when others have failed or are not feasible [30].

The authors' personal experience comprises more than 4,000 cases, treated in various animal species, in more than 10 years of practice. That experience also includes the treatment of human patients in the clinical setting, allowing us to be aware of the relevant differences that the treatments have. It also relies on the organization of the Latin American workshops on ECT, the delivery of 9 courses to more than 150 veterinarians from Latin America and Spain, as well as the service to many users through different online platforms to answer their questions (https://vetoncologia.com/ect).

This guide provides updated practical and useful information to the veterinarians. Here we brought detailed information about important aspects that

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should be considered for treating the wide spectrum of patients that encompass veterinary medicine.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics Statement

All regulations from the Consejo Profesional de Médicos Veterinarios (Argentina) were followed. This work was approved by the IACUC of the School of Veterinary Sciences, University of Buenos Aires, Argentina. Protocol Number: 2018/31. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author Contributions

All authors equally contributed to the design, writing, proofreading of the manuscript, and approved the submitted version.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.868989/full#supplementary-material

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Edited by: Carlos Eduardo Fonseca-Alves, Paulista University, Brazil

Reviewed by: Elisabetta Razzuoli, Experimental Zooprophylactic Institute for Piedmont, Liguria and Valle d'Aosta (IZSTO), Italy Zeinab Shankayi, Tarbiat Modares University, Iran

*Correspondence:
Felipe Maglietti,
felipemaglietti@gmail.com

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PEQUEÑOS ANIMALES

Tabla de contenido:

Tres suplementos alimentarios que ayudan a mantener la función cardíaca

La adición de determinados suplementos aminoacídicos y componentes derivados de aminoácidos a la dieta de las mascotas puede ayudar al tratamiento de determinadas patologías. Además, la deficiencia de algunos de ellos puede ser la causa directa de enfermedades del corazón.

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PEQUEÑOS ANIMALES

Taurina

La taurina es uno de los aminoácidos libres más abundantes. Se encuentra en elevadas concentraciones en los tejidos del músculo cardiaco, músculo esquelético, sistema nervioso central y plaquetas. Actúa en numerosos procesos metabólicos, ejerciendo diversas funciones:

- Antioxidación
- Actividad en las células fotorreceptoras de la retina
- Estabilización de las membranas neuronales
- Desarrollo del sistema nervioso
- Reducción de la agregación plaquetaria
- Reproducción
- Actividad miocárdica[1]:
  - Modulación de las concentraciones de calcio en los tejidos y su disponibilidad.
  - Inactivación de los radicales libres y cambio de la osmolaridad celular.
  - Efectos en la osmorregulación del miocardio.
  - Otros mecanismos específicamente relacionados con la función miocárdica incluyen la N-metilación de los fosfolípidos de la membrana celular, efectos directos en las proteínas contráctiles e interacciones con el sistema renina-angiotensina-aldosterona.

¿Qué ocurre en casos de deficiencia de taurina?

En **gatos**, la taurina es un aminoácido esencial y su deficiencia puede causar miocardiopatía dilatada (MCD), degeneración de la retina y anomalías reproductivas. Existen evidencias de que la MCD causada por su deficiencia puede ser reversible con la suplementación de este aminoácido[2,3].

En **perros**, hasta hace unos años, la taurina no se consideraba un aminoácido esencial ni se conocía su papel en el desarrollo de la MCD[4]. Sin

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embargo, diversos estudios han demostrado que sí lo es en perros alimentados con dietas restrictivas en proteína y que, al igual que los gatos, pueden desarrollar MCD secundaria a la deficiencia de taurina[5].

L-carnitina

La L-carnitina es un derivado aminoacídico que se obtiene de la proteína de la dieta o por síntesis endógena en el hígado, siendo la lisina y la metionina los aminoácidos precursores. La síntesis requiere hierro, vitamina C y vitamina B6 como cofactores. El músculo esquelético y el cardiaco son los lugares donde se almacena hasta el 95-98% de la carnitina del cuerpo.

Entre las funciones de la carnitina, la más importante es la de cofactor de algunas enzimas necesarias para el transporte de ácidos grasos de cadena larga al interior de las mitocondrias, donde se oxidan para la generación de energía para el corazón, que obtiene de esta manera aproximadamente el 60% de su producción de energía total.

¿Qué es la miocardiopatía dilatada (MCD)?

Se trata de una enfermedad del corazón muy habitual, progresiva y, en gran medida, irreversible, que puede conducir a fallo cardiaco congestivo o muerte súbita. Es la segunda enfermedad cardiaca más habitual en perros, con una prevalencia superior al 50% en algunas razas10. La nutrición está actualmente aceptada como un importante adyuvante a la terapia médica en perros y gatos con MCD.

¿Qué ocurre en casos de deficiencia de L-carnitina?

La deficiencia de L-carnitina puede ser un trastorno primario o secundario.

- Las deficiencias primarias pueden aparecer por defectos genéticos en la síntesis, transporte, absorción o degradación. En personas se han asociado con cardiomiopatías.
- Las deficiencias secundarias son más comunes en pacientes que siguen dietas restrictivas

Se ha demostrado en perros que la deficiencia de L-carnitina puede favorecer el desarrollo de MCD en perros. Además, varios estudios[6,7,8,9,10] han puesto de manifiesto que suplementar con carnitina mejora el tiempo de supervivencia de perros con MCD.

Hidrolizado de levadura de cerveza

La levadura de cerveza es un subproducto de la industria cervecera que puede ser un ingrediente beneficioso en la alimentación de las mascotas, ya que aporta el contenido nutricional que necesitan los perros y gatos[11]. Esterilizada y sin poder leudante, es una levadura inactiva compuesta por el organismo unicelular Saccharomyces cerevisiae.

Su administración tiene efectos beneficiosos sobre la salud intestinal y la función inmune de los perros, estimulando las respuestas Th1 y, en consecuencia, la inflamación. Además, mejoran la palatabilidad de las dietas[12]. Esto resulta especialmente útil para los perros con poco apetito a consecuencia de una enfermedad crónica.

Es una fuente proteínica rica en aminoácidos esenciales y vitaminas del grupo B:

- Los aminoácidos ayudan a la mascota a construir y mantener sus músculos, huesos, sangre, órganos, sistema inmunitario y pelaje y uñas. En particular, la arginina es un aminoácido esencial que reacciona con el oxígeno para producir óxido nítrico. El óxido nítrico relaja los músculos lisos de los vasos sanguíneos y reduce la presión arterial[13]. La hipertensión puede contribuir a las cardiopatías y a la insuficiencia cardíaca crónica, por lo que es conveniente controlar la tensión arterial de cualquier perro sospechoso de padecer una cardiopatía.
- Las vitaminas del grupo B contribuyen a la función cerebral, la fuerza muscular, la producción de glóbulos rojos y la digestión de los animales.

Por otro lado, se ha demostrado que estimula la producción de determinados marcadores de defensa antioxidantes, lo que ayuda a mejorar la salud cardiovascular de los animales[14].

- A medida que progresa la insuficiencia cardiaca congestiva, aumenta el daño a las células cardiacas por la formación de radicales libres. Los estudios realizados en perros con insuficiencia cardíaca congestiva han demostrado que estos pacientes presentan un aumento de oxidantes reactivos y una disminución de antioxidantes a medida que progresa la enfermedad[15].
- En perros con fallo cardiaco, la oxigenación y el metabolismo celular no funcionan de forma apropiada, lo que conlleva la producción de elevadas cantidades de radicales libres. Los radicales libres son responsables de los principales daños celulares, lo que se denomina estrés oxidativo[16].

Adicionalmente, la levadura de cerveza contiene sodio, calcio, magnesio y potasio. Muchos de los medicamentos utilizados para tratar las cardiopatías disminuyen los niveles sanguíneos de potasio y magnesio.

o Unos niveles inadecuados de potasio y magnesio pueden favorecer las arritmias cardiacas y debilitar las contracciones del músculo cardiaco[17].

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Importancia de una **dieta adecuada** para cachorros con **problemas gastrointestinales**

Agustín Fernández Velasco.
Veterinario.
Comunicación Científica Royal Canin.

Las enfermedades gastrointestinales representan un problema frecuente en el perro especialmente en la etapa de cachorro, donde un 10-25% presenta algún problema digestivo durante el primer año de vida.

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El veterinario con un enfoque de tratamiento multimodal, puede prescribir una dieta como herramienta terapéutica con una estrategia y un perfil nutricional muy específico, abordando la patología y garantizando cubrir las necesidades especiales de crecimiento del cachorro (**Figura 1**).

Muchas de las diarreas crónicas suelen controlarse adecuadamente con tratamiento dietético[1], con posibilidad de evitar problemas asociados al uso prolongado de antibióticos (por ej., alteración del microbioma gastrointestinal) o de fármacos inmunomoduladores (por ej. Alteraciones del sistema inmune y riesgos de infecciones secundarias).

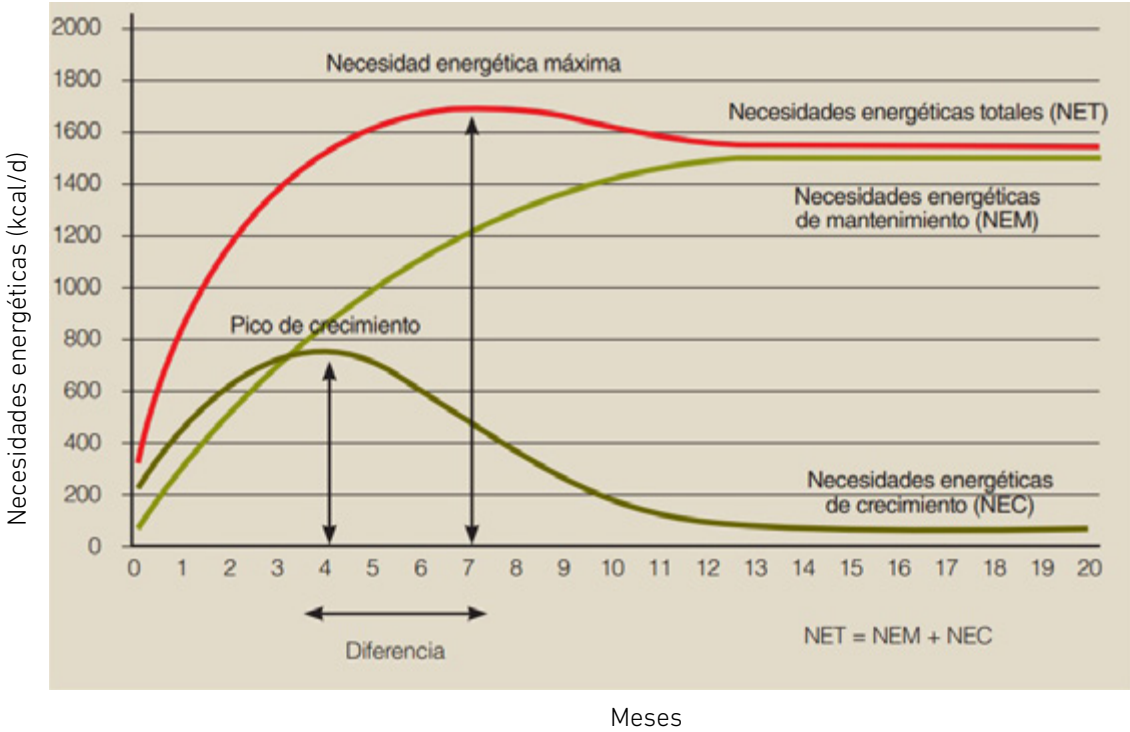

Figura 1. *Necesidades energéticas de los cachorros.*

Perfil nutricional de las dietas gastrointestinales para cachorros

La principal característica que debe tener una dieta GI diseñada para cachorros es su ***alta digestibilidad,*** especialmente cuando tratamos patologías agudas.

El perfil nutricional debe asegurar la inclusión de un porcentaje adecuado de energía, proteína, calcio y fósforo, que garanticen cubrir las necesidades nutricionales en periodos de crecimiento[2,3]. La inclusión de EPA/DHA, prebióticos (FOS, MOS)[4,5,6], antioxidantes (vitamina D, vitamina C, taurina, luteína)[7], betacarotenos[8,9], betaglucanos y arginina también ayudaran en esta etapa de vida.

Deben contener ***proteínas altamente digestibles***[10]. La proteína no digerida promueve el crecimiento de bacterias consideradas perjudiciales y puede aumentar el riesgo de aparición de una alergia inducida por el alimento, sobre todo si la barrera gastrointestinal está dañada. Los restos de proteína no digerida son fermentados por las bacterias del colon, lo que da lugar a productos de putrefacción como bioaminas, mercaptanos, indoles, etc. que causan un olor fuerte en las heces y pueden provocar inflamación, producción de toxinas, así como una reacción de hipersensibilidad. Las proteínas de origen vegetal como, el aislado de proteína de soja, el gluten de maíz y el gluten de trigo son fuentes de proteína purificada de muy **alta digestibilidad**, mayor incluso que las proteínas de origen animal.

También son **energéticamente concentradas** y por lo tanto ***altas en grasa,*** lo que ayuda a reducir el volumen de la ración, los perros y los gatos tienen gran capacidad para digerir las grasas.

Son más digestibles (digestibilidad superior al 90%) que los carbohidratos y que las proteínas, y aportan 2,25 veces las calorías para la misma cantidad, además, las proteínas necesitan más energía para ser utilizadas.

Estas características permiten ofrecer raciones más pequeñas, especialmente interesante en cachorros cuando queremos evitar la sobrecarga intestinal como en el caso de gastritis y enteritis. Reducir la ración e incrementar la frecuencia de las comidas (de 5 a 6 comidas por día)[11] ayuda a restaurar las funciones del intestino, facilita el trabajo de la digestión, favorece una buena absorción de nutrientes y se traduce en una mejor recuperación. Un alto nivel de grasa refuerza ***la palatabilidad del alimento*** y ayuda a estimular el apetito de animales enfermos que a menudo están anoréxicos.

La grasa retrasa el vaciado gástrico y prolonga la digestión, las bacterias pueden metabolizar la grasa no digerida en el intestino y esto puede conducir a una diarrea secretora de intestino grueso. Además, también deconjugan los ácidos biliares alterando la digestión y la absorción de los lípidos. La grasa no se absorbe directamente al torrente sanguíneo, sino que pasa de los enterocitos a la circulación linfática.

Dentro de la fuente de hidratos de carbono ***el arroz*** es ideal para el manejo de estas enfermedades. Tiene una gran digestibilidad por su contenido limitado de amilopeptina y muy bajo de fibra alimentaria; raramente está involucrado en reacciones adversas al alimento; mejora la digestibilidad de las dietas secas y contiene factores solubles que inhiben la diarrea secretora.

Importancia de la textura

La textura de presentación de la dieta jugará un papel primordial a la hora de ofrecerla a los cachorros con objeto de estimular su ingesta y facilitar al máximo su consumo. La posibilidad de ofrecer un ***alimento seco*** que por su diseño de croqueta sea ***fácilmente rehidratable*** (no todas las croquetas lo son) y poder obtener una papilla homogénea, resulta muy atractivo para los cachorros. Al humedecer el alimento con agua caliente se desprenden sustancias volátiles responsables de los aromas que estimulan, especialmente a los animales jóvenes, la ingesta de alimento. La presentación ***húmeda (mousse)*** en pequeñas cantidades y varias tomas también puede estar más indicada cuando se quiere despertar el apetito del cachorro, los alimentos húmedos suelen resultar más atractivos, sobre todo si se calientan hasta alcanzar la temperatura corporal. La combinación de texturas seca y húmeda ***(mixfeeding)*** es una muy buena *herramienta dietética para poder tratar estas patologías.*

En cualquier caso, si no se logra cubrir las necesidades nutricionales de forma voluntaria, hay que recurrir a ***la alimentación enteral*** asistida. En estos casos concretos (por ej. parvovirosis) podremos administrar **por un tiempo limitado**, ***dietas líquidas específicas*** completas y equilibradas para alimentación por sonda. Aportan los niveles nutricionales necesarios, ingredientes altamente digestibles, un alto contenido energético y de grasa y antioxidantes que ayudan a la más rápida recuperación de perros[12,13] que requieran nutrición enteral asistida.

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