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Original research paper

Effect of chemically modified tetracycline-8 (CMT-8) on hematology, blood chemistry, cytokines and peripheral blood lymphocyte subsets of healthy dogs

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Abstract

Tetracyclines are antibiotics widely used in human and veterinary medicine. Effects on the immune system and inflammatory response, including effects on blood leukocytes proliferation and function and in cytokines synthesis, have been described Chemically modified tetracyclines (CMT) have lost their antimicrobial activity, but maintain these other properties. This study analyzes the effect of chemically modified tetracycline-8 (CMT-8) on the evolution of complete blood count, blood chemistry, the mRNA expression of selected cytokines and peripheral blood lymphocyte subpopulations distribution in healthy dogs. CMT-8 at a dose of 10 mg/kg once daily was administered per os to six healthy dogs. A control group of five healthy dogs, living in the same conditions than dogs treated with CMT-8, received placebo with an identical therapeutic regime. When given at the doses used in this study, no side effects of CMT-8 were detected, suggesting a good tolerance and a limited toxicity of the drug. Dogs treated with CMT-8 showed a gradual increase in mean corpuscular hemoglobin. The administration of CMT-8 in healthy dogs did not affect blood mRNA expression of IFN-γ, TNFα, IL-4, IL-6, IL-10, IL-12 p40 and IL-13. However, the lymphocytes expressing class II MHC on their surface decreased during the first two weeks of CMT-8 treatment and subsequently increased for the next three months. Considering the absence of antimicrobial properties of the drug, the effects of CMT-8 detected in this studyseem to be unrelated to the classical antimicrobial activity attributed to tetracyclines.

Keywords: CMT-8; tetracycline; class II MHC; cytokines; flow cytometry

Introduction

Tetracyclines have activity against a wide variety of Gram-positive and Gram-negative, aerobic and anaerobic, intracellular and extracellular bacteria (Papich and Riviere, 2017). Due to their broad-spectrum antimicrobial activity, tetracyclines, and specifically doxycycline, are among the most widely prescribed antibiotics in veterinary medicine (Regula et al., 2009; Sainz et al., 2015; Palma et al., 2020).

Tetracyclines also have different immunomodulatory and/or anti-inflammatory properties, associated with effects on blood leukocyte proliferation and function, and cytokine synthesis (Gabler and Creamer, 1991; Griffin et al., 2010; Kogawa and Salgado, 2012), being capable of exerting an inhibitory effect on matrix metalloproteinases (MMPs), enzymes involved in numerous physiological processes (Lee et al., 2009; Zeng et al., 2011). Specifically, the anti-inflammatory and immunosuppressive effects of different tetracyclines, including CMTs, are thought to be mediated by several mechanisms, including the inhibition of different proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-12 (Cai et al., 2010; Gu et al., 2011; Leite et al., 2011; Ataie-Kachoie et al., 2013; Alyousef et al., 2017), down-regulation of MHC class II expression (Nikodemova et al., 2007), or suppression of T cell proliferation and activation (Kloppenburg et al., 1996; Popovic et al., 2002; Giuliani et al., 2005).

Deregulation of these mechanisms also participate in pathological processes, including periodontal disease and rheumatoid arthritis (Griffin et al., 2010).

Doxycycline has also been recently proposed as an anti-cancer agent in humans and in dogs (De Francesco et al., 2017; Tang et al., 2017; Hume et al., 2018).

Information about the non-antibiotic effects of tetracyclines in dogs is scarce. To the author's knowledge, only a previous study evaluated the effects of doxycycline on hematology, blood chemistry and blood lymphocyte subsets of healthy dogs (Villaescusa et al., 2015). Several studies have supported their therapeutic potential in practice in dogs with osteoarthritis (Nganvongpanit et al., 2009), periodontal disease (Kim et al., 2013), dermatological diseases (Mueller et al., 2003), corneal ulcers (Chandler et al., 2010) or lung injury (Zhang et al., 2014). In fact, doxycycline is reported as the most frequently prescribed antibiotic with no documented evidence of infection (Wayne et al., 2011).

The long-term administration of antimicrobial doses of antibiotics in non-infectious diseases is controversial due to the potential decreased susceptibility of clinically significant bacteria. Categorization of antibiotics in the European Union has been recently updated, being tetracyclines included into the category D ("prudence") (EMA, 2020). The use of subantimicrobial dosage of doxycycline has been proposed in order to decrease the resistance risk (Thomas et al., 2000; Kim et al., 2013), but the selection of resistance at low doses of antibiotics is being recently highlighted (Sandegren, 2014; Raymond, 2019).

Small animal clinical isolates of bacteria do usually express resistance to doxycycline (Kroemer et al., 2014; Thungrat et al., 2015; Ventrella et al., 2017). Specifically, when comparing *Staphylococcus aureus* and *Enterococcus* spp. isolates before and after doxycycline treatment, increased resistance to this drug has been detected (Tejedor-Junco et al., 2018).

An alternate approach is the use of chemically modified tetracyclines (CMTs), developed without antimicrobial properties (Golub et al., 1987; Acharya et al., 2004). CMT-8 was one of the first drugs derived from doxycycline to be developed and seems to be one of the most potent CMTs when inhibiting the secretion of inflammatory mediators, cytokines and MMPs (Uitto et al., 1994; Ramamurthy et al., 2002; Sandler et al., 2005; Salo et al., 2006; Ghangurde et al., 2017).

To the author's knowledge, CMTs have not been evaluated in dogs to date. The aim of this work is to evaluate the safety of CMT-8 administration in healthy dogs and its effects on hematology, blood chemistry, mRNA expression of selected cytokines and peripheral blood lymphocyte subsets.

Materials and Methods

Animals

Eleven healthy Beagle dogs were included in the study and distributed into two different groups: group CMT composed by six dogs treated with CMT-8, and placebo

group with five dogs receiving placebo. Stratified random sampling by sex and age was used for the distribution of dogs in groups. Dogs were housed at the facilities of the Veterinary Medical Teaching Hospital, Complutense University of Madrid. All dogs had to have normal physical examination, cell blood counts and blood biochemistry, and absence of clinical signs or any medication during three months before starting the study. All dogs were living in the same environmental conditions.

The study was approved by the Animal Experimentation Committee of the Complutense University of Madrid (reference number 31/12).

Treatment schedule and sample collection

Dogs included in group CMT received CMT-8 at a dose of 10 mg/kg of body weight, *per os* (p.o.), once a day, for 28 days. CMT-8 was synthesized by removing the dimethylaminogroup from the fourth carbon of doxycycline. The source of CMT-8 was Organistry (Bergondo, Spain).

Dogs included in placebo group received only the excipient (lactose) used in the CMT-8 formulation, with the same therapeutic protocol than in group CMT.

Physical examination was daily performed and samples were taken at days: 0, 15, 30, 60, 90, 120 and 180, considering day 0 the first day of CMT-8/placebo administration.

147 Blood samples were drawn by venipuncture and collected in tubes with EDTA
148 (for hematology, flow cytometry, and cytokine analysis) and heparin (for blood
149 biochemistry). All samples for flow cytometry were processed within 2 h of obtaining
150 the blood. Samples for biochemistry were centrifuged at 1500 g for 10 min before
151 analysis.
152
153 *Hematology and blood chemistry analyses*
154 Hematological parameters were analyzed using the flow cytometer ADVIA 120
155 (Siemens Healthcare Diagnosis, Erlangen, Germany). Blood biochemistry parameters
156 were analyzed using colorimetric enzymatic techniques (Bradford Diagnostics, Sigma–
157 Aldrich, St. Louis, Missouri, USA, and Thermo Scientific, Waltham, USA), and protein
158 concentration was determined by electrophoresis (Hydrasys, Sebia, Paris, France).
159
160 *Cytokine analyses*
161 Interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4),
162 interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 p40 (IL-12 p40), and
163 interleukin-13 (IL-13) were quantified using molecular biology techniques. Quantitative
164 reverse transcription polymerase chain reaction (RT-qPCR) was used to quantify the
165 mRNA expression of cytokines from blood samples immediately added after extraction
166 in special RNA stabilization tubes (500 µl RNAprotect Animal Blood Tubes®, Qiagen,
167 Hilden, Germany), with total RNA extraction performed using the RNeasy protect
168 Animal Blood kit® (Qiagen), following the manufacturer's recommendations.
169
170 Once obtained, the RNA samples were maintained at -80ºC until further
171 processing. The concentration and purity of the extracted RNA were determined using

the ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Retrotranscription through the enzyme reverse transcriptase (RT) was carried out with the Prime Script™ RT Reagent kit (Takara Bio Inc., Kusatsu, Japan) following manufacturer's instructions.

Two cell housekeeping genes, namely GAPDH (glyceraldehyde-3-phospate dehydrogenase) and B2M (β-2-microglobulin), were evaluated as a control. The sequences of the primers that were used in the study are presented in Table 1.

qPCR reactions were performed in duplicate on a plate in a total volume of 20 µl, with 1µl cDNA, using SYBR-Green PCR Master Mix 2X (Applied Biosystems, Foster City, USA). A reaction with RNA instead of cDNA was prepared as a negative control for each sample in the same conditions. A further negative control with water only was also included on each plate. The plates were sealed with an optical foil and read in the ABI PRISM 7900HT Fast Real-Time PCR system (Applied Biosystems). The PCR protocol consisted of a sample incubation at 95ºC for 15 min, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 min, and final dissociation at 95ºC for 15 seconds, 60ºC for 1 min and 95ºC for 15 seconds.

The efficiency of each primer pair was evaluated by serial dilution of cDNA according to the protocol developed by PE Applied Biosystems. The amount of target RNA was standardized to that of the GAPDH and B2M control genes and expressed according to the 2-ΔCt method. The results obtained were analyzed with the SDS 2.1 software associated to the ABI system (Applied Biosystems).

197 *Flow cytometry analyses*

198 A multiparametric flow cytometric study was performed to analyze the
199 distribution of different lymphocyte subsets in each sample, following methodology
200 previously described (Villaescusa et al., 2015). Briefly, monoclonal antibodies to canine
201 lymphocyte cell surface antigens were obtained from AbD Serotec (Oxford, UK) and
202 included: anti-canine CD3 (clone CA17.2A12) conjugated with fluorescein
203 isothiocyanate (FITC), anti-canine CD4 (clone YKIX302.9) conjugated with
204 phycoerythrin (PE), anti-canine CD8 (clone YCATE55.9) conjugated with AlexaFluor
205 647, anti-canine CD21 (clone CA2.1D6) conjugated with PE and anti-canine MHC
206 class II (clone YKIX334.2) conjugated with FITC. The use of this panel of antibodies,
207 exposing 100-µL aliquots of whole blood in EDTA for every dog to three combinations
208 of monoclonal antibodies, allowed the characterization of different lymphocyte
209 populations: T (CD3+ lymphocytes); T helper or Th (CD3+CD4+ lymphocytes); T
210 cytotoxic or Tc (CD3+CD8+ lymphocytes); B (CD21+ lymphocytes); CD3-CD21-
211 lymphocytes; and those lymphocytes that express MHC class II (MHCII+
212 lymphocytes). Ratio CD4/CD8 was calculated based on the previous analyses of both
213 populations. Absolute values for each subset were calculated using white blood cell
214 counts combined with flow cytometry analysis.

215

216 *Statistical analysis*

217 Normality of distribution was confirmed using Kolmogorov-Smirnov tests. Chi
218 square test and T test using Levene's test to assess the homogeneity of variance were
219 used to evaluate a correct distribution of dogs in groups by sex and age, respectively.

Comparison of the evolution of each parameter throughout the study period was analyzed using a two-ways ANOVA for repeated measurements. The level of statistical significance was established at $p < 0.05$, using the Bonferroni correction, when appropriate. The results were statistically analyzed using the software SAS, version 9.4 (SAS Institute, Cary, USA).

Results

Distribution of dogs in groups

Four males and two females were treated with CMT-8, and three males and two females were treated with placebo. Distribution of dogs in groups by sex was not statistically significant (p=0.514). Age of dogs treated with CMT-8 and placebo (4.67±1.63 and 6.40±2.19, respectively) was not different (p=0,266). Average weight at day 0 was similar in both groups (10.67±0.73 and 10.41±1.09, respectively; p=0,446).

Clinical findings and hematology parameters

No clinical signs were detected in any of the dogs in both groups throughout the study. Systematic physical examinations of all dogs were normal during the study. All the hematology parameters of dogs treated with CMT-8 were within the reference values.

Hematology results at the different follow-ups are shown in Table 2. No significant differences were found between groups at day 0 for all the hematological parameters.

Regarding red blood cell parameters, mean corpuscular hemoglobin showed a significantly different evolution during the study time between dogs that received CMT-8 and dogs that received placebo, based on the interaction between time and treatment. Specifically, dogs treated with CMT-8 showed a progressive increase in mean corpuscular hemoglobin during the treatment and a subsequent decrease when stopping the therapy, while this parameter did not change in dogs treated with placebo. Other red blood cell parameters such as mean corpuscular volume and mean corpuscular hemoglobin concentration significantly changed during the different follow-ups in both groups of dogs, but without statistical significant differences between treatment groups. Specifically, mean corpuscular volume increased at the end of the study, while mean corpuscular hemoglobin concentration decreased.

With regard to white blood cell parameters, basophil counts showed a significantly different evolution between groups when analyzing the interaction between time and treatment. Specifically, basophil counts decreased until day 60 in dogs treated with CMT-8, while this decrease was only significant at the end of the therapy in the placebo group. White blood cell, neutrophil, lymphocyte and monocyte absolute counts significantly changed homogeneously during the different follow-ups in both groups of dogs due to a time effect, but without statistical differences between treatment groups. A progressive decrease throughout the study was observed in white blood, neutrophil and monocyte counts. Oppositely, an increase in lymphocytes was also detected during the study.

The rest of hematological parameters, including platelet counts, did not change in none of the groups.

Biochemistry and serum protein electrophoresis

All the biochemistry and serum protein electrophoresis parameters of dogs treated with CMT-8 were within the reference values. No significant differences were found between groups at day 0 for all the biochemistry and serum protein electrophoresis parameters, with the exception of glucose (p=0.033).

For all biochemical parameters (Table 3), a time effect similar in both groups (no interaction) has been found, but no effect of the treatment has been detected. When analyzing the time effect, glucose increased and creatinine and γ-globulins progressively decreased during the therapy in both groups of dogs, showing an analogous pattern. Similarly, the concentration of proteins, albumin, globulins and, specifically, α 2- and β - globulins, showed a significant increase after therapy, especially until day 60, and a subsequent decrease, with the exception of α2-globulins that maintained high values until the end of the study. γ-globulins decreased until day 30, while the albumin/globulin ratio increased conversely in both groups.

No changes were detected in BUN and ALT during the study neither in dogs treated with CMT-8 nor in dogs included in placebo group.

Cytokines and peripheral blood lymphocyte subsets distribution

The expression of cytokines did not show significant differences among the different follow-ups (data not shown) in dogs treated with CMT-8 and placebo.

Regarding peripheral blood lymphocyte subsets distribution, results are shown in Table 4. No significant differences were found between groups at day 0 for all the peripheral blood lymphocyte subsets. When analyzing the interaction of time and treatment, dogs treated with CMT-8 had a decrease of MHC class II + lymphocytes at day 15 and a subsequent progressive increase of this population. In contrast, dogs included in placebo group did not suffer any significant modification in this subset throughout the study (Fig. 1).

Other parameters changed homogeneously with time, independently on the treatment. Specifically, time effect was related to a progressive increase of the count of Th CD3+CD4+ lymphocytes in dogs treated with CMT-8 and placebo, showing a similar pattern in both canine populations. At the same time, a decrease in the values of CD3-CD21- lymphocytes was also detected in dogs from both groups.

The counts of T CD3+, Tc CD3+CD8+, and B CD21+ lymphocytes, and the CD4/CD8 ratio were not modified neither in CMT group nor in placebo group during the study.

Discussion

The effects of doxycycline on hematology, blood chemistry, cytokines and peripheral blood lymphocyte subsets have been previously evaluated in dogs using a

313 similar therapeutic regimen to the used in this study (Villaescusa et al., 2015). This
314 regimen (10 mg/kg, p.o., once a day, for 28 days) is usually recommended when
315 treating ehrlichial diseases in dogs (Sainz et al., 2015). The regimen of CMT-8 was
316 chosen for this initial study in order to compare the effects of CMT-8 with the
317 previously obtained using doxycycline. Furthermore, CMT's have been previously
318 administered at the same dose than other tetracyclines in different mammalians, using
319 different routes of administration (Golub et al., 1987; Curci et al., 1998; De Bri et al.,
320 1998; Ramamurthy et al., 2002). When evaluating specifically non-antibiotic effects of
321 these drugs, studies in vitro suggest that both doxycycline and CMT's inhibit in a dose-
322 dependent manner both collagenolytic and gelatinolytic activities (Curci et al., 1998;
323 Raulo et al., 2006). In fact, the relative efficacy of CMT-8 seems to be 105% compared
324 with doxycycline, without significant differences when administered at the same dose
325 (Curci et al., 1998).

326 Side effects after the administration of CMT-8 were not detected in the dogs
327 included in this study. CMT administration has not been previously evaluated in dogs,
328 but other tetracyclines are currently used in veterinary medicine, and their side effects
329 have been widely described (Schulz et al., 2011). In human medicine, toxicity
330 associated to CMT therapy, especially photosensitivity and rash, has been described
331 (Acharya et al., 2004; Dezube et al., 2006; Richards et al., 2011). Photosensitivity and
332 other side effects were not detected in any of the dogs included in the study, suggesting
333 a good tolerance of CMT-8 when given at the doses used in this work.

334

335 Regarding the potential effects on hematology derived of CMT's administration,
336 anemia has been infrequently described in people treated with CMT-3 (Acharya et al.,

2004). Dogs treated with CMT-8 did not suffer anemia, but mean corpuscular hemoglobin decreased from the end of the therapy until day 60. Similar effects have been described when using doxycycline in dogs (Villaescusa et al., 2015), suggesting a potential effect of tetracyclines on erythrocytic mean corpuscular hemoglobin that should be thoroughly evaluated.

Administration of CMT-8 was associated with a decrease in the basophil counts. The lower reference interval is typically zero in most laboratories, and decreased concentrations of blood basophils are not well recognized in domestic animals, precluding detection of basopenia (Boes and Durham, 2017). The significance of decreased or increased concentrations of blood basophils has been involved in the immune system modulation, taking into account the role of basophils as regulatory cells in immunity (Chirumbolo et al., 2018). Mast cells and basophils can express IL-4, CD40 ligand and low levels of MHC class II (Voehringer, 2013), and CMTs have proven to induce apoptosis in mast cells (Sandler et al., 2005). However, some automated hematology analyzers have been demonstrated to be inaccurate for basophil counts in dogs and cats (Lilliehöök and Tvedten, 2011). Based on that, further studies using other techniques of basophil counts are required to properly confirm these results.

A time effect has been found when analyzing different hematology and blood chemistry parameters in this study, independently on the treatment. Previous reports have found a large variability for several routine parameters in clinically healthy adult small-sized dogs (Misbach et al., 2014). When evaluating homogeneous population of

Beagle dogs, even when inter- and intra-individual variability is low for different hematology analytes, intra-individual variability can be, at least, moderate for WBC and differential counts (Bourgès-Abella et al., 2015). These variations, along with the limited sample size of our study, could explain some of the differences found in the placebo group over time. However, it should be noted that all the parameters were within the reference interval.

A decrease in creatinine when using doxycycline in dogs has been previously described (Villaescusa et al., 2015), suggesting a potential effect of tetracyclines on nephrons. The inhibition of different MMP activities and serine proteases in the kidney tissues has been associated to the effect of doxycycline on the reduction of the oxidative injury and the promotion of renal repair in different diseases, and, as a consequence, on the reduction in serum creatinine (Ihtiyar et al., 2009; Kucuk et al., 2009; Cortes et al., 2018; Nakagawa et al., 2018). A similar decrease has also been detected in dogs treated with CMT-8 in the current study. However, these findings should be cautiously interpreted due to the detection of a similar evolution in dogs treated with placebo, which is related with a time effect more than with a treatment effect Furthermore, all the creatinine data were included into the reference interval in all dogs. However, further studies are required in order to evaluate renal function in a larger sample of dogs treated with this drug.

Electrophoretic protein profiles changed with time in both groups, showing an increase in alpha-2 globulins and a decrease in γ-globulins. Even when these changes

cannot be attributable to the treatment, similar findings have also been described in dogs treated with doxycycline (Villaescusa et al., 2015). α-2 globulins include different proteins, among them, α-2 macroglobulin, that inhibits serine proteases (Rehman et al., 2013) and is considered a major endogenous inhibitor of MMPs (Kucukguven and Khalil, 2013). The evaluation of the different proteins included into the α-2 globulin group would be useful to confirm the specific effects of CMT-8 on α-2 macroglobulin in dogs.

Non-antimicrobial tetracyclines are effective decreasing inflammatory mediators, including pro-inflammatory cytokines and MMPs in humans (Griffin et al., 2010; Tilakaratne and Soory, 2014; Golub and Lee, 2020). The evaluation of cytokines can be useful to estimate these effects based on these previous reports. However, in our study, administration of CMT-8 in healthy dogs apparently does not change the mRNA expression of cytokines in blood cells. These differences can be due to the fact that most studies have evaluated the cytokine profiles in states of disease where pro-inflammatory cytokines are usually increased, while our results have been found in healthy dogs. Furthermore, discordance between serum and tissue concentrations of some cytokines has been described (Fell et al., 2013; Zekeridou et al., 2019). Further studies are required in order to evaluate the potential effect of CMT's on cytokines in dogs with different inflammatory diseases, using both blood and tissue samples. Specifically, it should be interesting the evaluation of this drug in dogs with diseases that could be immunomediated and/or influenced by MMP activity, and where tetracyclines appear to be effective, such as osteoarthritis (Nganvongpanit et al., 2009), periodontal disease

406 (Kim et al., 2013), dermatological diseases (Mueller et al., 2003), corneal ulcers
407 (Chandler et al., 2010) or lung injury (Zhang et al., 2014).

408

409 Regarding the effects of CMT-8 on peripheral blood lymphocyte subsets, its
410 administration to dogs produces a decrease of MHC class II + lymphocytes. Similar
411 results have been found in dogs treated with doxycycline (Villaescusa et al., 2015),
412 suggesting an effect of tetracyclines on MHC expression on the surface of peripheral
413 blood lymphocytes that could partially explain the anti-inflammatory effects of these
414 drugs. Expression of MHCII is a characteristic of antigen presenting cells, including
415 monocytes, macrophages, dendritic cells, B-lymphocytes and activated T-cells
416 (Abcouwer et al., 2013). An experimental study using a model of retinal
417 neurodegeneration showed that minocycline inhibits the accumulation of MHCII+
418 subpopulations, suggesting that it acts to preferentially block the accumulation of
419 inflammatory leukocytes, and it may also produce a shift in MHCII expression
420 (Abcouwer et al., 2013). Concordantly, a down-regulation of MHC class II expression
421 in microglia and macrophages after minocycline administration has been described in
422 experimental models of allergic encephalomyelitis and chronic neuropathic pain
423 (Nikodemova et al., 2007; Li et al., 2016). Similar results have also been found in
424 models of burn injury (Cromer et al., 2018).

425

426 The immunosuppressive activity of minocycline has been found to be mediated
427 by the induction of regulatory dendritic cells (Kim et al., 2016). The suppressed antigen
428 presenting cell function linked to the administration of these drugs may be due to the

decreased expression of MHC class II and co-stimulatory molecules on the cell surface (Kim et al., 2016). In fact, these drugs could be potentially used to increase the production of regulatory dendritic cells for cell therapy to treat autoimmune disorders, allergy, and transplant rejection (Kim et al, 2016). Taking into account the similar results of MHC class II lymphocytes found in our work, further studies are required in order to evaluate if CMT-8 can have similar effects in dogs with analogous diseases.

An initial increase in B lymphocytes (CD21+) followed by a decline has been described in dogs treated with doxycycline (Villaescusa et al., 2015). The behaviour of different lymphocyte subsets when treating dogs with CMT-8 was not similar to the previously described with doxycycline. These differences could also be due to individual variations, as previously described (Faldyna et al., 2001; Miranda et al., 2007). Variations over time of lymphocyte subpopulations have been described in dogs (Villaescusa et al., 2012), so the inclusion of a control group in this type of studies is considered essential.

Changes in biomarkers when using tetracyclines could be due to direct effects of the drug or to antimicrobial effects of doxycycline on the normal microflora (Villaescusa et al., 2015), and could justify potential differences when comparing with CMT. Interestingly, the effects of CMTs can be more complex than initially expected and can reach the microbiota in a different way than antibiotics do. Recent studies have suggested that CMT-3 can beneficially restore certain gut microbial communities and

451 can attenuate pathological alterations in gut wall, through their impact on microglial
452 cells (Sharma et al., 2019).

453

454 **Conclusions**

455 To the best of the author's knowledge, this is the first study that evaluates the
456 administration of a CMT in healthy dogs. CMT-8 can have direct immunomodulatory
457 and/or anti-inflammatory effects that could be of interest for the treatment of different
458 diseases in dogs. Further studies employing CMTs in dogs suffering specific
459 inflammatory diseases could aid to clarify the potential benefits of these drugs in
460 practice.

461

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467

468 **Disclosure statement**

469 The authors report no other conflicts of interest. The authors alone are
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471

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Figure legends

Fig. 1. Evolution of MHC class II + lymphocytes/μl (mean value ± SD) in dogs treated with CMT-8 and placebo during the follow-up period of study.

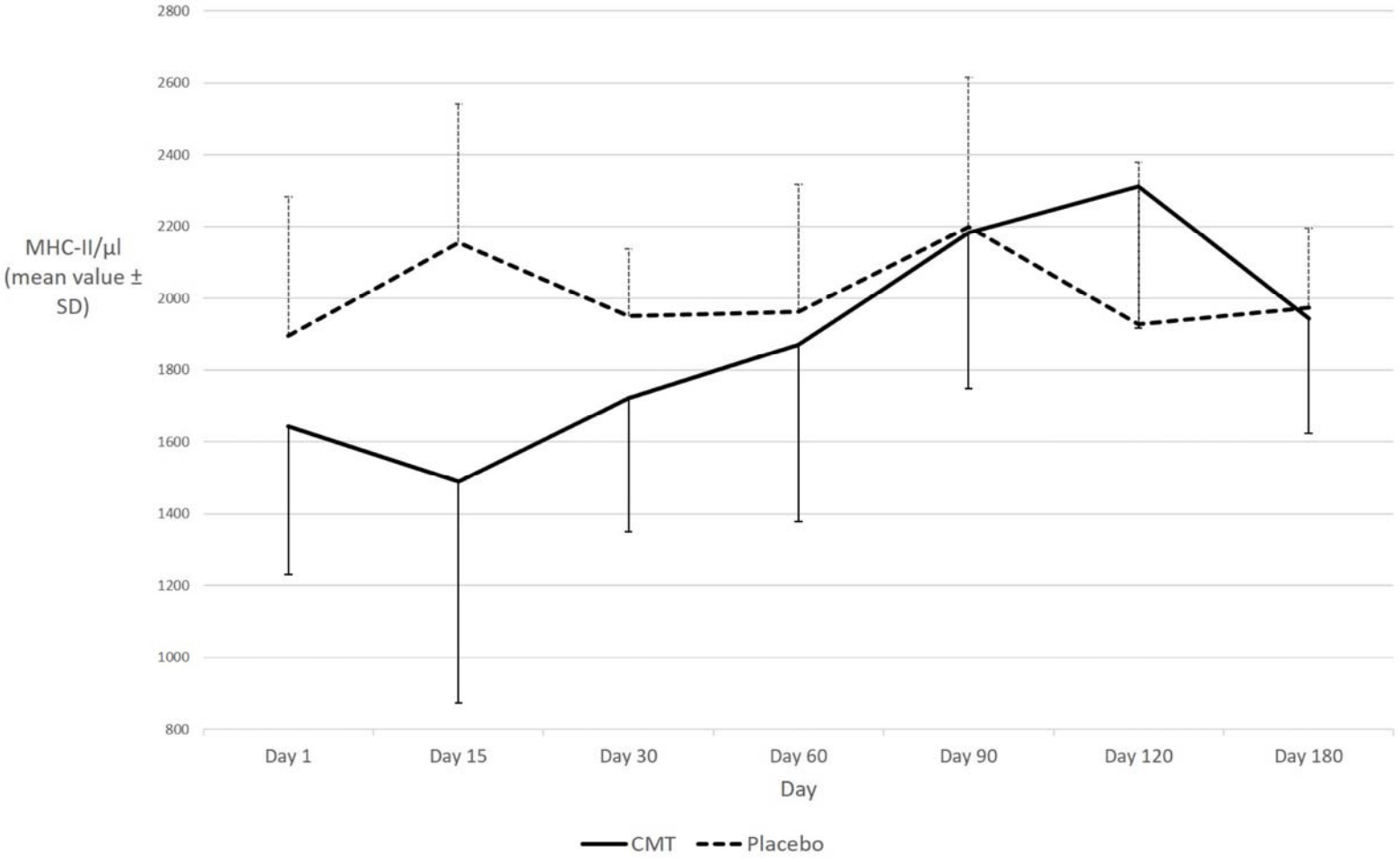
MHC-II/µl (mean value ± SD)
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
Day 1
Day 15
Day 30
Day 60
Day 90
Day 120
Day 180
Day
CMT
Placebo

Table 1

Characteristics of the primers used for the qPCR.

Target gene	GenBank accession number	Primer sequence (5'-3')	Product length (bp)
GAPDH[a]	XM_003435649.4	Forward ACTTTGTCAAGCTCATTTCC Reverse CATGTGGACCATGAGGTCC	77
B2M[b]	XM_003640047.2	Forward TCCTCATCCTCCTCGCT Reverse TTCTCTGCTGGGTGTCG	85
IFN-γ[c]	AF126247	Forward GCATTCCAGTTGCTGCCTACT Reverse ACCAGGCATGAGAAGAAATGC	138
TNF-α[d]	NM_001003244.4	Forward CCAAACCGACCCTTTGATCA Reverse CCAGCCCTGAGCCCTTAATT	83
IL-4 [c]	AF239917	Forward TAGCACTCACCAGCACCTTTGT Reverse CTTGACAGTCAGCTCCATGCA	118
IL-6[d]	NM_001003301.1	Forward CCCACCAGGAACGAAAGAGA Reverse CTTGTGGAGAGGGAGTTCATAGC	68
IL-10[e]	U33843	Forward ACCACGACCCAGACATCAAGA Reverse CCTGGAGCTTACTAAATGCGCT	153
IL-12p40[f]	XM_014112766.2	Forward GGACGTTTCACATGCTGGT Reverse CCACTCTGACCCTCTCTGCT	100-150

a Predesigned SYBR Green primer, Sigma Aldrich
b Piek et al., 2011
c Kobayashi et al., 2007
d Song et al., 2012
e Alves et al., 2009
f Veenhof et al., 2010

Table 2

Evolution of the hematological parameters in dogs treated with CMT-8 (CMT) and with placebo during the follow-up period of the study (mean ± SD).

		Follow-up time points									
		Day 0	Day 15	Day 30	Day 60	Day 90	Day 120	Day 180	Treatment (*p* value) [a]	Time (*p* value) [b]	Interaction (*p* value) [c]
Red blood cell count (x10^6/µl)	CMT	7.0 ± 0.8	6.7 ± 0.8	6.5 ± 1.0	7.1 ± 0.8	7.3 ± 0.7	6.7 ± 0.7	6.9 ± 0.5	0.599	0.387	0.261
	Placebo	7.1 ± 0.3	7.2 ± 0.3	7.2 ± 0.6	6.8 ± 0.5	7.2 ± 0.5	6.9 ± 0.5	7.1 ± 0.9			
Hemoglobin (g/dl)	CMT	16.1 ± 1.9	15.4 ± 1.7	15.4 ± 2.1	16.0 ± 1.7	16.5 ± 1.6	15.7 ± 1.5	16.2 ± 1.3	0.698	0.485	0.411
	Placebo	16.1 ± 1.1	16.3 ± 1.1	16.5 ± 1.3	15.6 ± 1.4	16.5 ± 1.0	16.2 ± 1.6	16.3 ± 1.8			
Hematocrit (%)	CMT	46.7 ± 6.3	44.8 ± 5.7	44.3 ± 6.5	47.1 ± 5.2	47.6 ± 4.4	45.6 ± 4.8	48.5 ± 3.8	0.584	0.356	0.382
	Placebo	47.2 ± 3.1	48.3 ± 2.5	48.3 ± 3.8	45.9 ± 3.5	48.0 ± 3.3	47.2 ± 4.7	49.2 ± 5.8			
Mean corpuscular volume (fl)	CMT	66.7 ± 1.6*	66.7 ± 0.9	68.1 ± 1.2	66.8 ± 1.8	65.4 ± 1.5*	68.0 ± 1.8	70.1 ± 1.3*	0.909	<0.0001	0.206
	Placebo	66.5 ± 1.9†	66.6 ± 1.4	67.5 ± 1.6	67.5 ± 0.8	66.9 ± 1.2†	68.0 ± 2.1	69.6 ± 1.1†			
Mean corpuscular hemoglobin (pg)	CMT	23.0 ± 0.4	23.0 ± 0.7	23.6 ± 0.7*	22.7 ± 0.7*	22.7 ± 0.6	23.4 ± 0.8	23.5 ± 0.5	0.017	0.0003	0.003
	Placebo	22.7 ± 0.8	22.5 ± 0.7	23.1 ± 0.6	22.9 ± 0.4	23.0 ± 0.7	23.3 ± 0.5	23.1 ± 0.6		0.083	
Mean corpuscular hemoglobin concentration (g/dl)	CMT	34.5 ± 0.9	34.5 ± 1.0	34.7 ± 0.5*	34.0 ± 0.8	34.6 ± 0.6*	34.4 ± 0.7	33.4 ± 0.5*	0.494	0.016	0.324
	Placebo	34.1 ± 0.2	33.8 ± 0.5	34.2 ± 0.3†	34.0 ± 0.6	34.4 ± 0.6†	35.1 ± 2.1	33.2 ± 0.4†			
Platelet count (x10^3/µl)	CMT	355 ± 105	345 ± 98	326 ± 96	289 ± 57	277 ± 53	299 ± 99	286 ± 42	0.965	0.196	0.445

	Placebo	352 ± 115	291 ± 84	306 ± 91	315 ± 126	302 ± 81	274 ± 92	322 ± 162			
White blood cell count (x10^3/µl)	CMT	11.1 ± 4.1*	6.4 ± 2.9*	8.0 ± 1.0	7.8 ± 1.2*	7.5 ± 1.2	8.7 ± 2.3	7.6 ± 1.4*	0.159	0.0003	0.461
	Placebo	13.4 ± 2.3†	8.5 ± 2.8†	9.0 ± 3.0	9.5 ± 1.1†	8.3 ± 1.5	7.9 ± 1.3	8.2 ± 0.9†			
Neutrophils/µl	CMT	8394 ± 3540*	4192 ± 1324*	5251 ± 1109	4877 ± 664	4399 ± 564*	5416 ± 2142	4761 ± 1083*	0.143	0.0002	0.691
	Placebo	10035 ± 2422†	5670 ± 2457†	5971 ± 2789	6266 ± 797	5045 ± 1020†	5024 ± 664	5316 ± 609†			
Lymphocytes/µl	CMT	1940 ± 583	1602 ± 636*	1926 ± 390	2074 ± 541	2395 ± 491*	2466 ± 369*	2136 ± 344	0.643	0.019	0.098
	Placebo	2257 ± 376	2038 ± 248†	2143 ± 163	2171 ± 347	2388 ± 454†	2043 ± 486†	2158 ± 187			
Monocytes/µl	CMT	509 ± 254*	297 ± 90	338 ± 55	305 ± 63*	324 ± 65*	371 ± 135	234 ± 40*	0.117	<0.0001	0.639
	Placebo	713 ± 214†	453 ± 248	449 ± 244	432 ± 108†	401 ± 145†	410 ± 116	350 ± 144†			
Eosinophils/µl	CMT	239 ± 152	307 ± 181	480 ± 185	471 ± 201	334 ± 132	400 ± 159	420 ± 201	0.985	0.088	0.256
	Placebo	389 ± 302	277 ± 93	349 ± 138	565 ± 302	425 ± 137	350 ± 170	308 ± 113			
Basophils/µl	CMT	27.69 ± 5.81*	13.30 ± 8.01*	17.58 ± 7.23	15.69 ± 7.47*	20.09 ± 7.28	21.59 ± 6.07	20.36 ± 7.62	0.009	0.004	0.012
	Placebo	34.06 ± 8.77†	26.57 ± 9.13	25.96 ± 10.14†	20.86 ± 4.81	26.56 ± 8.39	24.04 ± 8.36	25.85 ± 9.28		0.007	

[a] *p* value comparing the evolution of the corresponding parameter evolution between dogs treated with CMT-8 (CMT) and with placebo
[b] *p* value comparing the corresponding parameter value between the different follow-up time points
[c] *p* value evaluating the interaction between treatment and time
*Significant difference versus *group
†Significant difference versus †group

Table 3

Evolution of the blood chemistry and serum protein electrophoresis parameters in dogs treated with CMT-8 (CMT) and with placebo during the follow-up period of the study (mean ± SD).

		Follow-up time points							Treatment (*p* value) [a]	Time (*p* value) [b]	Interaction (*p* value) [c]
		Day 0	Day 15	Day 30	Day 60	Day 90	Day 180	Day 180			
Glucose (mg/dl)	CMT	86.7 ± 13.6*	78.0 ± 8.6	91.8 ± 11.8*	83.7 ± 9.5	109.3 ± 8.9*	87.2 ± 12.5	98.2 ± 8.8*	0.051	0.0008	0.382
	Placebo	68.0 ± 10.4†	69.6 ± 6.0	85.4 ± 7.3†	73.8 ± 6.8	98.2 ± 6.0†	66.0 ± 34.7	93.2 ± 7.5†			
BUN (mg/dl)	CMT	10.0 ± 3.5	11.1 ± 2.6	12.2 ± 3.9	11.9 ± 2.9	10.9 ± 2.0	11.5 ± 3.6	13.6 ± 5.2	0.626	0.335	0.418
	Placebo	14.5 ± 5.7	12.3 ± 3.9	11.7 ± 5.3	12.3 ± 3.6	11.1 ± 3.8	11.2 ± 2.0	14.2 ± 3.1			
Creatinine (mg/dl)	CMT	0.68 ± 0.1	0.73 ± 0.1*	0.63 ± 0.1*	0.75 ± 0.1	0.72 ± 0.1	0.77 ± 0.1	0.78 ± 0.1*	0.578	0.001	0.449
	Placebo	0.70 ± 0.1	0.74 ± 0.1†	0.64 ± 0.1†	0.70 ± 0.1	0.64 ± 0.1	0.76 ± 0.1	0.76 ± 0.1†			
ALT (IU/l)	CMT	69.5 ± 22.5	63.0 ± 35.7	64.2 ± 30.9	46.7 ± 6.0	46.5 ± 4.0	58.8 ± 17.0	47.5 ± 6.4	0.182	0.244	0.485
	Placebo	46.6 ± 25.3	45.6 ± 26.2	41.6 ± 20.0	35.8 ± 11.3	43.6 ± 13.8	55.0 ± 26.2	47.6 ± 11.9			
Total protein (g/dl)	CMT	6.53 ± 0.7	6.33 ± 0.3	6.27 ± 0.3*	6.92 ± 0.4*	6.25 ± 0.3*	6.28 ± 0.5	6.23 ± 0.5	0.398	<0.0001	0.542
	Placebo	6.88 ± 0.4	6.56 ± 0.2	6.46 ± 0.3†	7.06 ± 0.3†	6.28 ± 0.3†	6.40 ± 0.4	6.54 ± 0.3			
Albumin (g/dl)	CMT	3.19 ± 0.5	3.09 ± 0.3*	3.24 ± 0.3	3.45 ± 0.3*	3.03 ± 0.3*	3.14 ± 0.5	3.09 ± 0.4*	0.969	0.001	0.940
	Placebo	3.18 ± 0.6	3.13 ± 0.4†	3.25 ± 0.4	3.36 ± 0.4†	2.99 ± 0.3†	3.15 ± 0.4	3.12 ± 0.2†			

Globulins (g/dl)	CMT	3.34 ± 0.5	3.24 ± 0.2	3.03 ± 0.2*	3.46 ± 0.3*	3.22 ± 0.2	3.14 ± 0.2*	3.14 ± 0.3	0.283	<0.0001	0.443
	Placebo	3.70 ± 0.4	3.43 ± 0.4	3.21 ± 0.3†	3.70 ± 0.3†	3.29 ± 0.2	3.25 ± 0.4†	3.41 ± 0.4			
α1-globulins (g/dl)	CMT	0.27 ± 0.1*	0.27 ± 0.1	0.28 ± 0.1	0.29 ± 0.1	0.25 ± 0.1*	0.26 ± 0.1	0.26 ± 0.1	0.576	0.0002	0.498
	Placebo	0.29 ± 0.1†	0.26 ± 0.1	0.28 ± 0.1	0.31 ± 0.1	0.25 ± 0.1†	0.27 ± 0.1	0.28 ± 0.1			
α2-globulins (g/dl)	CMT	0.92 ± 0.3	0.91 ± 0.1	0.84 ± 0.1*	0.97 ± 0.2*	0.93 ± 0.1	0.96 ± 0.2	0.95 ± 0.2*	0.160	0.001	0.298
	Placebo	1.16 ± 0.3	1.11 ± 0.2	0.96 ± 0.2†	1.15 ± 0.2†	0.99 ± 0.1	1.10 ± 0.2	1.14 ± 0.2†			
β-globulins (g/dl)	CMT	1.67 ± 0.2	1.58 ± 0.2	1.51 ± 0.2*	1.71 ± 0.2*	1.60 ± 0.1	1.48 ± 0.1*	1.46 ± 0.2	0.408	<0.0001	0.540
	Placebo	1.79 ± 0.2	1.66 ± 0.2	1.59 ± 0.2†	1.81 ± 0.2†	1.65 ± 0.2	1.49 ± 0.1†	1.60 ± 0.2			
γ-globulins (g/dl)	CMT	0.48 ± 0.1*	0.49 ± 0.1	0.39 ± 0.1*	0.49 ± 0.1	0.44 ± 0.1	0.43 ± 0.1	0.47 ± 0.1	0.384	0.001	0.601
	Placebo	0.46 ± 0.1†	0.41 ± 0.1	0.37 ± 0.1†	0.42 ± 0.1	0.40 ± 0.1	0.40 ± 0.1	0.40 ± 0.1			
Albumin/Globulin ratio	CMT	0.96 ± 0.2*	0.96 ± 0.1	1.08 ± 0.2*	1.01 ± 0.2	0.95 ± 0.2	1.00 ± 0.2	0.99 ± 0.2	0.599	0.027	0.816
	Placebo	0.88 ± 0.3†	0.93 ± 0.2	1.03 ± 0.2†	0.92 ± 0.2	0.91 ± 0.1	0.98 ± 0.2	0.93 ± 0.1			

[a] *p* value comparing the evolution of the corresponding parameter evolution between dogs treated with CMT-8 (CMT) and with placebo
[b] *p* value comparing the corresponding parameter value between the different follow-up time points
[c] *p* value evaluating the interaction between treatment and time
*Significant difference versus *group

Table 4

Evolution of the absolute and relative values of peripheral blood lymphocyte subsets in dogs treated with CMT-8 (CMT) and placebo during the follow-up period of the study (mean ± SD).

		Follow-up time points							Treatment (*p* value) [a]	Time (*p* value) [b]	Interaction (*p* value) [c]
		Day 0	Day 15	Day 30	Day 60	Day 90	Day 120	Day 180			
Lymphocytes T (CD3+)/µl	CMT	1254 ± 491	1191 ± 611	1267 ± 453	1514 ± 498	1684 ± 393	1837 ± 383	1570 ± 316	0.598	0.053	0.142
	Placebo	1519 ± 345	1632 ± 197	1487 ± 120	1536 ± 261	1735 ± 399	1570 ± 387	1560 ± 246			
Lymphocytes Th (CD3+CD4+)/µl	CMT	574 ± 163	538 ± 220	574 ± 175*	696 ± 151*	749 ± 188	797 ± 121*	664 ± 111	0.620	0.006	0.471
	Placebo	686 ± 278	696 ± 162	624 ± 190†	712 ± 255†	787 ± 291	773 ± 258†	696 ± 218			
Lymphocytes Tc (CD3+CD8+)/µl	CMT	399 ± 263	402 ± 284	399 ± 223	511 ± 252	585 ± 285	651 ± 288	554 ± 230	0.935	0.224	0.154
	Placebo	486 ± 237	523 ± 236	477 ± 181	448 ± 112	547 ± 256	435 ± 72	514 ± 253			
Lymphocytes B (CD21+)/µl	CMT	287 ± 169	277 ± 174	305 ± 158	323 ± 167	365 ± 179	381 ± 147	293 ± 88	0.961	0.481	0.203
	Placebo	295 ± 152	334 ± 167	317 ± 99	328 ± 137	308 ± 177	300 ± 82	319 ± 79			
CD3-CD21- lymphocytes/µl	CMT	413 ± 161*	330 ± 135	262 ± 133	267 ± 44*	308 ± 108*	279 ± 89*	290 ± 69*	0.342	0.005	0.330

	Placebo	482 ± 133†	490 ± 198	314 ± 168	292 ± 91†	337 ± 77†	205 ± 37†	317 ± 89†			
Lymphocytes MHC II+/µl	CMT	1643 ± 413*	1487 ± 613*Δ	1720 ± 369	1869 ± 491	2182 ± 434*	2311 ± 394*	1943 ± 320	0.039	0.037	0.040
	Placebo	1895 ± 386	2154 ± 385Δ	1949 ± 187	1961 ± 354	2198 ± 416	1928 ± 449	1974 ± 219		0.260	
CD4/CD8 ratio	CMT	1.79 ± 0.97	1.65 ± 0.95	1.74 ± 1.01	1.70 ± 1.14	1.56 ± 0.94	1.51 ± 0.95	1.47 ± 0.92	0.853	0.559	0.233
	Placebo	1.76 ± 1.12	1.69 ± 1.02	1.59 ± 1.01	1.79 ± 1.03	1.77 ± 1.12	1.88 ± 0.89	1.73 ± 1.09			

[a] *p* value comparing the evolution of the corresponding parameter evolution between dogs treated with CMT-8 (CMT) and with placebo
[b] *p* value comparing the corresponding parameter value between the different follow-up time points
[c] *p* value evaluating the interaction between treatment and time
*Significant difference versus *group
†Significant difference versus †group
Δ Significant difference versus Δ group