Veterinary Parasitology 239 (2017) 50–56

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Randomized, allopurinol-controlled trial of the effects of dietary nucleotides and active hexose correlated compound in the treatment of canine leishmaniosis

Sergi Segarra[a,*], Guadalupe Miró[b], Ana Montoya[b], Luis Pardo-Marín[c], Noemí Boqué[d], Lluis Ferrer[e], José Cerón[c]

[a] R & D Animal Health Bioiberica S.A.U., Pça. Francesc Macià 7, 08029 Barcelona, Spain
[b] Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Avda. Puerta de Hierro s/n, 28040 Madrid, Spain
[c] Interlab-UMU, Campus de Excelencia "Mare Nostrum", University of Murcia, Campus Espinardo, 30071 Murcia, Spain
[d] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Avinguda Universitat 1, 43204 Reus, Spain
[e] Department of Clinical Sciences, Tufts Cummings School of Veterinary Medicine, 200 Westboro Road, North Grafton, MA 01536, USA

ARTICLE INFO

Keywords:
Canine leishmaniosis
Dietary nucleotides
AHCC
Allopurinol
Xanthine
Hyperxanthinuria

ABSTRACT

First-line treatment for canine leishmaniosis (CanL) is N-methylglucamine antimoniate (MGA) combined with allopurinol. However, in some dogs allopurinol may induce hyperxanthinuria leading to urolithiasis. Moreover, allopurinol resistance has recently been described in *Leishmania infantum* isolates from treated dogs with a relapse of the disease. Alternative treatments are thus needed. Since the type of host immune response strongly influences CanL progression and prognosis, dogs could benefit from treatments targeted at modulating such response, such as nucleotides and active hexose correlated compound (AHCC). The aim of this study was to evaluate the effects of an oral combination of nucleotides and AHCC in dogs with clinical leishmaniosis.

Sixty-nine dogs with naturally-occurring clinical leishmaniosis were included in this multicenter, open-label, positively-controlled clinical trial and randomized to receive 10 mg/kg allopurinol PO BID (allopurinol group) or 17 mg/kg AHCC plus 32 mg/kg nucleotides PO SID (supplement group) for 180 days. All dogs were also given 50 mg/kg MGA SC BID during the first 28 days. At the time points 0, 30, and 180 days of the trial, dogs underwent a clinical examination, and blood, urine, and bone marrow samples were submitted for analytical tests.

Final data analyses (allopurinol group: $n = 29$; supplement group: $n = 24$) revealed a significant improvement in both groups in clinical scores and ELISA-determined antibody titers after treatment. However, the supplement group showed a significantly lower clinical score ($P = 0.005$) and significantly higher antibody titers ($P = 0.032$) after 180 days, compared to the allopurinol group. RT-PCR parasite loads were reduced in groups (mean ± SD supplement: 0.38 ± 0.56 *vs* 5.23 ± 18.9; allopurinol: 0.45 ± 1.47 *vs* 3.09 ± 8.36 parasites/ng of DNA), but there were no significant differences over time or between groups. During the study, 12 dogs in the allopurinol group developed xanthinuria (41%) compared to no dogs (0%) in the supplement group ($P = 0.000$). Both treatments led to significantly increased CD4+/CD8+ ratio, and improvements in protein electrophoretic pattern and acute phase response.

In conclusion, 6-month oral treatment with nucleotides and AHCC in addition to MGA showed similar efficacy to the current first-line treatment for CanL, without producing xanthinuria. This combination could be a good alternative to MGA-allopurinol combination treatment for CanL, especially for dogs suffering allopurinol-related adverse events.

Abbreviations: CanL, canine leishmaniosis; MGA, N-methylglucamine antimoniate; APP, acute phase proteins; AHCC, active hexose correlated compound; ELISA, enzyme-linked immunosorbent assay; CBC, complete blood count; CRP, C-reactive protein; UPC, urinary protein/creatinine ratio; RT-PCR, real time-PCR; ANCOVA, analysis of covariance; ANOVA, analysis of variance; LSD, least significant difference

* Corresponding author at: R & D Animal Health Bioiberica S.A.U., Pça. Francesc Macià 7, 08029 Barcelona, Spain.
E-mail addresses: ssegarra@bioiberica.com (S. Segarra), gmiro@ucm.es (G. Miró), amontoya@ucm.es (A. Montoya), lpm1@um.es (L. Pardo-Marín), noemi.boque@ctns.cat (N. Boqué), Lluis.Ferrer@tufts.edu (L. Ferrer), jjceron@um.es (J. Cerón).

http://dx.doi.org/10.1016/j.vetpar.2017.04.014
Received 13 February 2017; Received in revised form 24 March 2017; Accepted 11 April 2017

1. Introduction

Currently, the most effective treatment against canine leishmaniosis (CanL) consists of subcutaneous N-methylglucamine antimoniate (MGA) for 4–6 weeks combined with oral allopurinol for at least 6–12 months (Manna et al., 2015; Solano-Gallego et al., 2009). Allopurinol is a purine analog of adenosine nucleotides that blocks RNA synthesis in *Leishmania* parasites, leading to inhibition of parasite multiplication. However, the use of allopurinol has several limitations mainly related to possible side effects (Denerolle and Bourdoiseau, 1999; Gómez-Ochoa et al., 2009; Neal et al., 1985; Nelson et al., 1979; Noli and Saridomichelakis, 2014; Solano-Gallego et al., 2009, 2011, 2013). Most dogs treated with allopurinol alone undergo clinical remission but without parasite elimination (Cavaliero et al., 1999; Koutinas et al., 2001; Ling et al., 1991; Nelson et al., 1979; Yasur-Landau et al., 2016). Allopurinol treatment in dogs can lead to increased urinary xanthine levels, which can develop as early as 3 weeks after starting treatment (Torres et al., 2016). Hyperxanthinuria may ultimately cause urolithiasis and renal mineralization (Bartges and Kirk, 2009; Feo et al., 2012; Ling et al., 1991; Miró, 2007; Osborne et al., 2009; Solano-Gallego et al., 2009, 2013; Torres et al., 2011, 2016). Furthermore, allopurinol resistance has recently been reported in *L. infantum* parasites isolated from dogs undergoing allopurinol treatment, and it was associated with clinical relapse (Yasur-Landau et al., 2016), becoming a major concern. There is thus a need for novel treatments that can be effectively and safely administered over the long term in dogs with leishmaniosis.

The type of host immune response raised against *L. infantum* plays a key role in disease progression and outcome. Dogs with subclinical infection (clinically healthy infected dogs) show a weak or absent Th2 (humoral) immune response and a stronger Th1 (cellular) response. These dogs usually show a low titer of anti-*Leishmania* antibodies, a low parasite load, and no clinical signs or clinicopathological abnormalities. Conversely, dogs with clinical leishmaniosis (sick dogs) are characterized by an exacerbated Th2 immune response and an absent or weak Th1 response, systemic parasite dissemination, low numbers of CD4+ T-helper cells, and immunosuppression. These dogs feature high anti-*Leishmania* antibody titers and clinical signs and/or clinicopathological abnormalities (Cortese et al., 2015; Locksley and Louis, 1992; Miranda et al., 2007; Paltrinieri et al., 2010; Solano-Gallego et al., 2011). Reductions in circulating levels of CD4+ and CD8+ lymphocytes and in the CD4+/CD8+ ratio have also been reported in sick dogs. Further, it has been established that protective immunity against the disease is mediated by a CD4+ T helper 1 (Th1) cellular response, which promotes macrophage intracellular clearance of *Leishmania* parasites (Bourdoiseau et al., 1997; Esch et al., 2013; Miranda et al., 2007). Moreover, CD4+ and CD8+ T cell exhaustion has been reported in sick dogs, related to a poorer response to treatment and to the onset of clinical manifestations (Esch et al., 2013).

Among the currently available methods, response to treatment in dogs with leishmaniosis can be monitored by evaluating changes in clinical signs, and by determining serum proteins and acute phase protein (APP) indexes, which are higher in infected dogs and tend to decline as they respond to treatment (Martinez-Subiela et al., 2011; Paltrinieri et al., 2010).

Some authors have argued that the future of CanL management may be a combination of parasiticidal and parasitostatic treatments to eliminate the parasite and immuno-modulators so as to elicit an appropriate and more efficient immune response against the parasite (Solano-Gallego et al., 2013). In effect, a recent study has shown that an immune system-modulating diet can improve the immune response in dogs with leishmaniosis under standard pharmacological treatment (Cortese et al., 2015).

Orally administered nucleotides modulate the immune response. These low molecular weight compounds positively influence lipid metabolism and immunity, and tissue growth, development and repair, and can be especially beneficial in situations when rapidly proliferating tissues, such as the intestine and the immune system, are unable to fulfill their nucleotide needs by *de novo* synthesis. Dietary nucleotides are considered potential immuno-modulatory compounds (Fontana et al., 2010; Gil, 2002). Active hexose correlated compound (AHCC), an α-glucan-rich dietary supplement extracted from Basidiomycota mushrooms, has shown a capacity for stimulating the immune system in humans. Among its effects, AHCC has been reported to increase Th1 cell response (Lee et al., 2012; Ulbricht et al., 2013; Yin et al., 2010), which could benefit dogs with *Leishmania* infection.

Given the key role of the immune system in CanL, we hypothesized that dietary nucleotides and AHCC might improve the immune response to CanL and thus be beneficial to the dogs. Based upon this hypothesis, the objective of this study was to assess the efficacy of a dietary supplement containing nucleotides and AHCC in treating dogs with clinical leishmaniosis, and to determine whether this supplement in combination with MGA could be a safe, effective alternative to allopurinol. To this end, dogs with confirmed leishmaniosis were randomized to receive either MGA plus allopurinol as the treatment recommended by the LeishVet group (Solano-Gallego et al., 2009), or MGA plus dietary nucleotides and AHCC. The two groups of dogs were matched in terms of clinical signs, age, and sex. A further objective was to assess the safety and tolerance of both treatment regimens, with special attention paid to the development of side effects related to the compounds.

2. Materials and methods

This was a multicenter, open-label, positively-controlled, randomized clinical trial conducted in Spain. The protocol was reviewed and approved by the Committee of Research Ethics of the University of Murcia, Spain. All dog owners gave their written informed consent. Client-owned dogs of any age, breed, or gender were enrolled at 10 veterinary practices. Main inclusion criteria were a positive serology for *Leishmania* by enzyme-linked immunosorbent assay (ELISA), a positive cytology or PCR result obtained in bone marrow or lymph node tissue, and at least two of the following clinical manifestations: apathy, weight loss, muscle atrophy, skin lesions, lymphadenopathy, splenomegaly, epistaxis, and ocular lesions. Dogs were excluded if they had been vaccinated against CanL, if they had received treatment with allopurinol in the three weeks prior to entering the study, or if they had been treated with MGA, miltefosine, domperidone, ciclosporin, or glucocorticoids two months prior to the study outset, or if they were receiving any kind of special diet or dietary supplements for improving their immune response. Pregnant and lactating bitches were excluded. Dogs could be withdrawn from the study at any time if they showed intolerance to the treatment or if requested by the owner.

Selected dogs were randomized by means of a computer-generated schedule to one of two treatment arms. Dogs in the allopurinol group (positive control) received 10 mg/kg allopurinol (Alopurinol Normon®, Laboratorios Normon, S.A., Madrid, Spain) orally twice daily for six months. Dogs in the supplement group were given a dietary supplement (Impromune®, Bioiberica S.A.U., Barcelona, Spain) containing 32 mg/kg dietary nucleotides (Nucleoforce® Dogs, Bioiberica S.A.U., Barcelona, Spain) plus 17 mg/kg AHCC (Amino Up Chemical Co. Ltd., Sapporo, Japan) orally once daily for six months. Treatment was started immediately after enrollment. During the first 28 days of treatment, all dogs were also administered 50 mg/kg MGA (Glucantime®, Merial Laboratorios S.A., Barcelona, Spain) subcutaneously every 12 h.

Clinical follow-up evaluations were conducted by each corresponding veterinarian on days 0 (day of enrollment), 30, and 180 of treatment. Each follow-up session consisted of a general physical exam and scoring for clinical signs associated with CanL using a scoring system adapted from Miró et al. (Miró et al., 2009) (Table 1). This score was the primary outcome measure. Apart from these follow-up visits, owners were contacted by phone after 60 and 120 days of treatment so

Table 1
Clinical scoring system used in this study. Miró et al., 2009.

Clinical signs	Score			
	0	1	2	3
Appetite	Normal	Slightly reduced (> half normal intake)	Markedly reduced (< half normal intake)	Anorexia
Apathy	Absent	Mild (active if stimulated)	Severe	Prostration
Polydipsia	Absent	Drinking less than 2× normal amount	Drinking between 2× and 4× normal amount	Drinking more than 4× normal amount
Temporal muscle atrophy	Absent	Mild	Severe	Temporal muscles not visible
General muscle atrophy	Absent	Mild (emaciation)	Severe	Cachexia
Lymphadenomegaly	–	1 or 2 enlarged lymph nodes	> 2 enlarged lymph nodes	Generalized lymphadenomegaly
Splenomegaly	–	–	Splenomegaly	–
Conjunctivitis and/or blepharitis	Absent	Mild unilateral	Bilateral/severe unilateral	Bilateral severe
Keratitis/uveitis	Absent	Mild unilateral	Bilateral/severe unilateral	Bilateral severe
Pale mucous membranes	Absent	Mild	Moderate	Severe
Oral mucosa	Normal	1 or 2 small ulcers and/or nodules	> 2 small ulcers and/or nodules	> ¼ surface ulcerated or with nodules
Nasal mucosa	Normal	–	Epistaxis	–
GI mucosa	Normal	Occasional vomiting/diarrhea	Frequent vomiting/diarrhea or occasional hematochezia	Frequent bloody vomiting/diarrhea or frequent hematochezia
Arthritis	Absent	1 joint affected	Polyarthritis in one limb	Polyarthritis in more than one limb
Skin erythema	Absent	< 10% body surface or generalized but mild erythema	< 25% of body surface or generalized moderate erythema	> 25% of body surface
Skin ulcers	Absent	1 or 2 ulcers	3 to 5 ulcers	More than 5 ulcers
Skin nodules	Absent	1 or 2 nodules	3 to 5 nodules	More than 5 nodules
Alopecia +/- scaling/exfoliation	Absent	< 10% of body surface or generalized but mild alopecia	< 25% of body surface or generalized moderate alopecia	> 25% of body surface
Onychogryphosis	Absent	Mild hypertrophy	Moderate hypertrophy	Severe hypertrophy

that they could report any clinical signs that might require an additional visit to the veterinary practice.

Blood samples were collected at 0, 30, and 180 days of the trial for complete blood count (CBC), serum biochemistry, serum protein electrophoresis, antibody titer (Leiscan® *Leishmania* ELISA Test), selected APP concentrations (C-reactive protein (CRP) and ferritin), and CD4+ and CD8+ lymphocyte counts. CD4+/CD8+ ratios were calculated from the latter counts. Urinalysis, including the urinary protein/creatinine ratio (UPC), urinary density, and urinary sediment analysis were also undertaken 0, 30, and 180 days after treatment onset on urine samples obtained by cystocentesis. Bone marrow or lymph node aspirates were taken before (day 0) and after (day 180) treatment to evaluate parasite load. Molecular diagnosis was performed on samples stored in 200 µl of buffer NET 10 (NaCl 10 mM, EDTA 10 mM, Tris 10 mM). The QIAamp® DNA Micro Kit (50) (Qiagen®, Hilden, Germany) was used to obtain DNA according to the manufacturer's instructions. *Leishmania* DNA was detected by PCR targeting internal transcribed spacers (ITS) 1 and 2 as described by Kuhls et al. (2005). The PCR amplification product size was 280–330 bp. The parasite DNA load was quantified by amplification of a 200-bp kinetoplast DNA fragment using real time-PCR (RT-PCR), as previously described (Mary et al., 2004), in a Corbett Rotor Gene 6000 thermal cycler (Qiagen®, Hilden, Germany). Results were expressed as parasites per ng of DNA.

During the course of treatment, any adverse events that could be related to the treatment, such as gastrointestinal disturbances or urinary abnormalities, were recorded.

Statistical analysis was performed by a biostatistician (NB) using the software package SPSS Statistics v19 (SPSS Inc., Chicago, IL, USA). A descriptive analysis of the data was performed according to the nature of the variables for each follow-up visit and assigned treatment. Quantitative variables are reported as means ± SD, and categorical variables as frequencies and percentages. Baseline differences were analyzed with a Student's *t*-test for quantitative variables and Fisher's exact test for categorical variables. Treatment effects were compared by analysis of covariance (ANCOVA) using baseline values as covariables for quantitative variables. Fisher's exact test was used for categorical variables. Changes over time were analyzed by repeated-measures analysis of variance (ANOVA) and *post hoc* tests of least significant difference (LSD). Linear relationships between key variables were tested using Pearson's correlation coefficients. The level of statistical significance was set at 5%.

3. Results

During the study period, 89 dogs were initially assessed for eligibility. Of these dogs, 69 were randomized into the two study groups (allopurinol group: $n = 38$; supplement group: $n = 31$). Sixteen dogs (23%) did not complete the study, mainly due to owner decisions. Two dogs in the allopurinol group were euthanized due to severe worsening of their clinical condition, one of them because of renal disease. The final numbers completing the trial were 29 dogs in the allopurinol group and 24 in the supplement group (Fig. 1). The baseline characteristics of the study population are provided in Table 2. The two groups were initially matched in terms of clinical scores, and blood, bone marrow, and urine test results. At the study's start, there were no significant differences between groups for any of the studied parameters ($P > 0.05$).

3.1. Clinical examination

The mean clinical scores recorded at each follow-up time in each group are shown in Fig. 2. After 180 days, the mean clinical scores fell significantly in both treatment groups ($P = 0.000$). In addition, a significant decrease in the clinical score was observed from 30 to 180 days of treatment only in the supplement group (supplement $P = 0.000$; allopurinol $P = 0.387$). When groups were compared, there were no significant differences in scores at 30 days but by the end of the study the scores were significantly lower in the supplement group (adjusted mean ± SE: 0.47 ± 0.46) compared to the allopurinol group (adjusted mean ± SE: 2.30 ± 0.42; $P = 0.005$). In addition, at the end of the study, a significantly higher proportion of dogs scoring below 5 was observed in the supplement group than in the allopurinol group (96% *vs* 72%; $P = 0.031$).

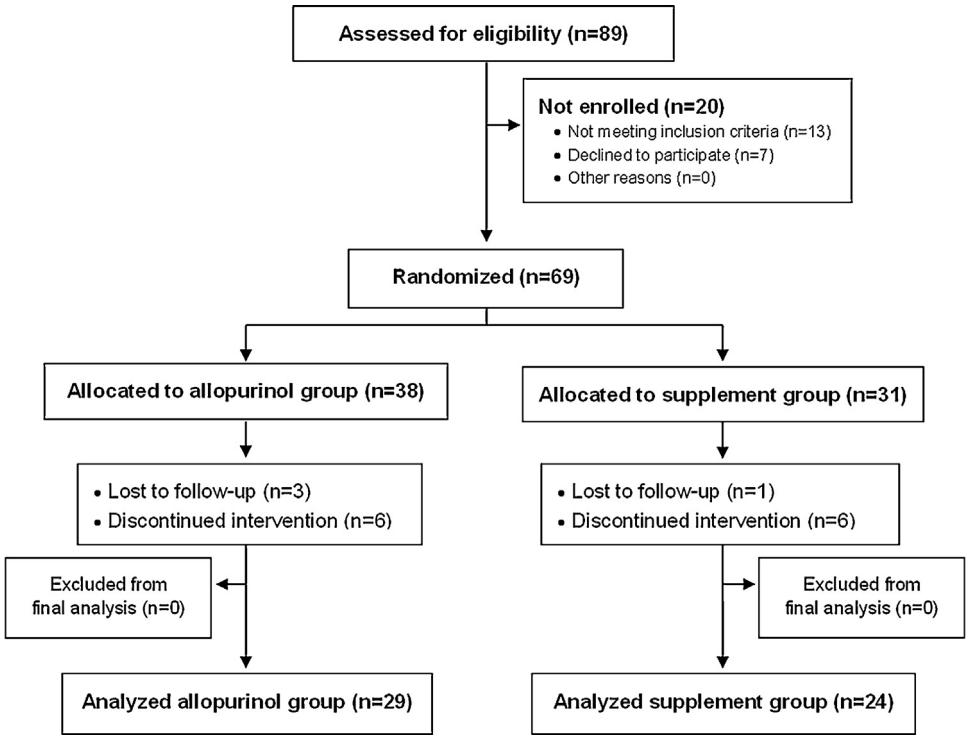

Fig. 1. Flow diagram showing the dogs with leishmaniosis in each stage of the clinical trial.

Table 2
Baseline characteristics of dogs assigned to each study group and homogeneity analysis data, expressed as mean ± standard deviation and frequencies (%).

Variable	Supplement group (n = 24)	Allopurinol group (n = 29)
Sex: female, n (%)	13 (54.2%)	14 (48.3%)
Age: months	55.3 ± 35.4	59.5 ± 35.5
(minimum-maximum)	6–142	7–132
Age groups: n (%)		
< 3 years	6 (25%)	6 (20.7%)
3 to 8 years	15 (62.5%)	18 (62.1%)
> 8 years	3 (12.5%)	5 (17.2%)
Clinical score: points	7.67 ± 3.84	6.93 ± 4.46
ELISA serology: arbitrary units	4.46 ± 1.57	4.32 ± 1.78
RT-PCR: parasites/ng of DNA	5.23 ± 18.9	3.09 ± 8.36
Body temperature: °C	38.4 ± 0.51	38.5 ± 0.59
Weight; kg	21.5 ± 11.7	20.7 ± 14.6

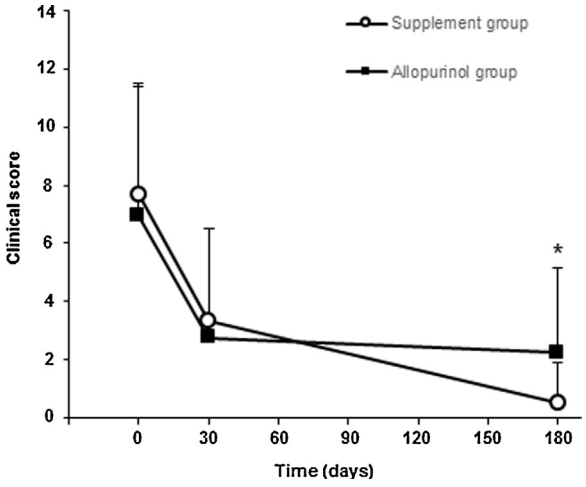

Fig. 2. Changes produced in mean clinical score in dogs with leishmaniosis treated with supplement or allopurinol for 180 days. Data reported as mean ± SD (n = 24 *vs* 29). * $P < 0.05$, supplement *vs* allopurinol (ANCOVA).

3.2. Blood tests

Initially, no significant differences ($P > 0.05$) were observed between groups for any of the blood variables determined (data not shown). Significant reductions ($P < 0.01$) in antibody titers (ELISA serology) were observed in both groups after 30 days (mean ± SD, supplement 3.68 ± 1.65; allopurinol 3.65 ± 1.42) and after 180 days of treatment (mean ± SD, supplement 3.41 ± 1.41; allopurinol 2.61 ± 1.28) compared to baseline values (Table 2). When groups were compared, the supplement group showed higher antibody titers at the end of the study ($P = 0.032$) (Fig. 3).

Compared to baseline, no significant variations were detected in urea and serum creatinine concentrations during the study in either treatment arm, and no significant differences were found at any time point between groups for these variables.

Serum ferritin and CRP levels fell during the course of treatment in both groups. The reduction observed in ferritin was significant ($P < 0.05$) for both treatments at 30 days and 180 days, whereas the decrease in CRP was significant for both treatments after 30 days ($P < 0.01$) but only at the end of the study for the allopurinol treatment (supplement $P = 0.056$; allopurinol $P = 0.003$).

Compared to baseline, both treatments led to significantly reduced total serum protein concentrations ($P < 0.01$) after 30 days and 180 days, and to increased albumin concentrations after 30 and 180 days ($P < 0.05$ and $P < 0.01$, respectively). After 30 days and 180 days, both treatments achieved significant reductions in the percentage of the gamma globulin fraction ($P < 0.01$).

At the study's end, CD4+ values increased significantly in both treatment groups compared to baseline ($P < 0.01$). Both groups showed an increasing trend in CD8+ lymphocytes at 180 days compared to baseline (supplement $P = 0.078$; allopurinol $P = 0.098$), with no significant differences between groups. Compared to baseline, a significant increase was observed in the CD4+/CD8+ ratio at the end

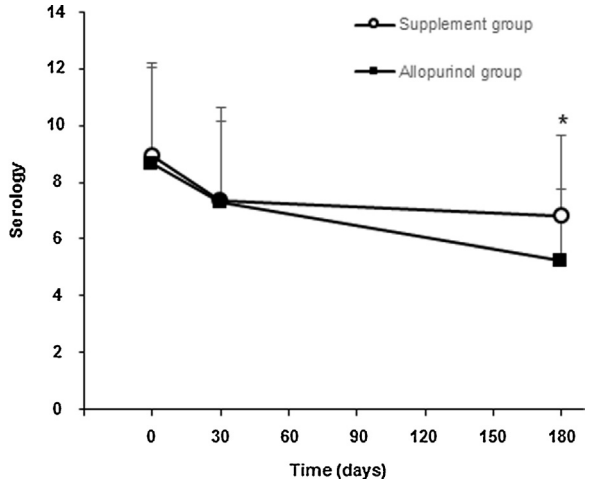

Fig. 3. Changes observed in mean ELISA antibody titers in dogs with leishmaniosis treated with supplement or allopurinol for 180 days. Data reported as mean ± SD (n = 24 *vs* 29). * $P < 0.05$, supplement *vs* allopurinol (ANCOVA).

of the study in the supplement (mean ± SD = 2.58 ± 1.00 *vs* 1.62 ± 1.33; $P = 0.034$) and allopurinol (mean ± SD: 3.14 ± 2.20 *vs* 2.05 ± 2.29; $P = 0.023$) groups, but no significant differences emerged between groups.

3.3. Urinalysis

The variations recorded in the urine variables examined are shown in Table 3. During the study, 12 dogs treated with allopurinol developed xanthinuria while none of the dogs in the supplement group did so (41% *vs* 0%, respectively; $P = 0.000$). In both treatment groups, UPC fell during the course of treatment, but significantly only in the allopurinol group (supplement $P = 0.075$; allopurinol $P = 0.036$). At the end of the study, dogs in the supplement group showed a higher urine density ($P = 0.038$), although no significant variations in time ($P > 0.05$) were found in urine density for any of the study groups. No significant differences were recorded for the other urine analytes.

Table 3
Changes produced over time in the urine variables determined in each treatment group.

Variable		Supplement group	Allopurinol group	*p* value
Leukocytes; positive, *n* (%)	0 days	12 (63.2%)	13 (56.5%)	
	30 days	7 (30.4%)	11 (37.9%)	0.770
	180 days	5 (23.8%)	10 (34.5%)	0.537
Cylinders; positive, *n* (%)	0 days	9 (37.5%)	8 (27.6%)	
	30 days	9 (39.1%)	6 (20.7%)	0.218
	180 days	10 (47.6%)	9 (31.0%)	0.255
Bacteria; positive, *n* (%)	0 days	4 (16.7%)	6 (20.7%)	
	30 days	4 (17.4%)	8 (27.6%)	0.513
	180 days	1 (4.8%)	7 (24.1%)	0.117
Struvite; positive, *n* (%)	0 days	10 (41.7%)	11 (37.9%)	
	30 days	13 (56.5%)	14 (48.3%)	0.588
	180 days	11 (52.4%)	11 (37.9%)	0.391
Xanthine; positive, *n* (%)	0 days	3 (12.5%)	0 (0.0%)	
	30 days	2 (8.7%)	10 (34.5%)	**0.046***
	180 days	1 (4.8%)	2 (6.9%)	1.000
Urine density; g/L	0 days	1.03 ± 0.01	1.03 ± 0.01	
	30 days	1.03 ± 0.01	1.03 ± 0.01	0.288
	180 days	1.04 ± 0.01	1.03 ± 0.01	**0.038***
UPC	0 days	2.31 ± 3.91	1.34 ± 2.32	
	30 days	1.91 ± 3.76	0.97 ± 2.40	0.655
	180 days	0.83 ± 1.45	0.70 ± 1.07	0.800

3.4. Parasite load

After 180 days, RT-PCR parasite loads were reduced in both groups, compared to baseline (mean ± SD supplement: 0.38 ± 0.56 *vs* 5.23 ± 18.9; allopurinol: 0.45 ± 1.47 *vs* 3.09 ± 8.36 parasites/ng of DNA), but there were no significant differences over time or between groups for any time point.

3.5. Adverse events

Both study compounds were well tolerated and no major side effects related to these treatments were reported in any dog.

4. Discussion

In this study, we compared the effects of MGA plus a supplement containing dietary nucleotides and AHCC, and MGA plus allopurinol as treatment for CanL. Disease outcome and treatment response were monitored using a comprehensive score, which grades the main clinical signs associated with CanL (Denerolle and Bourdoiseau, 1999; Paltrinieri et al., 2010; Solano-Gallego et al., 2009), and RT-PCR-determined parasite burden. Both treatments led to clinical improvement and reduced parasite burden, but a greater improvement in clinical signs was observed with the supplement. Such additional improvement could be the consequence of immune response modulation by the supplement, as reported in a study in which a nutraceutical with immune modulating properties improved the immune profile of sick dogs with leishmaniosis receiving MGA and allopurinol (Cortese et al., 2015).

In addition to clinical signs, various biomarkers commonly used to monitor CanL treatment were assessed in this study. To examine the humoral immune response, we measured antibody titers with ELISA testing. Although clinical signs and serology can vary accordingly in many cases (Solano-Gallego et al., 2016), changes in antibody titers should be interpreted with caution since a good clinical response to treatment is not always reflected by a rapid decline in antibody levels (Ferrer et al., 1995; Torres et al., 2011). Here, both treatment groups showed a significant reduction in antibody titers compared to baseline, although the supplement group showed relatively higher antibody titers at the end of the study, despite having lower clinical scores. This diversity in the dynamics of the decrease in antibody titers during allopurinol and MGA treatment has been well documented in the literature but its causes are poorly understood (Cavaliero et al., 1999; Manna et al., 2015; Torres et al., 2011). Further studies are needed to elucidate the cause of these findings, which could perhaps be related to the modulation of the immune response by dietary nucleotides and AHCC not following the same trend as allopurinol.

To assess the cellular response, CD4+ and CD8+ lymphocytes were quantified by flow cytometry. A significant increase in CD4+ values and CD4+/CD8+ ratio was observed after treatment in both groups, indicating an improved cellular immune response (Baneth et al., 2008; Bourdoiseau et al., 1997; Cortese et al., 2015; Miranda et al., 2007). Lower CD4+ counts have been associated with increased infectivity susceptibility of CanL-infected dogs to sand flies (Guarga et al., 2000). This improvement in the supplement group might be the outcome of the immunomodulatory effects of dietary nucleotides, possibly even enhanced by AHCC, since this compound has been reported to improve CD4+ and CD8+ T-cell immune responses in healthy elderly people (Yin et al., 2010).

Similar trends in electrophoretic and APP patterns were observed in both treatment groups. Over the course of treatment, gamma globulin fraction, ferritin, and CRP decreased, and albumin increased in both groups, indicating a positive response to both treatments (Martinez-Subiela and Cerón, 2005).

In our study, none of the cases that received the combination of dietary nucleotides with AHCC developed xanthinuria, while 41% did

with allopurinol. This percentage is higher than what was reported in a previous retrospective study (Torres et al., 2016). Both treatments led to reduced proteinuria and thus improved glomerular function. Remarkably, before treatment the dogs assigned to the supplement group showed a higher prevalence of xanthinuria. Although dogs had not been subjected to allopurinol treatment for at least three weeks before the study outset, xanthine crystals may remain in urine after allopurinol administration. Hyperxanthinuria induced by allopurinol leads to an increased risk of xanthine-associated nephrolithiasis, which could require surgical intervention. In effect, most xanthine uroliths in dogs are attributed to allopurinol treatment (Bartges and Kirk, 2009; Cavaliero et al., 1999; Feo et al., 2012; Ling et al., 1991; Osborne et al., 2009; Plevraki et al., 2006). In a recent retrospective study, a series of clinical cases of dogs that developed xanthinuria while receiving allopurinol treatment for CanL were reviewed. After confirming that urolithiasis and renal mineralization may occur in dogs receiving allopurinol treatment, the authors warned that dogs with leishmaniosis should be monitored for the development of urinary adverse effects from treatment onset (Torres et al., 2016). In addition, xanthine in urine is known to enhance the *in vitro* multiplication of *Leishmania* (Warburg et al., 2008). Using a lower dosage of allopurinol might have led to a lower incidence of xanthinuria in this study, although its clinical efficacy could then have also been affected. The significantly higher urine density values at the end of the study in the supplement group should not have any clinical significance.

Another problem associated with the use of allopurinol is the development of resistance by the parasite, which potentially could increase the risk of uncontrolled transmission of *Leishmania* infection from dogs to humans or to other dogs (Yasur-Landau et al., 2016). This association between clinical relapse and allopurinol-resistant parasites supports the need for alternative treatment options. This is especially relevant since CanL is a globally emerging disease in constant expansion. In the past few years, cases have been reported in non-endemic areas where this disease is still considered exotic, including the UK, Germany, and Poland (Baneth et al., 2008; Kaszak et al., 2015; Shaw et al., 2009). In effect, the combination of dietary nucleotides and AHCC tested here could be a safer option with similar efficacy to allopurinol.

Our data confirm that 180 days of treatment with dietary nucleotides and AHCC added to four weeks of MGA leads to a marked improvement in clinical and clinicopathological signs in dogs with leishmaniosis without increasing the risk of developing hyperxanthinuria. Our study has some limitations that should be mentioned. First, it was an open-label study and, although the clinical changes were evaluated using a comprehensive scoring system, some of the parameters could not be assessed in a completely objective manner. Although all dogs received a regular diet, different trademarks and formulations were used among dogs from the study. Therefore, whether the diet affected the immune system of these dogs cannot be ruled out. The occurrence of concomitant tick-borne infections was not assessed and this might have affected the immune response of the dogs form the study. The mechanism of action of this new therapeutic option is not yet known. The increased CD4+ levels observed here suggest it may improve the immune response and help rescue T cell exhaustion present in some sick dogs (Esch et al., 2013). However, further investigations are needed to determine which of the two compounds is responsible for this improvement, or whether they both act synergistically. To compare the specific effects of the supplement with those of allopurinol, the two treatments would need to be used as sole treatment. In addition, a longer follow-up period would be helpful to further characterize the effects of the combination tested, especially the efficacy of this treatment in preventing relapses of the disease, and also to verify that the clinical improvement is not solely attributable to the beneficial effects of MGA. Further prospective studies need to assess longer term treatment with the supplement combination and examine the long-term treatment response and its possible MGA-sparing effect.

5. Conclusion

The combined use of MGA with a supplement containing dietary nucleotides and AHCC for six months in dogs with clinical leishmaniosis lowered clinical scores and led to an overall improvement in the biomarkers used to monitor response to treatment, and showed similar efficacy to the current first-line treatment for CanL without producing xanthinuria. This supplement together with MGA could be a good alternative to MGA-allopurinol combination treatment for CanL, especially for dogs suffering allopurinol-related adverse events.

Funding

Bioiberica S.A.U. provided the compounds and funding for this clinical trial.

Conflict of interest statement

SS is an employee of Bioiberica S.A.U., Barcelona, Spain. LF and JC work as scientific consultants for Bioiberica S.A.U.

Acknowledgments

The authors would like to thank all the dog owners and veterinarians who participated in this study.

References

Baneth, G., Koutinas, A.F., Solano-Gallego, L., Bourdeau, P., Ferrer, L., 2008. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. 24, 324–330.

Bartges, J., Kirk, C., 2009. Interpreting and managing crystalluria, in: Kirk's Current Veterinary Therapy IVX. Saunders Elsevier, St. Louis, Missouri, pp. 850–854.

Bourdoiseau, G., Bonnefont, C., Hoareau, E., Boehringer, C., Stolle, T., Chabanne, L., 1997. Specific IgG1 and IgG2 antibody and lymphocyte subset levels in naturally Leishmania infantum-infected treated and untreated dogs. Vet. Immunol. Immunopathol. 59, 21–30.

Cavaliero, T., Arnold, P., Mathis, A., Glaus, T., Hofmann-Lehmann, R., Deplazes, P., 1999. Clinical, serologic, and parasitologic follow-up after long-term allopurinol therapy of dogs naturally infected with *Leishmania infantum*. J. Vet. Intern. Med. 13, 330–334.

Cortese, L., Annunziatella, M., Palatucci, A.T., Lanzilli, S., Rubino, V., Di Cerbo, A., Centenaro, S., Guidetti, G., Canello, S., Terrazzano, G., 2015. An immune-modulating diet increases the regulatory T cells and reduces T helper 1 inflammatory response in Leishmaniosis affected dogs treated with. BMC Vet. Res. 11, 295.

Denerolle, P., Bourdoiseau, G., 1999. Combination allopurinol and antimony treatment versus antimony alone and allopurinol alone in the treatment of canine leishmaniasis (96 cases). J. Vet. Intern. Med. 13, 413–415.

Esch, K.J., Juelsgaard, R., Martinez, P.A., Jones, D.E., Petersen, C.A., 2013. Programmed death 1–mediated T cell exhaustion during visceral leishmaniasis impairs phagocyte function. J. Immunol. 191, 5542–5550.

Feo, L., Planellas, M., Monné, J.M., Dalmau, A., Pastor, J., Espada, I., 2012. Alteraciones urinarias secundarias al tratamiento prolongado con alopurinol en perros con leishmaniosis. In: Proceedings of the Southern European Veterinary Conference - SEVC 2012. Barcelona, Spain. .

Ferrer, L., Aisa, M., Roura, X., Portus, M., 1995. Serological diagnosis and treatment of canine leishmaniasis. Vet. Rec. 136, 514–516.

Fontana, L., Martínez-Augustin, O., Gil, Á., 2010. Role of dietary nucleotides in immunity. Funct. Food Rev. 2, 91–100.

Gil, A., 2002. Modulation of the immune response mediated by dietary nucleotides. Eur. J. Clin. Nutr. 56 (Suppl. 3), S1–S4.

Gómez-Ochoa, P., Castillo, J.A., Gascón, M., Zarate, J.J., Alvarez, F., Couto, C.G., 2009. Use of domperidone in the treatment of canine visceral leishmaniasis: a clinical trial. Vet. J. 179, 259–263.

Guarga, J.L., Moreno, J., Lucientes, J., Gracia, M.J., Peribáñez, M.A., Alvar, J., Castillo, J.A., 2000. Canine leishmaniasis transmission: higher infectivity amongst naturally infected dogs to sand flies is associated with lower proportions of T helper cells. Res. Vet. Sci. 69, 249–253.

Kaszak, I., Planellas, M., Dworecka-Kaszak, B., 2015. Canine leishmaniosis – an emerging disease. Ann. Parasitol. 61, 69–76.

Koutinas, A.F., Saridomichelakis, M.N., Mylonakis, M.E., Leontides, L., Polizopoulou, Z., Billinis, C., Argyriadis, D., Diakou, N., Papadopoulos, O., 2001. A randomised, blinded, placebo-controlled clinical trial with allopurinol in canine leishmaniosis. Vet. Parasitol. 98, 247–261.

Kuhls, K., Mauricio, I.L., Pratlong, F., Presber, W., Schönian, G., 2005. Analysis of ribosomal DNA internal transcribed spacer sequences of the *Leishmania donovani* complex. Microbes Infect. 7, 1224–1234.

Lee, W.-W., Lee, N., Fujii, H., Kang, I., 2012. Active hexose correlated compound

promotes T helper (Th) 17 and 1 cell responses via inducing IL-1β production from monocytes in humans. Cell. Immunol. 275, 19–23.
Ling, G.V., Ruby, A.L., Harrold, D.R., Johnson, D.L., 1991. Xanthine-containing urinary calculi in dogs given allopurinol. J. Am. Vet. Med. Assoc. 198, 1935–1940.
Locksley, R.M., Louis, J.A., 1992. Immunology of leishmaniasis. Curr. Opin. Immunol. 4, 413–418.
Manna, L., Corso, R., Galiero, G., Cerrone, A., Muzj, P., Gravino, A.E., 2015. Long-term follow-up of dogs with leishmaniosis treated with meglumine antimoniate plus allopurinol versus miltefosine plus allopurinol. Parasit. Vectors 8, 289.
Martinez-Subiela, S., Cerón, J., 2005. Validation of commercial assays for the determination of haptoglobin, C-reactive protein and serum amyloid A in dogs. Arch. Med. Vet 37, 61–66.
Martinez-Subiela, S., Strauss-Ayali, D., Cerón, J.J., Baneth, G., 2011. Acute phase protein response in experimental canine leishmaniasis. Vet. Parasitol. 180, 197–202.
Mary, C., Faraut, F., Lascombe, L., Dumon, H., 2004. Quantification of Leishmania infantum DNA by a real-time PCR assay with high sensitivity. J. Clin. Microbiol. 42, 5249–5255.
Miranda, S., Martorell, S., Costa, M., Ferrer, L., Ramis, A., 2007. Characterization of circulating lymphocyte subpopulations in canine leishmaniasis throughout treatment with antimonials and allopurinol. Vet. Parasitol. 144, 251–260.
Miró, G., 2007. Leishmaniosis canina: situación actual en Europa, diagnóstico y control. Acta Sci. Vet. 35, s227–s229.
Miró, G., Oliva, G., Cruz, I., Cañavate, C., Mortarino, M., Vischer, C., Bianciardi, P., 2009. Multicentric, controlled clinical study to evaluate effectiveness and safety of miltefosine and allopurinol for canine leishmaniosis. Vet. Dermatol. 20, 397–404.
Neal, R.A., Croft, S.L., Nelson, D.J., Nelson, D.J., 1985. Anti-leishmanial effect of allopurinol ribonucleoside and the related compounds, allopurinol, thiopurinol, thiopurinol ribonucleoside, and of formycin B, sinefungin and the lepidine WR6026. Trans. R. Soc. Trop. Med. Hyg. 79, 122–128.
Nelson, D.J., Bugge, C.J., Elion, G.B., Berens, R.L., Marr, J.J., 1979. Metabolism of pyrazolo(3,4-d)pyrimidines in *Leishmania braziliensis* and *Leishmania donovani*. Allopurinol, oxipurinol, and 4-aminopyrazolo(3,4-d)pyrimidine. J. Biol. Chem. 254, 3959–3964.
Noli, C., Saridomichelakis, M.N., 2014. An update on the diagnosis and treatment of canine leishmaniosis caused by *Leishmania infantum* (syn. L. chagasi). Vet. J. 202, 425–435.
Osborne, C.A., Lulich, J.P., Swanson, L.L., Albasan, H., 2009. Drug-induced urolithiasis. Vet. Clin. North Am. Small Anim. Pract. 39, 55–63.
Paltrinieri, S., Solano-Gallego, L., Fondati, A., Lubas, G., Gradoni, L., Castagnaro, M., Crotti, A., Maroli, M., Oliva, G., Roura, X., Zatelli, A., Zini, E., 2010. Guidelines for diagnosis and clinical classification of leishmaniasis in dogs. JAVMA 236 1984-1191.
Plevraki, K., Koutinas, A.F., Kaldrymidou, H., Roumpies, N., Papazoglou, L.G., Saridomichelakis, M.N., Savvas, I., Leondides, L., 2006. Effects of allopurinol treatment on the progression of chronic nephritis in canine leishmaniosis (*Leishmania infantum*). J. Vet. Intern. Med. 20, 228–233.
Shaw, S.E., Langton, D.A., Hillman, T.J., 2009. Canine leishmaniosis in the United Kingdom: a zoonotic disease waiting for a vector? Vet. Parasitol. 163, 281–285.
Solano-Gallego, L., Koutinas, A., Miró, G., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2009. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. Vet. Parasitol. 165, 1–18.
Solano-Gallego, L., Miró, G., Koutinas, A., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Baneth, G., 2011. LeishVet guidelines for the practical management of canine leishmaniosis. Parasit. Vectors 4, 86–102.
Solano-Gallego, L., Villanueva Saz, S., Cardoso, L., Ordeix i Esteve, L., Miró Corrales, G., Fondati, A., Peña Giménez, M.T., Leiva Repiso, M., Naranjo Freixa, C., Dantas-Torres, F., Otranto, D., Grazia Pennisi, M., 2013. Tratamiento y pronóstico, in: Leishmaniosis Una Revisión Actualizada. Servet, Zaragoza, Spain, pp. 151–164.
Solano-Gallego, L., Di Filippo, L., Ordeix, L., Planellas, M., Roura, X., Altet, L., Martínez-Orellana, P., Montserrat, S., 2016. Early reduction of Leishmania infantum-specific antibodies and blood parasitemia during treatment in dogs with moderate or severe disease. Parasit. Vectors 9, 235.
Torres, M., Bardagí, M., Roura, X., Zanna, G., Ravera, I., Ferrer, L., 2011. Long term follow-up of dogs diagnosed with leishmaniosis (clinical stage II) and treated with meglumine antimoniate and allopurinol. Vet. J. 188, 346–351.
Torres, M., Pastor, J., Roura, X., Tabar, M.D., Espada, Y., Font, A., Balasch, J., Planellas, M., 2016. Adverse urinary effects of allopurinol in dogs with leishmaniasis. J. Small Anim. Pract. 57, 299–304.
Ulbricht, C., Brigham, A., Bryan, J.K., Catapang, M., Chowdary, D., Costa, D., Culwell, S., D'Auria, D., Giese, N., Iovin, R., Isaac, R., Juturu, V., Liu, A., Mintzer, M., Rusie, E., Shaffer, M., Windsor, R.C., 2013. An evidence-based systematic review of active hexose correlated compound (AHCC) by the Natural Standard Research Collaboration. J. Diet. Suppl. 10, 264–308.
Warburg, A., Gelman, S., Deutsch, J., 2008. Xanthine in urine stimulates growth of *Leishmania promastigotes* in vitro. J. Med. Microbiol. 57, 136–138.
Yasur-Landau, D., Jaffe, C.L., David, L., Baneth, G., 2016. Allopurinol resistance in *Leishmania infantum* from dogs with disease relapse. PLoS Negl. Trop. Dis. 10, e0004341.
Yin, Z., Fujii, H., Walshe, T., 2010. Effects of active hexose correlated compound on frequency of CD4+ and CD8+ T cells producing interferon-γ and/or tumor necrosis factor-α in healthy adults. Hum. Immunol. 71, 1187–1190.