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Research paper

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

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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

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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