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Short Communication

Comparison of Standard Protocols for the Treatment of Canine Leishmaniasis in an Endemic Area with and Without Zinc Oral Supplementation

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Abstract

Successful treatment of canine leishmaniasis (CanL) depends on an effective cellular immune response. Zinc is an essential trace element for the immune system and in dogs with clinical leishmaniasis low serum zinc levels have been reported. The aim of this work was to evaluate the effect of zinc oral administration during treatment of CanL.

Eighteen dogs from an endemic area were enrolled showing clinical signs of leishmaniasis and diagnosed by positive parasitological and serological tests. Dogs were subdivided in three treatment groups: MA, meglumine antimoniate 50 mg/kg SC for 30 days with allopurinol 10 mg/kg PO BID for 7 months; MZ, meglumine antimoniate 50 mg/kg SC BID for 30 days with zinc 2.2 mg/kg/die PO for 12 months; MAZ, same as MA group plus supplemented with zinc 2.2 mg/kg/die PO for 12 months. Each dog was monitored for 12 months using clinical and skin scores and some blood biochemical markers.

Dogs in MZ and MAZ group showed a better and earlier improvement of clinical and skin scores in comparison to control dogs (MA group). Among few blood markers studied (hemoglobin, albumin, γ globulins and A/G ratio) dogs in MAZ group did improve and earlier than other groups suggesting that zinc improves the condition where allopurinol is also present.

The supplementation of zinc in the treatment protocol for CanL increased the serum zinc concentrations. In addition, preliminary data showed in group MZ and MAZ dogs a faster response to therapy and the elongation of the disease-free interval time.

INTRODUCTION

Canine leishmaniasis (CanL) due to *Leishmania infantum* is endemic in Mediterranean countries where it has been estimated that 50–80% of the general canine population is infected by *L. infantum* and the prevalence of CanL varies from 2% to 5% [1,2]. The *L. infantum* frequently follows an insidious and chronic pattern of infection [3]. Therefore, CanL is a disease in which infection does not equal clinical illness resulting in a high prevalence of subclinical infection. A broad range of immune responses and clinical manifestations have been described in CanL [3]. Infection in dogs may be subclinical or manifested as a self-limiting disease, or a severe, and sometimes, fatal illness [4]; therefore, cellular immune response against disease is fundamental for leishmaniasis regression [5].

Despite the fact that new drugs have been licensed and new protocols investigated, in the last 30 years the therapy of CanL did not showed any significant progress and the clinical response to treatment of sick dogs is variable [6]. Clinical cure is often obtained associated with a reduction in parasite load and infectiousness, but clinical recurrence might occur and an appropriate life-long post-therapy follow-up should be maintained [3,7].

Successful treatment of canine leishmaniasis (CanL) depends on an effective cellular immune response [8]. Zinc is an essential trace element for the immune system and its deficiency can interfere with immune system cells function, including T cells. In particular, zinc deficiency leads to a selective reduction in Th1 cytokines and enhances humoral response (Th2), which results in decrease of IFN-production and clinical expression of leishmaniasis [9-11]. Low serum zinc levels have been reported in many parasitological diseases in humans including cutaneous [12] and visceral leishmaniasis [13]. In CanL low serum zinc levels have been registered in few studies as well [14-16]. Alterations in oxidative stress [15,5], trace elements, and biochemical parameters in dogs naturally infected by CanL Heidarpour and co-authors have been registered [15]. In human patients with visceral leishmaniasis [17] the variation in essential trace

elements concentration has been associated with the chronicity of the disease.

Based on these assumptions, it can be argued that zinc supplementation would be useful as a co-therapeutic agent for CanL, particularly in endemic areas. The aim of this work was to evaluate the effect of zinc oral administration during the treatment of CanL in order to suggest a novel therapeutic approach.

A prospective clinical trial was performed using three groups of symptomatic dogs naturally-infected by *L. infantum* and treated with standard protocols for CanL, with and without oral zinc supplementation. The effectiveness of zinc treatment was evaluated by clinical and clinico-pathological observations throughout 1-year follow-up or until the onset of clinical relapses.

MATERIALS AND METHODS

Animals

Eighteen dogs, all crossbreed except for three purebred, of both sexes and age ranging from 1 to 9 years old, weight ranging from 12 to 34 kg were enrolled. All dogs came from a Mediterranean endemic area for CanL, besides Southern Italy [18,19].

The dogs' owners have given their enlightened consent and have accepted to follow the protocol planning, in compliance with the admission criteria: clinical signs of leishmaniasis, serologically negative for vector borne disease (VBD) (detected by multitest kit including *Dirofilaria immitis, Anaplasma phagocytophilum, Anaplasma platys, Borrelia burgdorferi, Ehrlichia canis, Ehrlichia ewingii,* SNAP® 4Dx Plus, IDEXX), positive for *L. infantum* by direct observation of lymph-node smears, positive antibody levels toward *L. infantum* (Immuno-Fluorescence Antibody Test [IFAT]: cut-off for positive if ≥ 1/80), serum creatinine < 1.4 mg/dl, Urine Proteins to Creatinine ratio (UPC) ≤ 1 (belonging to Stage 2, sub-stages a and b according to the LeishVet clinical stages classification) [4].

Causes of exclusion from the trial were: pregnant or lactating mares, treatment for leishmaniasis within 3 months prior to inclusion or use of systemic long-acting corticosteroid, animals having concurrent disorders that may interfere with the evaluation of response to treatment, and dogs showing adverse events requiring treatment or follow-up interruption.

After the pre-inclusion evaluations (clinical examination, complete blood count, serum biochemistry, serum protein electrophoresis and complete urinalysis including UPC), dogs were randomly allocated in three experimental groups as follow:

MA group (6 dogs) – meglumine antimoniate 50 mg/kg subcutaneously (SC) (Glucantime®, Merial, Lyon, France), twice a day, for 30 consecutive days in association with allopurinol (Zyloric®, Teofarma srl, Pavia, Italy)10 mg/kg per os (PO) twice a day, for 7 months; MZ Group (5 dogs)- meglumine antimoniate 50 mg/kg SC, twice a day, for 30 consecutive days in combination with zinc supplementation at 2.2 mg/kg/die PO (Zincogen Pet® pearl, NBF-Lanes, Milan, Italy) for 12 months; MAZ Group (7 dogs) - same treatment of the MA group but integrated with zinc at 2.2 mg/kg/die PO for a total of 12 months (Zincogen Pet® pearl, NBF-Lanes, Milan, Italy).

The inclusion of dogs into the study did not alter the timing of preventive measures with repellent substances against ecto-endoparasites (i.e. imidacloprid 10% plus permethrin 50% spot on, Advantix®, Bayer, Milan, Italy; permethrin 54, 5% plus fipronil 6,1% spot-on, Effitix®, Virbac, Milan, Italy). Body weight was noted at each clinical examination and the drug dosages were adapted to body weight throughout the treatment period.

Procedures

Complete clinical examination and blood and urine sampling were performed in each enrolled dogs at day (D) D0, D30, D60, D90, D150, D210, and D360. A clinical score was recorded at each follow-up; the score was calculated by adding one point for each of the 26 clinical parameters (i.e. adenopathy, pallor of mucous membrane, epistaxis, ocular lesions, amyotrophy, arthritis, etc.) listed in the score system proposed by Mirò and co-authors [20]. Furthermore, a specific cutaneous score has been assigned based on the severity of lesions (0 absence, 1 mild, 2 moderate, 3 severe) of 10 cutaneous signs (i.e. erythema, ulcers, nodules, alopecia, dyspigmentation, etc.) (score ranging from 0 to 30) [20]. On blood samples collected the following analysis at each follow-up have been performed: complete blood count (CBC) by ADVIA® 2120 SIEMENS analyzer (Siemens Healthineers, Italy) and blood smear evaluation, serum total protein (TP) and albumin (Alb), serum protein electrophoresis, Albumin/Globulin ratio (A/G), serum γ-globulins and Zinc serum concentration (Zn). Differently, serum values of Alanine Amino Transferase (ALT), Alkaline Phosphatase (ALP), Urea and Creatinine have been investigated only at D0, D30, and D60. IFAT has been performed at D0 and D360 and the percentage of reduction of IFAT titre has been determined in each group. Serum zinc concentration has been determined by using a Direct Colorimetric Method (Giesse Diagnostic SRL, Roma, Italy). For all biochemical determinations, including Zinc concentration, HITACHI 912A analyzer (Diamond Diagnostics, USA) have been used.

Changes in the clinical score (collected by all clinicians involved in the study) and laboratory parameters have been monitored in each group throughout the study time.

The time of response to treatment was monitored in each group along with number and time of relapses.

Statistical analysis

A 3 × 7 (group x follow-up time) checks was performed for CBC parameters, serum TP, Alb, A/G, γ-globulin, and Zn concentration, along with total clinical and skin score. Differently, a 3 × 3 (group x follow up time) checks were performed for serum ALT, ALP, Urea, and Creatinine. The data set was subjected to the two-way ANOVA test using the general linear model (SAS Inst. Inc., Cary, NC, USA, 2011). Each treatment protocol was then analyzed separately by applying the Turkey's post-hoc test for repeated measures in order to assess the differences between the times of each parameter investigated. All data were expressed as quadratic means values. The significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

In Figure (1) values of both totals clinical and skin score were reported. In all groups, the total clinical score decreased at D60

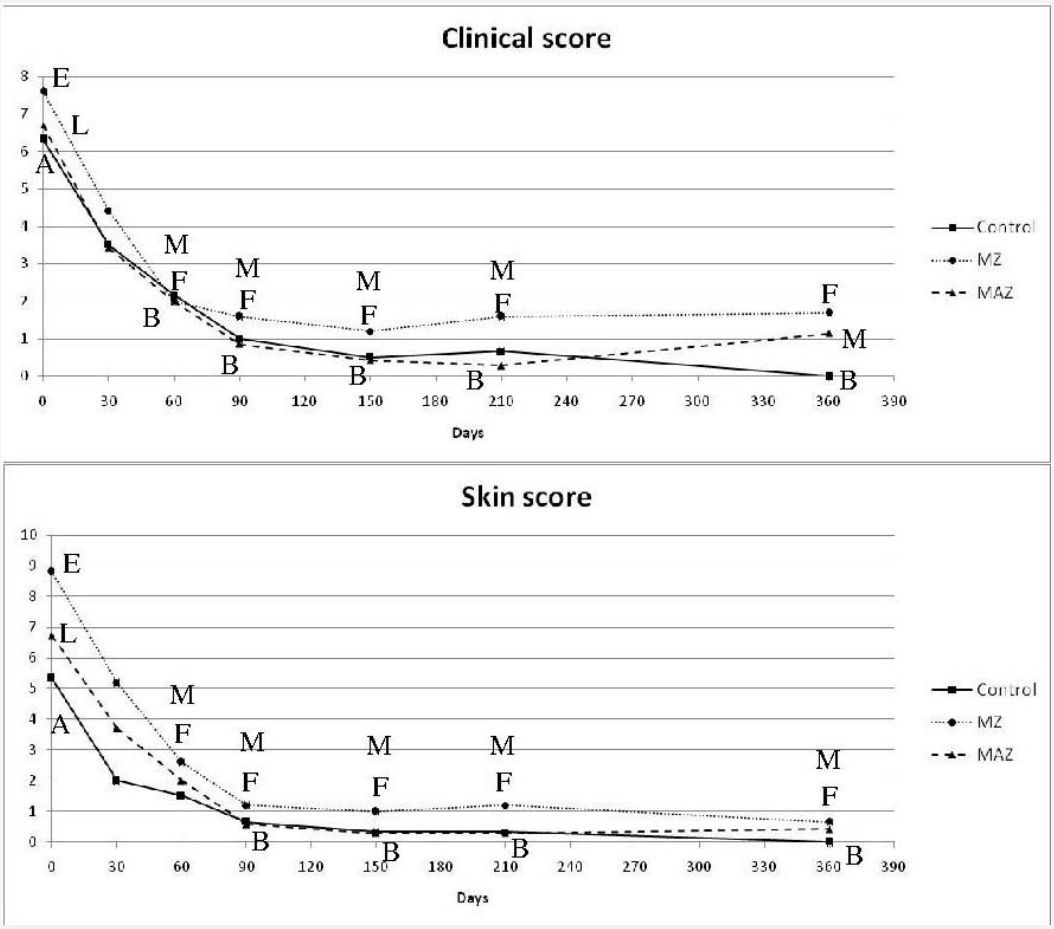

Figure 1 Clinical Score and Skin score in the three treatment groups at DO and in each follow-up (D30, D60, D90, D150, D210, and D360).Different letters in the same experimental group show statistical differences: Control group (MA): A, B = $p < 0.01$; MZ group: E, F = $p < 0.01$; MAZ group: L, M = $p< 0.01$.

($p < 0.01$) and remained constantly lower than D0 until D360 ($p < 0.01$). Also the skin score decreased showing a similar pattern. In particular, the MA group showed lower values than D0 ($p < 0.01$) from D90 up to D360, while MZ and MAZ groups showed lower values than D0 starting from D60 up to D360 ($p < 0.01$).

In Figure (2) results about hematocrit (HCT) and hemoglobin (Hb) values only in the three groups involved in the trial were reported. Other blood count parameters were also evaluated (such as RBC count, RBC indices, reticulocyte count, WBC total and differential count, PLT count) but they were not reported as no significant differences during the trial time and between the groups were noted ($p > 0.05$). HCT values showed an increase in D90 if compared to D0 ($p < 0.01$) in both MA and MAZ groups, thereafter values remained quite constant. Differently, HCT values did not change in MZ group during the trial ($p > 0.05$). Hb values showed an increase in the MA group with values at D90 higher than D0 and D30 ($p < 0.01$). In MAZ group Hb showed higher values than D0 ($p < 0.01$) at D60, while in MZ group no significant difference was observed ($p > 0.05$).

Figure (3) showed TP, Alb, A/G and γ-globulin concentrations. MA group at D0 showed TP values higher from D60 up to D 360 ($p < 0.01$). In MAZ group, the TP at D0 were higher than D90 up to D360 ($p < 0.05$). No differences for TP were observed during the trial in MZ group ($p > 0.05$). Albumin values of MA group from D90 up to D360 were higher than D0 ($p < 0.05$). Moreover, in MZ group Alb showed higher values than D0 from D60 up to D150 ($p < 0.05$). In MAZ group Alb values increased from D60 up to D360 if compared to D0 and D30 ($p < 0.01$). In MA group the A/G ratio at D60 ($p < 0.05$) and D150 ($p < 0.01$) showed higher values than D0. In MAZ group, at D30 ($p < 0.05$), at D60 ($p < 0.01$) and from D90 up to D360 ($p < 0.01$) the A/G ratio was higher than D0 ($p < 0.01$). No significant differences were observed in the A/G ratio of MZ group ($p > 0.05$). In MA group, γ-globulin levels from D90 up to D360 were lower than D0 ($p < 0.01$) and yet at D60 were lower than D0 ($p < 0.05$). In MAZ group, the γ-globulin levels from D60 up to D360 were lower than D0 ($p < 0.01$). No significant differences were observed in the MZ group about γ-globulin ($p > 0.05$).

ALT serum values at D30 were higher than D0 and D60 ($p < 0.05$) only in MA group. The other parameters (ALP, Urea and Creatinine) did not show any significant differences (data not shown).

Figure (4) showed the Zn serum concentration in the three groups involved in the trial. Serum zinc concentration at D0 was

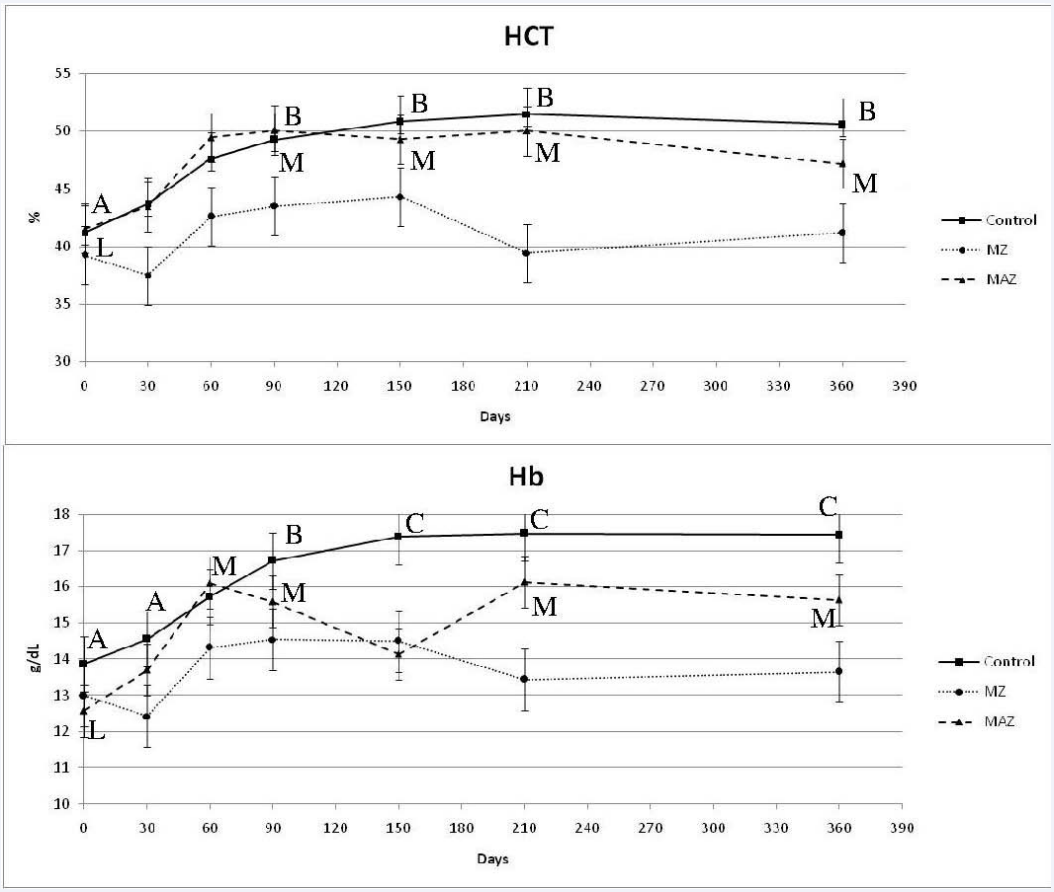

Figure 2 HCT (as %) and Hb (as g/dl) values registered in the three treatment groups at DO and in each follow-up (D30, D60, D90, D150, D210, and D360).Different letters in the same experimental group show statistical differences: Control group (MA): A, B, C = $p < 0.01$; MAZ group: L, M = $p < 0.01$.

quite similar in all groups. In MZ and MAZ groups Zn serum levels increased at D30 ($p < 0.05$) and remained higher than D0 for the entire trial period. Differently, no significant differences during experimental period were observed in MA group ($p > 0.05$). Moreover, from D30 up to D360, on each experimental time, Zn level in the MZ and MAZ groups were higher than MA group ($p < 0.01$).

The percentage of reduction of IFAT titre from D0 to D360 resulted in 4/7 dogs (57%) MAZ group, in 3/5 dogs (60%) MZ group, and in 1/6 dogs (16.7%) MA group.

Two dogs, one from MA group and one from MAZ group, showed clinical and laboratory signs of relapse of leishmaniasis, respectively at D210 and D360.

Results of this study show that the selected oral zinc supplementation induces an increase in serum zinc concentration. In fact, despite serum zinc concentration is comparable in all groups at D0, a significant increase at D30 and D60 was registered only in dogs receiving zinc supplementation, remaining constant throughout the study time.

Zinc supplementation in addition to the standard therapeutic protocol for CanL (MAZ group treated with meglumine antimoniate plus allopurinol) resulted in a faster response to treatment and prolongation of disease free interval time in treated animals. The positive effects of zinc integration could be due to various biological functions in which zinc is involved, especially immunological, inflammatory and antioxidant processes; a direct leishmanicidal activity has been observed as well [21,22].

Macrophages infected by *Leishmania* spp. generate highly toxic molecules such as reactive oxygen species (ROS) to kill the parasite [23]. To protect against ROS damage, vertebrate hosts possess a variety of antioxidant defenses that include antioxidant enzyme systems (i.e. Cu-Zn superoxide dismutase-SOD) requiring trace elements including Zn for their activity [15] .The importance of Zn, Cu, Se and Fe on the immune response to CanL, and their redistribution during the course of infection is well documented [14,15,24]. Although studies of human forms of leishmaniasis as well as in experimental animal models show an association between trace element serum levels and oxidative stress, only few reports of trace elements associated with CanL are available [16]. In recent study Souza and co-authors [16] found lower serum levels of Zn, Fe and Se and higher serum Cu in all infected dogs, especially those symptomatic, compared to controls, but they did not observe a correlation of Cu or Zn serum levels with SOD activity in any group. Changes in trace mineral metabolism are an integral aspect of acute-phase response and have been shown in CanL [14,24], possibly related to host susceptibility [25,12].

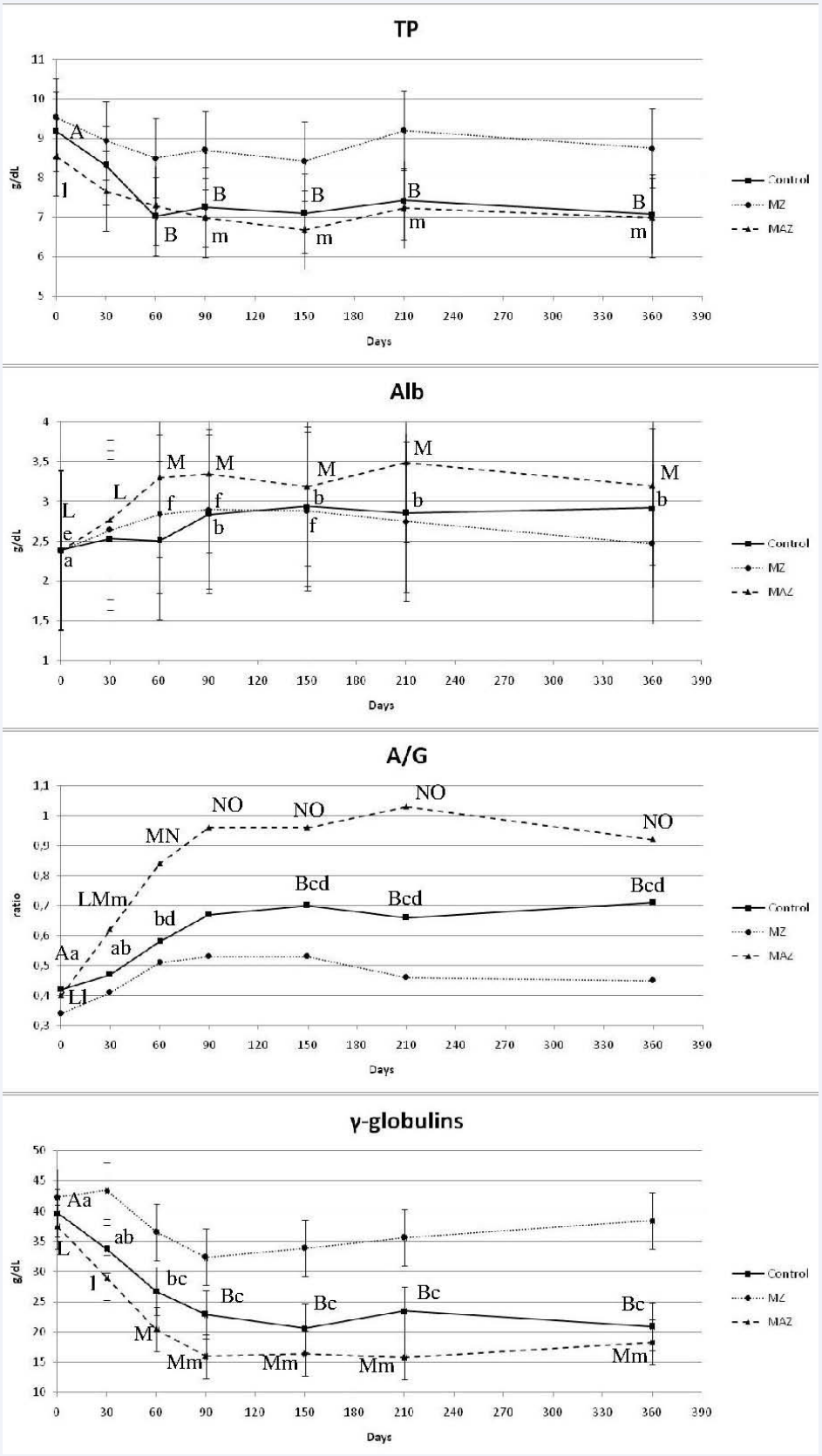

Figure 3 Total proteins (TP), albumin (Alb), albumin/globulin (A/G) ratio and γ-globulins serum concentrations (as g/dl) registered in the three treatment groups at DO and in each follow-up (D30, D60, D90, D150, D210, and 360).Different letters in the same experimental group show statistical differences: Control group (MA): A, B = $p < 0.01$; a, b, c, d = $p < 0.05$; MZ group: e, f =$p < 0.05$; MAZ group: L, M, N, O = $p < 0.01$; l, m =$p < 0.05$.

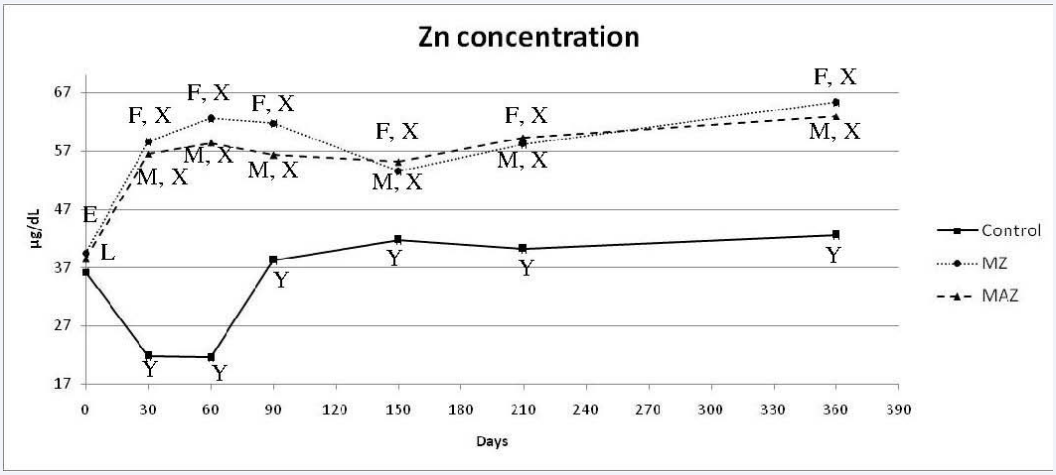

Figure 4 Zinc serum concentration at DO and in each follow-up (D30, D60, D90, D150, D210, and 360) in the three treatment groups. Different letters in the same experimental group show statistical differences: Control group (MA): $p > 0.05$; MZ: E, F = $p < 0.01$; MAZ: L, M = $p < 0.01$. Different letters in the same experimental days showed statistical differences between the groups (X, Y: $p < 0.01$).

The skin score registered showed a significant decrease between D0 and D90 in MA group and between D0 and D60 in both MZ and MAZ groups. Thus, dogs receiving oral zinc supplementation showed a faster improvement of cutaneous lesions associated to *L. infantum* infection if compared with dogs did not receiving zinc supplementation. Cutaneous lesions are common in CanL and often are the main sign of disease [26,18]. The beneficial effect of zinc in the treatment of lesions in human patient affected by cutaneous leishmaniosis has been previously reported [27].

Furthermore, in dogs receiving oral zinc supplementation associated to the standard protocol a faster improvement and/or reverse to normal of some laboratory parameters was registered. In this regard, in MAZ group significant changes ($p < 0.01$) in Hb, Alb, γ globulins and A/G were registered at D60. In MA group significant differences ($p < 0.01$) were observed at D90 for HCT, Hb, and γ globulin. The reverse to normal of A/G ratio, along with a reduction of the γ globulin concentrations, was obtained in MAZ group one month before the MA group. These parameters have been recognized as good indicators for disease development [28]. It is possible to assume that zinc immune-modulatory action was implicated in such results.

The positive effect of zinc supplementation to the standard protocol for leishmaniasis on the hemoglobin mean values increase is noticed, despite most of the dogs showed normal values at D0. It is accepted that anemia is not a constant finding in CanL [18].

Differently, the supplementation of zinc to animals treated only with meglumine antimoniate (group MZ) did not allow a good control of the disease pointing out that zinc supplementation need to be considered not as substitutive to allopurinol but as ancillary to it.

The ALT serum value shows a severe increase from D0 to D30 (i.e. during treatment with meglumine antimoniate) in MA group suggesting a possible consequence of the infection and/or of the treatment as previously reported [15,29,30]. Differently, ALT serum value remains unchanged in the two groups that receive oral zinc supplementation (MAZ and MZ). It could be argued a possible protective role of zinc on the hepatic damage.

Serological negativity is not expected after treatment. Treatment of sick dogs is often accompanied by a decrease in the specific antibody levels [4,31-33]. However, in other cases clinical improvement has not been associated with a decrease in the titer of specific antibodies [34]. In our study we had a percentage of reduction of IFAT titer higher in MAZ and MZ compared to MA suggesting the beneficial effects of zinc supplementation on immune response.

In this study clinical and laboratory signs of relapses were observed in two dogs, respectively at D210 in the MA group and at D360 in the MAZ group suggesting that zinc supplementation allowed an elongation of disease free interval time.

CONCLUSION

Results of this study encourage the opportunity for a supplementary oral intake of zinc in order to enhance and improve the effectiveness of treatment of CanL, nevertheless further data on a larger population are advisable to support this statement. Indeed, the supplementation of zinc PO in the standard therapeutic protocol for CanL results in the increase in serum zinc concentration with possible advantages in terms of faster response to therapy and prolongation of disease free interval time in treated animals.

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