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Levamisole enhances global and differential leukocyte numbers in peripheral blood of dogs with ehrlichiosis

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Abstract: Ehrlichiosis is a disease caused by *Ehrlichia*, a genus of obligatory intracellular bacteria that parasitize leukocytes and provoke several hematological alterations. In this work, we investigated the effect of levamisole associated with doxycycline on peripheral leukocytes in dogs PCR-positive for ehrlichial DNA. Twenty-one *Ehrlichia*-infected dogs were randomly distributed in 2 groups: a control group (n = 10) and a levamisole-treated group (n = 11). Blood samples were collected from each animal for hematological and DNA analysis both before initiating the experiment and 5 days later. All dogs were treated with doxycycline (10 mg/kg body weight), cimetidine (5 mg/kg of body weight), and fluid therapy for 5 consecutive days. In addition to receiving antibiotic therapy, the levamisole-treated dogs received a single subcutaneous injection (1 mg/kg body weight) of levamisole on the first day of the experiment. The results show that levamisole associated with doxycycline significantly enhanced global leukocyte, lymphocyte, and monocyte numbers in the peripheral blood of dogs with ehrlichiosis. Those data suggest that the combination of doxycycline with levamisole may improve canine ehrlichiosis treatment through impairment of leukopenia, which is not controlled by antibiotic therapy alone.

Key words: *Ehrlichia canis*, ehrlichiosis, levamisole

1. Introduction

Ehrlichiosis is an emergent tick-borne bacterial disease that affects several animal species, including livestock and companion animals (1). Moreover, ehrlichiosis is an important public health problem since *Ehrlichia canis* and *E. chaffeensis* are causes of human monocytic ehrlichiosis (2–4). *E. canis* is the primary agent of canine monocytic ehrlichiosis (5) but *E. canis* and *E. ewingii* have been identified as causes of canine ehrlichiosis in Brazil (6,7). Based on experimental infection of dogs with *E. canis*, canine monocytic ehrlichiosis is divided into acute, subclinical, and chronic phases (8–12). Accordingly, in the acute phase, leukopenia, thrombocytopenia, fever, anorexia, and depression are observed. During the subclinical phase, clinical signs are minimal, but leukopenia, anemia, and thrombocytopenia can still be observed (13). The chronic phase of canine monocytic ehrlichiosis may be severe or mild with recurrent clinical and hematological signs that include lymphocytosis, monocytosis, pancytopenia, hemorrhage, and weight loss (8,10,14–18). Ehrlichial diseases are remarkable for their uniform susceptibility to doxycycline but failures to treat *Ehrlichia*-infected dogs have unfortunate and sometimes tragic outcomes, mainly due to hematological alterations that are not controlled by antibiotic therapy (19). Since ehrlichiosis causes severe abnormalities in the immune response of dogs (12), we reasoned that treatment of naturally *Ehrlichia*-infected dogs would be improved through up-regulation of the immune response with levamisole, which is a potential Th1-biased compound (20,21). To test this hypothesis, we evaluated the effects of levamisole on the global and differential number of peripheral leukocytes in dogs with ehrlichiosis and showed that combining doxycycline therapy with levamisole significantly increased peripheral leukocytes and promoted a better recovery based on several clinical signs.

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2. Materials and methods

2.1. Animals

Twenty-one *Ehrlichia*-infected dogs were randomly distributed in 2 groups: a control group (n = 10) and a levamisole-treated group (n = 11). This study included female and male dogs of several breeds and ages that were naturally infected with *Ehrlichia* spp. and were PCR-positive for ehrlichial DNA. Dogs that had recently received a blood transfusion or were taking corticosteroids, as well as those that were newborn, pregnant, breast-feeding, or had any concomitant disease, were excluded from this study. The experiment was conducted in accordance with the institutional guidelines for animal welfare (protocol #23108.021390/08-4).

2.2. Polymerase chain reaction (PCR)

Blood samples from dogs suspected of having clinical ehrlichiosis were collected into plastic tubes containing EDTA and a DNA extraction of whole blood was carried out as previously described (22). The DNA samples were initially processed using a PCR protocol designed to amplify a 409-bp fragment of the dsb gene of the genus *Ehrlichia* (23) using primers 330 forward (5' GATGATGTCTGAAGATATGAAACAAAT 3') and 728 reverse (5' CTGCTCGTCTATTTTACTTCTTAAAGT 3)'. *Ehrlichia* spp. DNA was detected in the peripheral blood of dogs by using PCR amplification of a genus-specific disulfide bond formation protein (23). PCR products were resolved on 2% agarose gels and visualized using ethidium bromide staining under UV transillumination. DNA from a São Paulo strain of *E. canis* was used as the positive control and water as the negative control.

2.3. Clinical assessment

Before initiating the experiment, dogs that were PCR-positive for ehrlichial DNA were assessed for clinical signs such as lethargy, icteric mucous membranes, splenomegaly, abdominal petechiae, lymphadenopathy, anorexia, epistaxis, uveitis, and hydration condition. A rectal temperature check was also performed, and pulse and respiration were noted. Infected dogs included in this study were held in the veterinary hospital of the University of Cuiabá (UNIC, Cuiabá, MT, Brazil) for 5 days. During the experiment, all dogs were monitored daily for signs of illness and at the end of the treatment they were clinically assessed again.

2.4. Hematologic evaluations

Peripheral blood was collected in sterile tubes containing anticoagulant (EDTA) and assessed for global leukocyte and erythrocytes counts using a Neubauer chamber and differential blood cells and platelets on smears stained with a fast panoptic stain (Laboclin®).

2.5. Treatment

The antibiotic therapy for *Ehrlichia*-infected dogs consisted of doxycycline (Adoxa®) (10 mg/kg of body weight, orally) and cimetidine (Tagamet®) (5 mg/kg of body weight, intravenously). Both medicines were administered once a day for 5 consecutive days. When it was required, animals also received fluid therapy. Before starting the treatment, 11 *Ehrlichia*-infected dogs also received a subcutaneous injection of levamisole (Ripercol® L Solution; 1 mg/kg of body weight) and the other 10 *Ehrlichia*-infected dogs were subcutaneously injected with saline as the control.

2.6. Statistical analysis

An experimental model with 2 groups and at least 10 repetitions was used. The results obtained from the hematological evaluations were treated with one-way analysis of variance (ANOVA) followed by the Tukey test using SAEG 5.0 software. $P \leq 0.05$ was considered significant.

3. Results

3.1. Effect of levamisole on the global number of leukocytes in dogs with ehrlichiosis

To verify whether levamisole would increase the global number of leukocytes in dogs naturally infected with *Ehrlichia*, blood samples were collected from each animal before starting the treatment and after a 5-day doxycycline therapy associated or not associated with levamisole, and they were assessed for complete blood counts as described in Section 2. As shown in Table 1, at the end of the experiment, the levamisole-treated dogs reached significantly higher levels of global leukocytes than the control animals ($P = 0.04$). It should be noted that before initiating the treatment, 1 dog of the control group and 2 animals of the levamisole-treated group had leukopenia, but there was no statistical difference between the 2 groups ($P = 0.1$). Notably, by the end of the experiment, those 3 animals had recovered from leukopenia, but 3 other dogs of the control group and 1 animal of the levamisole-treated group developed leukopenia (data not shown).

3.2. Evaluation of the levamisole effect on subsets of leukocytes in dogs naturally infected with *Ehrlichia*

As levamisole significantly augmented the global number of leukocytes in dogs with ehrlichiosis, we then evaluated leukocyte subsets on blood smears stained with the fast panoptic stain. At the end of the experiment, the levamisole-treated dogs reached significantly higher counts of lymphocytes compared to the control group (Table 1; $P = 0.02$). Before initiating the experiment, 8 out of 11 animals of the levamisole-treated group and 3 out of 10 dogs of the control group had lymphopenia, but that difference was not significant ($P = 0.34$). At the end of the treatment, only 1 dog of the levamisole-treated group did not return to normal values of lymphocytes, while 5 animals of the control group were lymphopenic (data not shown). Our results corroborate data from Sousa (24), who showed that levamisole increases the global levels of leukocytes and absolute numbers of lymphocytes in mice.

Table 1. Total and subsets of peripheral leukocytes in dogs with ehrlichiosis. Different superscript letters indicate statistical differences.

Peripheral leukocytes ($\times 10^6$/mL)	Control group		Levamisole-treated group	
	Day 0	Day 5	Day 0	Day 5
Total leukocytes	5.200 ± 970	8.450 ± 1.374	4.327 ± 1.228[a]	10.627 ± 2.757[b]
Lymphocytes	1.104 ± 176	1.705 ± 346	957 ± 433[a]	2.793 ± 1.229[b]
Mature neutrophils	4.109 ± 1.121	5.924 ± 1.306	3.312 ± 1.450	7.419 ± 2.555
Immature neutrophils	40 ± 41	123 ± 59	55 ± 61[a]	63 ± 69[b]
Monocytes	58 ± 61	132 ± 36	41 ± 35[a]	219 ± 99[b]

As shown in Table 1, after a 5-day doxycycline therapy, all of the levamisole-treated dogs returned to normal value ranges of neutrophils and only 1 dog of the control group remained neutropenic, but that difference was not significant (P = 0.1). Notably, before starting the treatment, 5 out of 10 dogs of the control group and 7 animals of the levamisole-treated group were neutropenic. However, that difference was not statistically significant (P = 0.18). Even though levamisole associated with doxycycline did not significantly enhance the number of mature neutrophils, we evaluated the number of peripheral immature neutrophils and observed that the levamisole-treated dogs had significantly lower counts of peripheral immature neutrophils than the control animals (Table 1; P = 0.05). Therefore, we postulate that levamisole may stimulate the immune response, which in turn would impede the massive neutrophil deaths caused by *Ehrlichia*, and consequently a lower number of immature neutrophils would be output from the bone marrow.

In this work, we also analyzed the number of monocytes on blood smears from dogs naturally infected with *Ehrlichia*. The data presented in Table 1 show that 5 days after treatment, the levamisole-treated animals reached significantly higher numbers of monocytes than the control dogs (P = 0.02). It should be noted that before starting the treatment, there were no animals with monocytosis. Instead, 8 out of 10 dogs of the control group and all animals of the levamisole-treated group had decreased numbers of monocytes.

3.3. Evaluation of the levamisole efficacy on erythrocyte and platelet counts and hematocrit values in dogs naturally infected with *Ehrlichia*

It is well known that besides causing several abnormalities in white cells, *Ehrlichia* also provokes anemia and thrombocytopenia in infected dogs (19). Therefore, in this work we evaluated erythrocyte and platelet counts and hematocrit values in dogs with ehrlichiosis. Before starting the experiment, 7 out of 10 dogs of the control group had anemia, and after 5 days of doxycycline therapy, 3 dogs remained anemic (Figure). Before initiating the treatment, 7 out of 10 dogs of the control group also had reduced levels of hematocrit, and 2 of them remained with low values 5 days later (data not shown). At the beginning of the experiment, 8 out of 11 of the levamisole-treated group were anemic (data not shown). Five days later, only 3 of the 11 dogs that received levamisole did not recover from the anemia. At day 0, 9 dogs that would receive levamisole had low hematocrit values, and at the end of the treatment, only 2 animals of the levamisole-treated group did not return to normal hematocrit values. Considering anemia and hematocrit values, there was no statistical difference between the levamisole-treated animals and the control dogs (P = 0.4). Since *Ehrlichia* also decreases platelets counts in infected-dogs, we also evaluated the numbers of platelets. Before starting the therapy, all of the dogs with ehrlichiosis had reduced platelets counts (data not shown). However, the dogs that would receive levamisole had significantly lower numbers of platelets than the control animals (P = 0.001). At the end of the experiment, 5 dogs of the control group and 8 animals of the levamisole-treated group continued to have low numbers of platelets (data not shown).

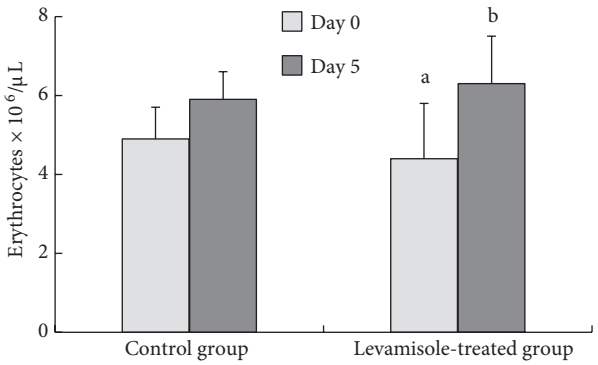

Figure. Levamisole's effect on erythrocytes in dogs naturally infected with *Ehrlichia*. Peripheral blood was collected from control animals and from levamisole-treated dogs and assessed for erythrocytes counts using a Neubauer chamber. Data are shown as mean ± SD.

3.4. Clinical signs of dogs in which ehrlichial DNA was detected by PCR

The animals included in this study entered the veterinary hospital of the University of Cuiabá (UNIC) presenting the following clinical signs: apathy, anorexia, abdominal petechiae, dehydration, icteric mucous membranes, lymphadenopathy, and splenomegaly (Table 2). Some animals also presented epistaxis, uveitis, and fever (data not shown). PCR amplification of peripheral blood samples detected ehrlichial DNA in 21 out of 40 dogs clinically suspected of having ehrlichiosis. On day 5, when the last data were recorded, all levamisole-treated dogs had recovered from anorexia and abdominal petechiae while some control animals were still presenting those clinical signs (Table 2).

4. Discussion

Levamisole, a synthetic phenylimidazothiazole, is a potent anthelmintic agent that was introduced in 1966 (25) and has been used to improve the treatment of colon cancer (26). Current studies have been focused on the effect of levamisole on the immune response as a potential Th1-biased compound (20,21). Regarding levamisole as a Th1-enhancing agent, it was useful in the treatment of leprosy based on an Indian clinical trial (27) and has been used to treat and prevent atopic diseases in the mouse model (28). In this work, we evaluated the effect of levamisole associated with doxycycline on peripheral leukocytes in dogs PCR-positive for ehrlichial DNA and showed that doxycycline plus levamisole significantly enhanced global leukocyte, lymphocyte, and monocyte numbers in the peripheral blood of dogs naturally infected with *Ehrlichia*. The mechanism(s) by which levamisole enhances peripheral leukocytes in dogs with ehrlichiosis was not determined, but one could postulated that levamisole may reinforce the *Ehrlichia*-lipopolysaccharide effect on macrophages and dendritic cells to secrete IL-12 to help T cell activation. As is well known, IL-12 promotes Th1 immune response by inducing the production of large amounts of INF-γ by T and NK cells (29). The hypothesis that levamisole acts on the macrophages and dendritic cells of dogs is supported by a previous report showing that levamisole increased the production of IL-10 and IL-12 by human monocyte-derived dendritic cells (20). As *Ehrlichia* are intracellular bacteria that mainly infect the monocytes/macrophage phagocytic system, an immune response polarized toward the Th1 profile would be more protective for dogs with ehrlichiosis.

As shown, overall levamisole increases global leukocyte, lymphocyte, and monocyte numbers in the peripheral blood of dogs with ehrlichiosis; however, one dog of the levamisole-treated group developed leukopenia during the treatment. Because of that, we postulate that other doses of levamisole and different frequencies of administration of this medicine should be tested to standardize a protocol that could be used in association with antibiotic therapy for successful treatment of ehrlichiosis in dogs. Independent of the remarkable susceptibility of *Ehrlichia* to doxycycline, 3 dogs of the control group developed leukopenia during the treatment. These results reinforce the postulation that leukopenia may progressively continue until the potential elimination of the *Ehrlichia* infection in dogs.

Levamisole associated with doxycycline did not alter the number of mature neutrophils. Nevertheless, our results are in agreement with previous studies carried out in caprines that reported a lack of effect of levamisole on the absolute number of mature neutrophils (30). However, the dogs treated with doxycycline associated with levamisole had fewer immature neutrophils compared to the control group. The mechanism(s) by which levamisole enhances

Table 2. Effect of levamisole on clinical signs in dogs with ehrlichiosis.

Clinical signs	Control group		Levamisole-treated group	
	Day 0	Day 5	Day 0	Day 5
Apathy	9/10	4/10	9/11	3/11
Pallid mucous	2/10	0/10	3/11	0/11
Splenomegaly	5/10	3/10	7/11	3/11
Petechial hemorrhage	2/10	1/10	4/11	0/11
Lymphadenopathy	5/10	3/10	7/11	2/11
Anorexia	7/10	3/10	4/11	0/11
Epitasis	3/10	0/10	1/11	0/11
Uveitis	0/10	0/10	1/11	0/11
Dehydration	3/10	0/10	3/11	0/11

lymphocyte and monocyte numbers and decreases immature neutrophils in dogs with ehrlichiosis was not studied but we hypothesize that levamisole may activate lymphocytes, which via cytokine production would activate monocytes and macrophages for the elimination of *Ehrlichia*, which in turn would prevent neutrophils from dying.

According to our data, it seems that levamisole associated with doxycycline does not change the usual profile of anemia, platelet counts, and hematocrit values observed in *Ehrlichia*-infected dogs. It has been shown that *Ehrlichia*-infected dogs slowly return to normal numbers of platelets from the seventh week after infection (31). The slow recovery from thrombocytopenia is attributed at least in part to *Ehrlichia* activation of a factor that inhibits platelet migration from the bone morrow to the peripheral blood, faster platelet death, and production of antibodies against platelets (19).

Besides increasing peripheral leukocytes, levamisole also improved some clinical signs in dogs with ehrlichiosis. As far as we know, we are showing for the first time that levamisole enhances peripheral leukocytes and improves the antibiotic treatment of dogs with ehrlichiosis. In general, our data indicate that using levamisole associated with doxycycline to treat dogs with ehrlichiosis may improve animals' immune response, which in turn would contribute to a better clinical recuperation from ehrlichiosis.

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