

Original Article

Hematological profile in natural progression of giardiasis: kinetics of experimental infection in gerbils

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Abstract

Introduction: The clinical manifestations of giardiasis and its impact are harmful to children, and may cause deficits in their physical and cognitive development. The pathogenic mechanisms are usually unknown and the available reports can be controversial.

Methodology: The present study aimed to know, for the first time, the evolution of the hematological profile of the gerbils, experimentally infected with *Giardia lamblia*, up to the infection's resolution. Hematological variables have been tested.

Results: White blood cells have not presented meaningful alterations during the course of the infection. A significant reduction in the number of red blood cells ($p = 0.021$), in the concentration of hemoglobin ($p = 0.029$) and in the value of the hematocrit ($p = 0.016$) has been observed, starting from the second week of infection, ratifying an anemia related to giardiasis. Reduction in the level of serum iron starting from the third week of infection, despite not being significant, could suggest the participation of iron in the anemia. However, the weight of the animals was kept and the hematimetric parameters started to return to the basic values after the parasitological cure without iron reposition.

Conclusions: The outcomes found suggest the idea that not only malabsorption but also other mechanisms such as chronic inflammation may be implicated in iron deficiency anemia in giardiasis and may explain how asymptomatic patients may have anemia without malabsorption. In this context, considering the highlighting character of the anemia in our study, we believe that anemia should be investigated in children with giardiasis. And in the cases of anemia without a definite etiology, giardiasis should also be investigated.

Key words: *Giardia lamblia*; experimental giardiasis; hematological profile; anemia.

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Introduction

Giardia lamblia, the causal agent of giardiasis, is one of the most frequent parasites in animals as well as humans, in both developed and underdeveloped countries. The prevalence ranges from 2% to 30% [1,2]. Among the infected ones, 50% can develop symptoms, which may vary in acute and chronic infections, even causing a deficit in the physical and cognitive development of children [3]. Among the symptoms, we highlight diarrhea and malabsorption, which can be caused by the parasites' induced enterocyte apoptosis [4]. In this case, it can cause an increase in the intestinal permeability and shortening of the epithelial microvilli, reducing the secretion of disaccharidases [5] and hypersecretion of anions, contributing to losses in the

absorption of lipids [6], fat-soluble vitamins [7], zinc, iron [8] and sodium [9]. Asymptomatic infections often resolve spontaneously [10]. As in every infection, the clinical variability is related to the host and parasite's characteristic factors. There are many controversies among the available studies [11]. The fundamental questions of the disease's pathogenesis are still unanswered and there is a lot more to be known and brought to light, especially concerning the natural progression of the disease. The recognition of the dysfunctions caused by *G. lamblia* may provide insights in identifying the markers of the infections' severity, to eliminate the parasite, as well as to treat the effects caused by it. In this context, what calls for attention is that the hematological profile of the infection is

unknown - at both the experimental and human infection level. In this study, the natural progression of the infection by *G. lamblia* and its alterations in the hematological profile of gerbils infected experimentally was evaluated.

Methodology

Experimental, longitudinal and prospective studies of male gerbils infected with *Giardia lamblia* were conducted for 5 weeks (until spontaneous parasitological cure), which assessed clinical variables of the animals (weight and appearance of pelage and feces) and hematological variables.

Animals

Eight male gerbils (*Meriones unguiculatus*), aged 4-6 weeks were obtained from the bioterium of the Department of Parasitology, Federal University of Minas Gerais. The animals were maintained in individual cages, under standard laboratory conditions: a 12:12 light/dark cycle, 40% humidity, and controlled temperature ($23 \pm 3^\circ\text{C}$). The gerbils were given filtered water and autoclaved rodent chow, *ad libitum*.

The animal studies were approved by the local Ethics Committee for Animal Experiments (CEUA-UFMG, protocol number 342/2015).

Parasite and growth conditions

G. lamblia infection was performed using trophozoites Portland strain (ATCC 30888), kept at 37°C in axenic culture in medium TYI -S -33 modified [12] and supplemented with bovine bile.

Experimental infection determination

The animals were infected by gavage using a cannula DELVO N° 15 coupled to a 1 mL syringe, with 0.3 mL suspension of 1×10^6 *G. lamblia* trophozoites. To prove infection, the fecal material was collected every two days and processed for morphological identification of cysts or trophozoites of *G. lamblia* by optical microscopy. All fecal material examined under a microscope was also evaluated for fecal antigens of *Giardia* by coproantigens method. Once the stool test came back negative for coproantigens and microscopy in three consecutive trials, no further collection of feces was done and the infection was considered resolved. The aspect and consistency of the feces were also evaluated.

Collection of blood samples

Blood was obtained by lateral tail vein puncture. These animals had blood samples taken at time zero

(control group) and after inoculation of *G. lamblia* trophozoites, every seven days until the thirty-fifth day. The gerbils were administered ketamine (100 mg/kg) and Xylazine (12 mg/kg), both from SESPO Industry, (São Paulo, Brazil). The euthanization was confirmed with cervical dislocation.

Histological analysis

The proximal portion of the small intestine was fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections were cut into 4 μm slices and stained with hematoxylin and eosin for morphological observation.

Hemogram determination

Samples of blood collected in EDTA were used to carry out the complete hemogram. Red and white blood cell counts were determined in an automated veterinary hematology analyzer (pocH-100iV Diff – Sysmex, Paraná, Brazil). The hematimetric indices of mean globular volume (MGV), mean globular hemoglobin (MGH) and mean globular hemoglobin concentration (MGHC) were calculated as previously described [13]. The differential leukocyte count was processed to establish the percentages of each cell type (relative values) and transform them into absolute numbers. The automatic cell counter was calibrated to rodent's samples.

Real-time reverse transcription-polymerase chain reaction analysis for mRNA expression of IL-6

mRNA expression of IL-6 was assessed using real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA isolated from the proximal portion of the small intestine homogenate was reverse transcribed into cDNA and used for PCR with Mongolian gerbil-specific primers (forward: AGGATCCAGGTCAAATAGTC and reverse: CGTCTGTGACTCCAGTTTC). Real-time RT-PCR reactions were prepared using SYBR Green PCR Master Mix reagents (Thermo Fisher Scientific, Waltham, USA). The GAPDH gene was amplified in the same reaction and served as the reference gene.

Statistical analyses

We used the Shapiro-Wilk test on the analysis of normality of the variables, all of them presenting normal distribution. Thus, between the groups, we used the t-Student test for paired samples or parametric ANOVA for repeated measures with Mauchly's sphericity test, and multiple comparison test or parametric ANOVA one-way with Tukey's test,

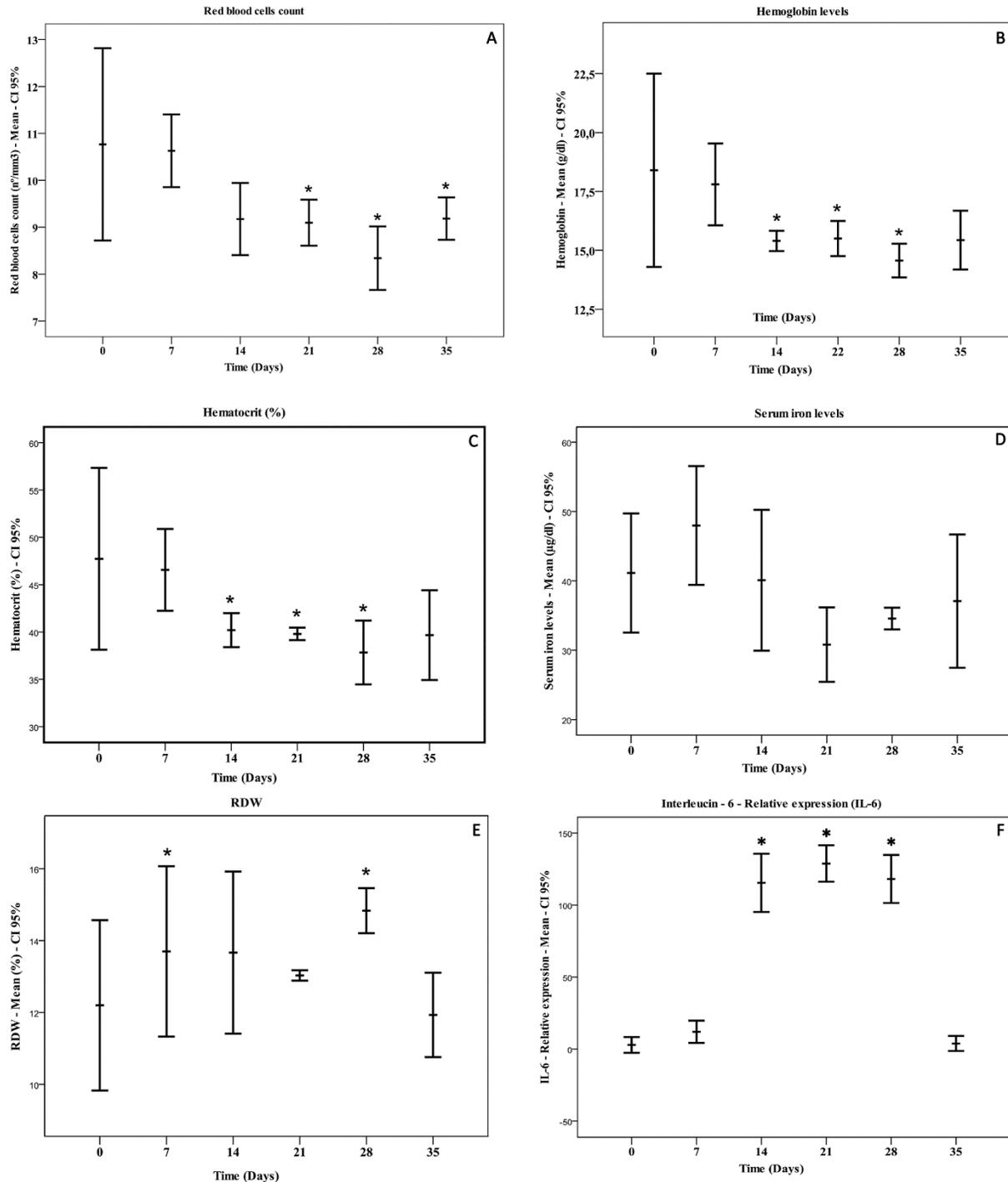
depending on the analyzed situation. Categorical data is shown as proportions and percentages, whereas continuous variables are described as the mean ± standard deviation. Correlation analysis was performed between continuous variables in the evaluation of their evolution over time. Data was analyzed using SPSS 23

(IBM, New York, USA) and $p < 0.05$ was considered significant.

Results

All experimental animals were evaluated before *Giardia* infection by stool examination and *Giardia*

Figure 1. Giardiasis progression in experimentally infected gerbils. Mean of red blood cell (A), hemoglobin (B), hematocrit (C), serum iron (D), RDW (E) and IL-6 (F) values before infection (time 0) and after different times of infection (7 = 7 days, 14 = 14 days, 21 = 21 days, 28 = 28 days, 35 = 35 days). *Significant difference with baseline values (time 0).



coproantigens detection. None of them was tested positive for any enteroparasite or *Giardia*. Cysts and coproantigens were detected in the feces of all animals on the fourth day post infection (DPI). Some coproantigens were negative by day 21 post infection. By 35 DPI, no animal was found positive. Cysts could be easily identified in the feces by direct examination, which also became negative around 32 DPI. The aspect and consistency of the feces were normal at macroscopic examination.

Significant anemia was observed in infected animals. In Figure 1A, we can observe a drop in red blood cells (RBC) counts from 10.48 ± 0.39 in the non-infected group with a nadir of 8.68 ± 0.23 at 28 DPI ($p < 0.05$). Anemia was detected only after the second week of infection and it was present during the observation, beginning to rise by the 35 DPI.

Similar variations were observed for hemoglobin and hematocrit levels. Hemoglobin (Figure 1B) was found at 17.82 ± 0.73 in the control animals and displayed decreasing levels after infection with a nadir of 15.04 ± 0.32 at day 28 ($p < 0.05$).

Hematocrit (Figure 1C) was at a baseline of 46.12 ± 1.8 and decreased significantly until reaching 37.80 ± 0.91 at day 28 ($p < 0.05$). Hemoglobin and hematocrit begin to return to baseline levels at 35 DPI.

Despite a trend of decreased levels of serum iron after the third week of infection (Figure 1D), we could not observe statistically significant variations ($p = 0.069$) of this parameter. The levels of serum iron returned to baseline at 28 DPI.

Red blood cell distribution width (RDW) was at a baseline of $12.08 \pm .32$ and increased until 14.68 ± 0.17

at day 28. RDW returned to baseline levels at 35 DPI (Figure 1E).

Significant increase in IL-6 values was observed from the second week of infection, and it remained elevated throughout the infection period. (Figure 1F)

Other blood parameters like (VCM, HCM, CHCM e platelets counts) as well as leukogram did not show significant alterations (Table 1).

The histological aspect of the duodenum of the animals infected with *G. lamblia* showed intestinal villus shortening, increased lamina propria, at the expense of intense inflammatory infiltrate, edema and hyperplasia of the intestinal crypts (Figure 2B), compared to the duodenum of animals without infection (Figure 2A).

Discussion

Up to now, the reports on hematological alterations induced by the giardiasis are rare [14-16]. There are no studies evaluating the complete blood count, from infection up to the spontaneous cure. Aiming to diminish this gap, we have monitored the hematological parameters of gerbils during *G. lamblia* infection.

The leukocytes have increased in the course of infection. However, no significant differences in the relative and absolute numbers were observed. These results would be expected if we consider that *Giardia* does not invade tissues and will not induce significant cell infiltration [17]. However, the parasite can induce local and systemic inflammatory response [18]. The intensity of this response could relate to the ability of certain strains in injure tissues. Some studies have shown trophozoites of *Giardia* invading the mucosa and submucosa [19-22]. These findings may partly

Figure 2. Morphological evaluation of duodenum of gerbils stained by hematoxylin and eosin. (A) Animals without infection. Duodenum with normal histological aspect, showing the lamina propria and intestinal crypt with its normal cellularity. (B) Animals infected with the Portland strain (ATCC 30888). Duodenum with shortening of intestinal villi, increase of lamina propria area at the expense of intense inflammatory infiltrate and edema. Hyperplastic intestinal crypts. Bar = 200 μm. muscularis mucosae (mm); intestinal villi (v).

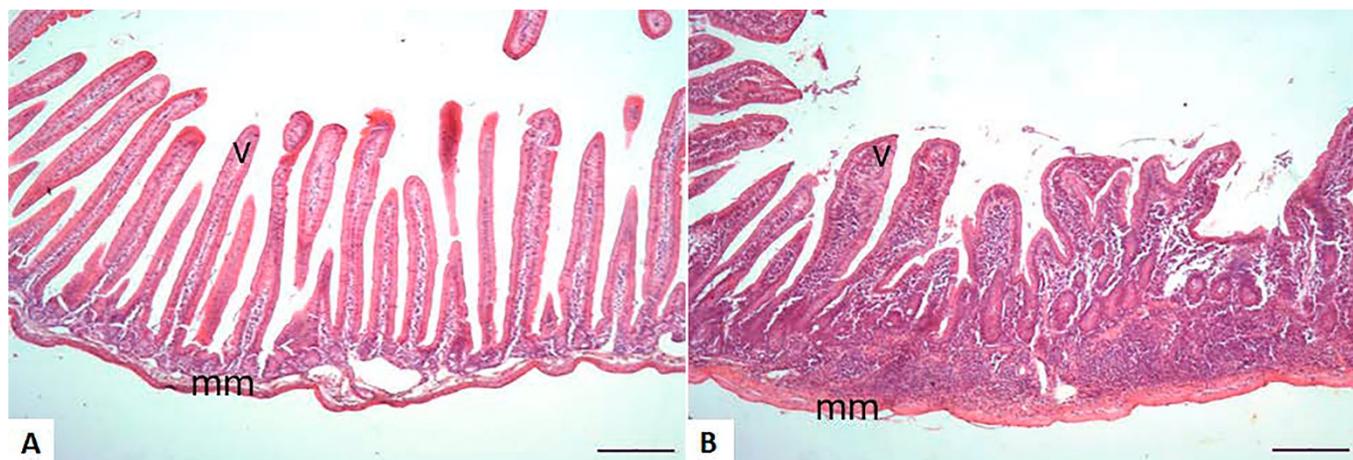


Table 1. Hematological values before and after *Giardia* infection of gerbils.

Hematological variables*	Progression of giardiasis infection (days post infection –DPI)					
	Baseline	7 DPI	14 DPI	21 DPI	28 DPI	35 DPI
RBC × 10 ⁶ mm ³	10.48 ± 0.39	10.63 ± 0.18	9.38 ± 0.19	8.89 ± 0.15	8.68 ± 0.23	8.92 ± 0.28
Hb g/dL	17.82 ± 0.73	17.8 ± 0.40	15.62 ± 0.17	15.32 ± 0.15	15.04 ± 0.32	15.63 ± 0.37
Hct %	46.12 ± 1.80	46.57 ± 1.00	40.84 ± 0.54	39.28 ± 0.34	37.8 ± 0.91	38.55 ± 1.36
MCV fL	44.02 ± 0.26	43.81 ± 0.75	43.88 ± 0.41	44.19 ± 0.41	45.08 ± 0.45	43.22 ± 0.56
RDW %	12.08 ± 0.32	13.7 ± 0.55	13.46 ± 0.34	13.34 ± 0.25	14.68 ± 0.17	12.13 ± 0.27
MCH μm ³	17.0 ± 0.9	16.75 ± 0.32	16.81 ± 0.23	17.23 ± 0.14	17.35 ± 0.22	16.97 ± 0.20
MCHC g%	38.63 ± 0.14	38.22 ± 0.7	38.31 ± 0.18	39.00 ± 0.19	38.47 ± 0.21	39.29 ± 0.56
WBC (×10 ³ mm ³)	10.58 ± 1.26	11.63 ± 2.71	12.76 ± 1.88	11.72 ± 1.07	13.24 ± 2.5	12.7 ± 2.37
SN (×10 ³ mm ³)	14.9 ± 8.8	6.19 ± 2.2	6.47 ± 1.1	7.38 ± 0.64	8.68 ± 2.09	5.41 ± 1.01
BN (×10 ³ mm ³)	0.31 ± 00.8	0.27 ± 0.02	0.198 ± 0.07	0.205 ± 0.05	0.49 ± 0.18	0.0
LYMP (×10 ³ mm ³)	3.78 ± 0.50	3.60 ± 0.98	5.56 ± 1.09	3.33 ± 0.96	3.34 ± 0.72	6.82 ± 1.33
MONO (×10 ³ mm ³)	0.36 ± 0.04	0.50 ± 0.15	0.32 ± 0.09	0.46 ± 0.07	0.39 ± 0.10	0.30 ± 0.08
EOS(×10 ³ mm ³)	0.24 ± 0.05	0.33 ± 0.10	0.22 ± 0.04	0.32 ± 0.08	0.42 ± 0.13	0.15 ± 0.04
PLT (×10 ³ mm ³)	993.2 ± 70.78	595.0 ± 102.5	913.8 ± 106.6	795.2 ± 131.9	801.0 ± 85.9	643.2 ± 64.1
SI (μ/dL)	41.14 ± 3.09	47.99 ± 3.08	40.09 ± 3.66	30.81 ± 1.93	34.57 ± 0.56	37.08 ± 3.46

Data are expressed as mean ± SD; *RBC: red blood cell; Hb: hemoglobin; Hct: hematocrit; MCV: mean corpuscular volume; RDW: red blood cell distribution width; MCH: mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; WBC: leukocytes; SN: segmented neutrophils; BN: band neutrophils; LYMP: lymphocytes; MONO: monocytes; EOS: eosinophil; PLT: platelets; SI: serum iron; Basophils, myelocytes and metamyelocytes were not detected.

explain the variation between symptomatic and low response induction in asymptomatic. We believe that the strain used produced asymptomatic infection in animals, because it did not observe changes in the consistency of the feces and the weight of the animals. We did not observe evidence of tissue invasion by that strain through immunohistochemistry, observing only trophozoites in the lumen and in the border in the brush of infected gerbils [23].

Eosinophil levels showed no significant increase, peaking at 4 weeks of infection. Increase of eosinophil levels is observed especially in parasitic infections with a pulmonary cycle or with tissue invasion. Hematological indices evaluation in children and adults with giardiasis, revealed eosinophilia only in adults [24]. Despite the non-significant increase in eosinophil values in this model, we believe that further studies should be conducted to clarify their involvement in the resolution of the infection.

The infected animals showed significant reduction in the number of red blood cells, in hemoglobin concentration and hematocrit values (from the second week of infection), confirming an anemia related to giardiasis. Reduction in hemoglobin concentration and the hematocrit value was also observed in experimental infection with *Giardia* [15, 25]. These results are in agreement with previous reports [14, 26] from evaluated patients with giardiasis. It is believed that, in intestinal infections the etiology of anemia is associated mainly with malabsorption syndrome or reducing food intake.

The reduction in serum iron level, although not significant, from the third week of infection, suggests

their involvement in the process of anemia. Some authors believe that anemia is primarily due to iron deficiency, secondary to giardiasis, only to be resolved after iron replacement [16,26-28], reinforcing the hypothesis of poor intestinal absorption of the mineral. In our model, it is interesting to note that in the second week there was a decrease in hemoglobin and hematocrit values with no significant change in serum iron levels, apparently opposing its direct participation in the process. Moreover, the weight of the animals did not change significantly in the course of infection. Thus, the reduction of iron may not be attributed to reduced food intake.

Significant change in the morphology of red blood cells from the measurement of RDW occurred in the first week after infection and remained until the fourth week, returning to normal values only after the animals were healed. This finding is common in anemia, including iron deficiency, being known that the RDW rises before hematimetric and serum iron changes [16]. These results suggest there are other ways in the process of anemia associated with giardiasis is interfered.

The *Giardia* infection leads to release and activation of several cytokines, such as TNF-α, INF-γ, IL-2, IL-4, IL-5, IL-6, IL-17 [29], with consequent metabolic changes in pro hypercatabolism, and stimulating increasing production of hepcidin by the liver, the regulating hormone of iron metabolism. In this case, anemia could not be characterized by a deficiency in the intake of the mineral, but rather for its catabolism induced by activation of systemic inflammation. Importantly, these findings are of an experimental model to evaluate the natural progression of giardiasis

and the consequences thereof on the hematological profile of the animals. This generates evolutionary information from infection, until its spontaneous cure, which occurred in this model. Note that, the evaluated parameters begin to return to baseline values after healing, emphasizing that the inflammatory and metabolic changes induced by *Giardia* were related to the hematological abnormalities observed. The increased IL-6 production from the second week of infection and intestinal histopathology corroborates the idea that inflammation could be subsidizing anemia in our model. Also corroborating with the hypothesis of inflammation financing the anemia in giardiasis, are the studies that found a strong correlation of the infection with the presence of mucus in the stool [30, 31].

Giardia infection is associated with several medical conditions and anemia is not always the outcome of the disease. The different clinical manifestations can be related to nutritional and immune status of the patient, the strain of the parasite, and the infection time [32]. In this context, despite the inherent differences in the complexity of human and rodent organisms, we clarify that gerbils (*Meriones unguiculatus*) allow to obtain good results mainly due to the similarity between the infection compared to humans [10, 33]. It is an animal model that can be used to analyze the behavior of the infection and the new intervention strategies in the disease [23, 34]. Thus, considering that the infection in our model was apparently asymptomatic, with spontaneous parasitological cure, we believe that anemia is a hallmark of giardiasis. Considering also the great number of individuals with giardiasis and anemia in the world, children with giardiasis with or without symptoms, anemia should be investigated. In addition, in cases of anemia without a definite etiology, giardiasis should also be investigated.

Conclusion

The fact that the serum iron levels were not significantly reduced, the weight of the animals was retained, and the hematimetric values returned to baseline without replacement of this ion, suggests the idea that not only malabsorption but also other mechanisms such as chronic inflammation may be implicated in iron deficiency anemia in giardiasis and may explain how asymptomatic patients may have anemia without malabsorption. Clarifying the mechanism of this anemia can be the target for more efficient therapeutic ways to control the symptoms of the disease.

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