

## **Alterations in Oxidative Stress Activities and Trace Elements Levels on Experimental *Blastocystis***

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### **ABSTRACT**

**AIM:** The present study was intended to assess the impact of the *Blastocystis* infection in mice on oxidative stress markers, in particular, malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) and the serum concentrations of vital trace elements; zinc, iron, and copper.

**Methods:** At a dose of 10<sup>4</sup> cysts/mouse, *Blastocystis* were inoculated orally. Feces from all mice were microscopically examined after infection and after metronidazole (MTZ)

treatment for assessment of the intensity of *Blastocystis* infection. Measurement of antioxidant enzymes activities in the liver and determination of serum zinc, iron, and copper were established.

**Results:** The greatest infection intensity was reported in the third week of *Blastocystis* reaching 48.6 vegetative forms/field and 186.2×10<sup>3</sup> cysts/gm. Treatment with metronidazole after three weeks of infection disclosed a decrease of about 60 percent and 83 percent in the amount of *Blastocystis*. *Blastocystis* infection significantly enhanced lipid peroxidation in the liver while SOD, CAT, and GSH were significantly reduced. The altered values of MDA, SOD, CAT, and GSH tend to be standardized compared to normal values by using antiparasitic drug; MTZ. Serum zinc and iron levels in mice infected with *Blastocystis* declined significantly however, there was a notable increase in serum copper levels. Treatment of *Blastocystis*-infected mice with metronidazole standardized the altered levels of serum zinc, iron and copper compared with normal groups.

**Conclusion:** Our research suggests that other than inflicting oxidative stress, *Blastocystis* infestation prompts biochemical alterations and interfering with micronutrient absorption in mice.

**Key words:** *Blastocystis*, oxidative stress, trace elements, metronidazole

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### **INTRODUCTION**

*Blastocystis* is a unicellular, anaerobic, eukaryotic<sup>[1]</sup>, that lives in the intestinal tract of diverse hosts, including humans<sup>[2]</sup> and has been associated with common gastrointestinal illnesses<sup>[3]</sup> such as diarrhea and abdominal pain<sup>[4]</sup>, irritable bowel syndrome (IBS)<sup>[5]</sup> as well as skin disorders<sup>[6-8]</sup>. *Blastocystis* has a significant role in enhancing carcinogenesis by resulting in damage to the intestinal epithelium and promoting oxidative damage in *Blastocystis* infected rats<sup>[9]</sup>.

The organism occurs in various morphological forms with each

form displaying considerable variations in size<sup>[10]</sup> and is transmitted through the fecal-oral route<sup>[11]</sup>. Due to presence of specific isolates, it is strongly suggested that *Blastocystis* is a pathogenic parasite<sup>[12]</sup>. Due to conflicting pathogenicity reports<sup>[13]</sup>, its pathogenic potential remains contentious. The behavior of *Blastocystis* in humans is consistent with that of *Giardia* and *Entamoeba histolytica*<sup>[14]</sup>.

*Blastocystis* is distributed globally<sup>[15]</sup> comprising great health concerns<sup>[16]</sup>, as it is one of the most commonly detected parasites in human fecal samples with an incidence of up to 60% in tropical, subtropical and developing countries<sup>[13,17]</sup> affecting children and adults<sup>[18]</sup>.

Numerous studies have revealed the existence of oxidative stress due to parasitic infections in humans and animals<sup>[19,20]</sup>. High levels of oxidative damage were recorded in *Blastocystis* infection<sup>[19]</sup>. Oxidative stress is associated with damage to all biomolecules (polynucleotides, proteins, lipids, and sugars)<sup>[21]</sup>, which can lead to a critical failure of biological functions and cell death<sup>[22]</sup>. However, long-lasting oxidative stress could lead to illnesses including cancer, cardiovascular illnesses, and diabetes<sup>[23-25]</sup>. Oxidative stress may result from ROS reactive oxygen species being overproduced. ROS is generated through cellular metabolic activity and environmental variables<sup>[26]</sup> and is critical to the elimination of intracellular pathogens in many infections<sup>[27]</sup> as well as in the commencement and progression of some gastrointestinal diseases<sup>[28]</sup>. Lipid peroxidation is one of the best ROS level indicators that caused systemic biological damage from parasitic infection<sup>[29,30]</sup>. MDA is the final product of these modifications. It is used as a lipid peroxidation marker and oxidative stress presence<sup>[31]</sup>. To neutralize ROS, humans, and livestock have an antioxidant defense mechanism consisting of enzymes SOD, CAT, and non-enzymatic antioxidants including non-protein thiols, particularly GSH<sup>[32,33]</sup>. In long term infections, when the defenses of the organism are insufficient for neutralizing the ROS, oxidative damage can occur, exposing the host to other illnesses<sup>[34]</sup>.

Trace elements such as zinc, iron, and copper have an important role to play in metabolic function and tissue maintenance<sup>[35]</sup>. Alterations in serum levels are frequently discovered in parasitic gastrointestinal diseases<sup>[36-38]</sup>. Zinc is an essential component for maintaining the structure, function, and many enzyme operations of the membrane<sup>[39]</sup>. In addition, zinc was also regarded as a powerful antioxidant defense involving in the scavenging of free radicals, the causative factors of oxidative stress<sup>[40]</sup>. Iron is required for the production of hemoglobin, the red blood component that carries oxygen to all parts of the body. It is also essential for good development of the nervous system, and for infection control wall<sup>[41]</sup>. Copper is crucial for the production of red blood cells, the formation of hemoglobin and iron absorption, and the activity of different enzymes. Zinc and copper are cytosolic superoxide dismutase cofactors and their alteration effects on cytosolic superoxide dismutase activity<sup>[42]</sup>. The antioxidant enzymes generally depend on the presence of trace components as cofactors and therefore on the protection against oxidative stress<sup>[43,44]</sup>.

The objective of the present study was to explore the effect of the *Blastocystis* infection on levels of oxidative stress biomarkers as well as the serological levels of three vital elements in the body; zinc, iron, and copper in experimental animals.

## MATERIALS AND METHODS

### Animals

For the research, male Swiss albino mice, weighing 18-20 gram bred at *Theodor Bilharz Institute's* Experimental Research Center,

Cairo, Egypt, were used. The animals were kept in well-aerated plastic cages with free access to conventional laboratory chow (El Nasr Lab Chem. Co, Egypt) and tap water. Animal experiments were conducted on the basis of animal ethics committee and conducted at TBRI in accordance with rules on animal ethics that are globally applicable.

### Infection of animals

Fresh stool samples were gathered from patients attending *Theodor Bilharz* Research Institute (TBRI) outpatient clinic. A wet smear with iodine solution and merthiolate iodine formaldehyde concentration method (MIFC)<sup>[45]</sup> instantly examined the stool samples for intestinal parasites. Harvested *Blastocystis* were washed in sterile saline and further incubated at room temperature for 48 hours in saline supplemented with 100 unit/ml penicillin-streptomycin and stored. In order to eliminate possible bacterial contamination, these steps were repeated 2-3 times before the inoculation phase. A hemocytometer was used to enumerate isolated cysts. At a dose of 10<sup>4</sup> cysts/mouse, *Blastocystis* were inoculated orally in each mouse.

### Detection of cysts shedding in feces and vegetative forms in intestinal contents

Feces from all mice were microscopically examined at different periods (1, 2 and 3 weeks) after infection and 1 week after treatment. The quantitative assessment of the infection intensity of *Blastocystis* in the stool samples in mice under the microscope was performed according to the method described by<sup>[46]</sup> to verify for the existence and burden of the parasite infection. In each experiment, the mean number of cysts/gm stool was determined. Vegetative forms (*trophozoite*) of *Blastocystis* viability was assessed using Eosin Brilliant cresyl blue stain. Also, the number of *trophozoites* in intestinal contents was counted in five successive high power fields per animal and then the average was calculated.

### Study design

This experiment was performed in five groups on Fifty Swiss albino mice.

Group 1: 10 non-infected mice kept as a control group.

Group 2, 3, and 4: 30 infected non-treated mice. Each group comprised of ten mice. They were sacrificed for one, two, and three weeks of infection at three intervals.

Group 5: 10 mice infected and treated with metronidazole after the third week of infection.

Treatment was given to experimental animals; conventional drug, MTZ in tablet form (Amriya Pharm. Industry, Alexandria, Egypt). MTZ stock solution was prepared by dissolving 600 mg in 10 ml distilled water to provide a final stock solution of 60 mg/ml and stored at 4°C in the dark<sup>[47]</sup>. It was administered orally for 5 successive days in a dose of 10 mg/kg/day as the dose often used for relevant mouse reports<sup>[48]</sup>.

### Measurement of antioxidant enzymes activity

Animals were decapitated; livers were rapidly excised to estimate lipid peroxidation alterations; MDA, antioxidant enzymes such as SOD and CAT as well as non-enzymatic antioxidants such as GSH.

### Lipid peroxidation

Hepatic lipid peroxidation was evaluated using a colorimetric assay<sup>[49]</sup> to determine the MDA content of live homogenate.

### Liver superoxide dismutase (SOD) activity

Superoxide dismutase activity was determined by the method of<sup>[50]</sup>.

**Liver Catalase (CAT) Activity**

Tissue catalase was determined according to the method of<sup>[51]</sup>.

**Liver glutathione (GSH) content**

A spectrophotometric method using Elman’s reagent of GSH was used for the estimation of GSH content<sup>[52]</sup>.

**Biochemical parameters**

Blood samples were gathered from all groups to determine serum zinc, iron, and copper. Until the test, Sera was stored at -20°C. A polarized atomic absorption spectrophotometer (Z8000 polarized Zeeman Absorption Spectrophotometer, Hitachi Ltd., Tokyo, Japan) was used to determine the concentrations of zinc, iron, and copper.

**Statistical Analysis**

Analyses were made with the Student’s *t-test*, using Graph Pad Prism Software (Graph Pad, San Diego, CA, USA).

**RESULTS**

The intensity of *Blastocystis* infection in intestinal content and mice stool gradually increased over time in this research. The greatest infection intensity was reported in the third week of *Blastocystis* infection reaching 48.6/field in intestinal content (Figure 1) and 186.2×10<sup>3</sup> cysts/gm stool (Figure 2). Treatment with MTZ after three weeks of infection disclosed a reduction of about 60 percent and 83 percent in the vegetative forms of *Blastocystis* and the number of cysts in gram stool relative to the infected group at the same time (Table 1).

Table 2 shows the study’s oxidative stress and that’s Figure 3 The lipid peroxidation, measured by the examination of MDA in the liver, significantly increased to 3.13 ± 0.07 (18%) at *p* < 0.05 after two weeks of infection, whereas it increased to reach 3.51 ± 0.1 (*p* < 0.001) after three weeks of infection by about 32%. The amount of MDA showed no significant improvement (*p* > 0.05) relative to standard level after treatment with MTZ at the third week of infection.

**Table 1** Blastocystis number in intestinal contents (vegetative forms) and stool (cysts) of infected mice before and after treatment with metronidazole. Values are expressed as means ± standard error.

Parasitological parameters	1week	2weeks	3weeks	treated
Vegetative forms in intestinal contents/field	33.2±4.8	45.6±3.7	48.6±2.8	19.6±1.6
Cysts/gm stool×10 <sup>3</sup>	60.6±3.4	83.4±4.0	186.2±6.1	31.4±2.3

**Table 2** Effect of Blastocystis infection on hepatic activities of MDA, SOD, CAT, and GSH before and after treatment with metronidazole. Values are expressed as means ± standard error.

P arameters	Normal	1week	p value	2weeks	p value	3weeks	p value	treated	p value
MDA nmole/mg	2.66 ± 0.12	2.92 ± 0.10	p >0.05	3.13 ± 0.07	P <0.05	3.51 ± 0.1	p <0.001	3.09 ± 0.10	p >0.05
SOD u/mg	9.76 ± 0.27	9.10 ± 0.35	p >0.05	7.60 ± 0.36	P <0.01	4.80 ± 0.35	p <0.001	7.84 ± 0.32	p <0.01
CAT u/mg	6.01 ± 0.14	5.95 ± 0.21	p >0.05	4.75 ± 0.42	P <0.05	4.94 ± 0.28	p <0.01	5.02 ± 0.47	p >0.05
GSH nmole/mg	96.8 ± 2.1	82.0 ± 3.1	p >0.05	73.4 ± 2.0	P <0.05	65.6 ± 1.8	p <0.001	86.0 ± 3.8	p <0.05

treatment with metronidazole. Values are expressed as means ± standard error.

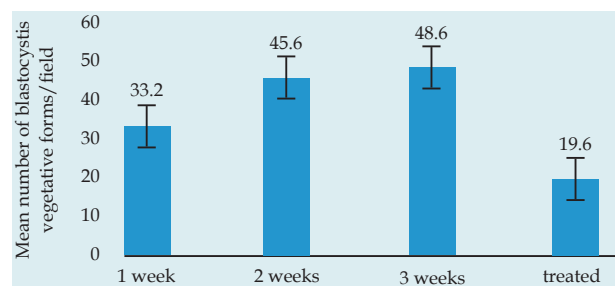
**Table 3** Effect of Blastocystis infection on serum concentration of zinc, iron, and copper before and after treatment with metronidazole.

P arameters	Normal	1week	p value	2weeks	p value	3weeks	p value	treated	p value
Zinc Mg/ml	0.99 ± 0.05	0.92 ± 0.08	p >0.05	0.75 ± 0.02	p <0.01	0.67 ± 0.03	p <0.001	0.92 ± 0.05	p >0.05
Iron Mg/ml	1.95 ± 0.10	1.78 ± 0.04	p >0.05	1.62 ± 0.06	p <0.05	1.34 ± 0.05	p <0.001	1.73 ± 0.05	p >0.05
Cop per Mg/ml	1.10 ± 0.06	1.00 ± 0.03	p >0.05	1.27 ± 0.04	p <0.05	1.39 ± 0.06	p <0.01	1.20 ± 0.04	p >0.05

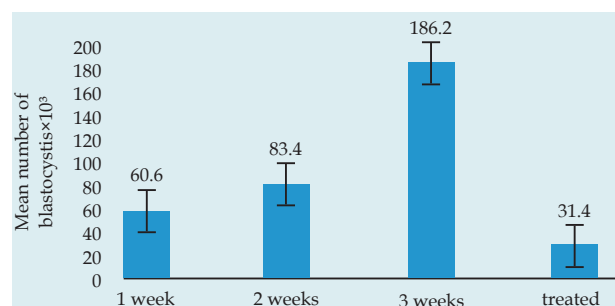
Values are expressed as means ± standard error.

The activities of antioxidant enzymes, i.e., SOD and CAT, were decreased significantly two (*p* < 0.01 and *p* < 0.05) and three (*p* < 0.001 and *p* < 0.01) weeks post-infection respectively. The maximal decline was observed after the third week of infection reaching 51% and 18% respectively. By using antiparasitic drug; MTZ three weeks after infection, the altered values of SOD (Table 2 and Figure 4) and CAT (Table 2 and Figure 5) tending to be standardized (*p* < 0.01 and *p* > 0.05) compared to normal values respectively.

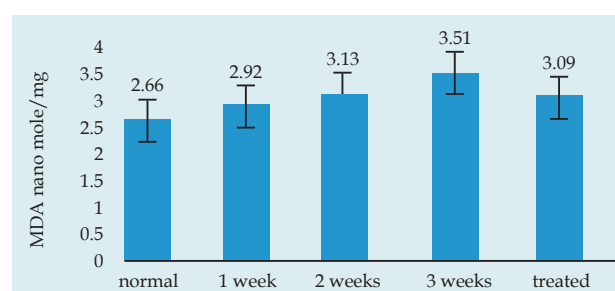
As shown in Table 2 and Figure 6, The mean titer of GSH reduced significantly in the hepatic tissues of *Blastocystis* infected mice



**Figure 1** Blastocystis vegetative forms in intestinal contents/field before and after treatment.



**Figure 2** Blastocystis shedding per gram of feces×10<sup>3</sup> before and after treatment.



**Figure 3** Effect of blastocystis infection on hepatic MDA content.

after two weeks to  $73.4 \pm 2.0$  with a decrease of 24% compared to standard control ( $p < 0.05$ ), whereas it was  $65.6 \pm 1.8$  ( $p < 0.001$ ) in the third week of infection with a decrease of 32% compared to control. Mice treated with MTZ ( $p < 0.05$ ) significantly improved the GSH level relative to the control group.

The results presented in table 3 and Figure 7 showed that serum zinc and iron levels decreased significantly in mice infected with *Blastocystis* compared to healthy controls two weeks, ( $p < 0.01$ ,  $p < 0.05$ ) amounting to 24% and 17% respectively, and three weeks ( $p < 0.001$ ) reaching to 32% and 31% respectively post-infection. There was a significant increase in serum copper levels after two weeks ( $p < 0.05$ ) and three weeks ( $p < 0.01$ ) post-infection by about 16% and 26% respectively. Treatment of *Blastocystis* infection with metronidazole after three weeks tended to normalize the altered levels of serum zinc, iron and copper ( $p > 0.05$ ) compared with normal groups.

## DISCUSSION

The current study was conducted to demonstrate the impact of *Blastocystis* infection on oxidative stress markers in mice's liver and to evaluate levels of serum trace elements; zinc, iron, and copper; three of the key components that play important roles in body health in parasitized and treated animals.

Oxidative stress is becoming increasingly relevant as a significant clinical and biochemical mechanism of disease pathogenesis<sup>[53,54]</sup>. Oxidative stress functions against the infection as a first line of defense or immune response. It results from the high metabolic rate of the rapidly growing and multiplying parasite which produces large quantities of toxic redox-active by-products. The observed elevation in MDA values of infected mice in this study is in concordance with the findings observed in *Blastocystis*<sup>[19,55-57]</sup> zinc, and copper and their demolishing effects against lipid peroxidation in chronic giardiasis. Serum iron, zinc and copper levels, erythrocyte cytosolic superoxide dismutase activity, and malondialdehyde levels were measured in 34 children with chronic giardiasis and were compared with controls. The serum iron and zinc levels and erythrocyte superoxide dismutase activity were significantly lower, and malondialdehyde levels were significantly higher among the children with chronic giardiasis compared to the control group ( $p < 0.001$ , *Giardiasis*<sup>[58,59]</sup>, *hydatidosis*<sup>[60]</sup>, *ascariasis*<sup>[61]</sup>, *Malaria*<sup>[62]</sup> and *Schistosomiasis*<sup>[63]</sup>.

Parasites are known to generate free radicals, inducing lipid peroxidation in organs, tissues, and cells<sup>[64]</sup>. The generation of free radicals was regarded as an immune response against parasitic infection<sup>[65]</sup>. The end product of free radical reactions on membrane fatty acids is called MDA. one of the main reasons for high MDA levels in mice infected with *Blastocystis* could be decreased defense system activity protecting tissues from free radical damage caused by *Blastocystis*-infected activated phagocytes<sup>[66]</sup>. An increase in MDA concentration showed that *Blastocystis* infection evokes significant changes in the oxidative status of infected hosts<sup>[67]</sup>.

Previous studies reported the depletion of liver SOD, due to parasitic infections<sup>[60,68]</sup>. These results were consistent with our study. the reduction in these activities was significantly recorded in the second and the third weeks after infection. SOD, CAT and GSH act as mutually supportive antioxidant enzymes that protect against reactive parasite-induced oxygen species<sup>[69-72]</sup>. The reduction in the activity of these enzymes may be as a consequence of the exhaustion of the host SOD, CAT and GSH in neutralizing the free radicals generated by the parasites as they are used as scavengers<sup>[73]</sup>, stated that excess ROS

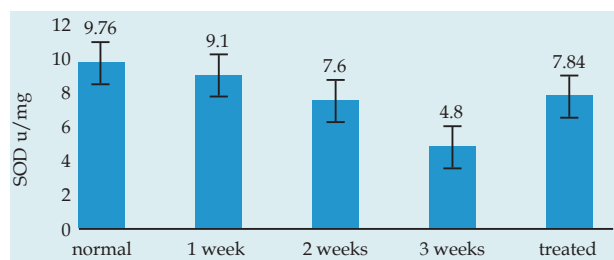


Figure 4 Effect of blastocystis infection on hepatic sod activity.

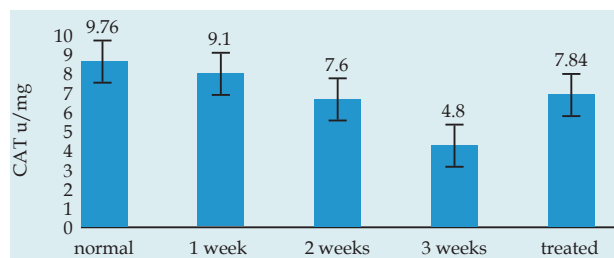


Figure 5 Effect of blastocystis infection on hepatic cat activity.

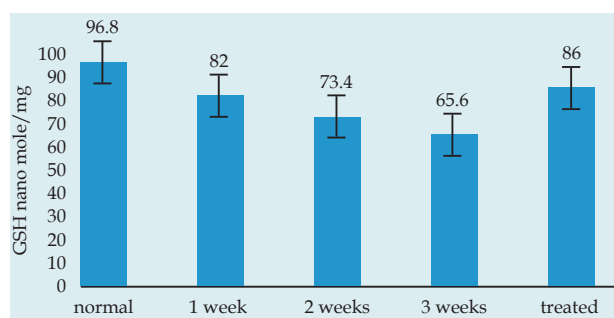


Figure 6 Effect of blastocystis infection on hepatic gsh content.

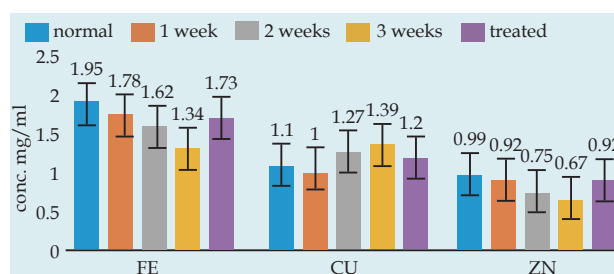


Figure 7 Effect of blastocysts infection on serum concentration of zinc, iron, and copper before and after treatment.

probably inactivate these antioxidant enzymes. This observation is in line with the findings of<sup>[74]</sup>, who linked the reduced activities of SOD and CAT in *Plasmodium berghei* infection to excessive generation of ROS. Reduction in these enzymes' activities may be a consequence of decline in levels of micronutrients recorded (Table 2 and Figure 6). The concentration of these trace elements has an important impact on antioxidant enzyme activity and therefore on the protection against oxidative stress<sup>[24,75]</sup>, whereas, trace elements are components of antioxidant enzymes involved in antioxidant mechanisms; copper and zinc are an essential part of the group of superoxide dismutase enzymes (Cu/Zn SOD)<sup>[76]</sup> and iron ions are an integral component of catalase (CAT)<sup>[77]</sup>.

Metronidazole is the most frequently suggested agent for treating human *Blastocystis*<sup>[47,78,79]</sup>. However, in the liver of *Blastocystis*-infected mice treated with metronidazole within seven days, the tendency of normalization was noted in oxidative stress markers. The drug may directly affect *Blastocystis* or it may act by destroying



the bacterial flora necessary for its growth or both<sup>[79]</sup>. This suggests that upon the elimination of the parasite from the host, (Table 1 and Figure 1 and 2), oxidant and antioxidant equilibrium is attained on its own.

Minerals are important molecules that take part in sustaining physiological functions and protecting organisms against disease (Karagül *et al.*, 2000). Even a tiny shift in the level of trace elements in the tissues creates a metabolism disruption, leading to many diseases<sup>[75]</sup>. In the present study, the serum zinc and iron levels in parasitic mice were significantly lower compared to normal controls. While serum copper was significantly higher in response to *Blastocystis* infection. Coincident decrease in serum zinc level and an increase of serum copper were more prominent among *Giardia lamblia* and *Cryptosporidium* patients<sup>[81]</sup>, in malaria-infected mice<sup>[82]</sup> and in *Leishmania major* infection<sup>[83]</sup>.

During protozoan and helminth infections, serum zinc concentrations were generally smaller<sup>[84-86]</sup>. As regard, the significant decrease in serum zinc levels in the infected group was in agreement with<sup>[86-88]</sup>. Zinc cannot be stored in the body so its serum level could be easily dropped. However, Mineral, in specific Zn, Fe, and Cu<sup>[89]</sup>, may influence animals' capacity to deal with parasitic infection. Some trace mineral deficiencies may weaken host protection and result in elevated parasite numbers<sup>[90]</sup>. This is corroborated in our work by a decrease in serum concentration of Zn by incline in the number of the parasite in the host. In this context<sup>[91,92]</sup>, identify the relationship between zinc deficiency and intestinal parasitic infections among children. Confirming this term<sup>[93]</sup> evidenced that zinc can have protective impacts specific to pathogen.

Deficiencies in micronutrients may be associated with malabsorption owing to intestinal affection. In particular, *Blastocystis* may have intestinal lesions<sup>[8,9,94,95]</sup> that can significantly impair the absorption of intestinal zinc and iron<sup>[96]</sup>. In addition, the intestinal parasites use the host's carbohydrates, lipids, minerals, vitamins, and other food sources to obtain vital life cycle energy<sup>[37]</sup>.

Our findings were in line with<sup>[55,86]</sup> and<sup>[97]</sup>, which discovered that Cu level was significantly increased due to intestinal parasitic infections. While the mechanism is unknown, this rise in the amount of Cu in these infections could possibly be explained by the increase of Cu containing enzyme systems. Elevation of serum copper levels is standard in most of the acute and chronic parasitic infections, which is related to the increases in ceruloplasmin, which is mainly the storage binding form of copper in various infections<sup>[98]</sup>.

One of the main influences of infections in the hemopoietic system is concerning iron metabolism. Iron is crucial for cell differentiation and growth and is a critical enzyme element for the functioning of immune cells engaged in cytokine manufacturing and action regulation<sup>[99]</sup>. Some reasons in this study may be responsible for the low in serum iron concentration of infected mice, such as a combination of reduced intake due to deficient absorption and utilization of iron by the parasite, which is essential for their growth and multiplication<sup>[100]</sup>. Some studies not only showed that patients with parasitic infections had micronutrients deficiencies, but also their treatment with anti-parasitic medications had improved their serum levels<sup>[101]</sup>. It was ascribed to the restoration of impaired intestinal mucosa from infection by using antiparasitic drugs<sup>[102]</sup> and eradication of infection.

## CONCLUSION

From our study, it tends to be reasoned that other than causing oxidative stress, *Blastocystis* infestation induces biochemical

alterations and interfering with micronutrient absorption in mice. This may contribute somewhere in the pathogenesis of *Blastocystis* infection. Treatment with metronidazole effectively clears *Blastocystis*, restores oxidative imbalance and has a positive impact on the micronutrient status in *Blastocystis*-infected mice.

## AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Azza Fahmy, Rabab Zalat and Amany Hegab, and Wafaa Wafy. The first draft of the manuscript was written by Azza Fahmy and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## ETHICAL APPROVAL

Animal experiments were conducted on the basis of animal ethics committee and conducted at TBRI in accordance with rules on animal ethics that are globally applicable.

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