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Levels of oxidative stress markers: correlation with hepatic function and worm burden patients with schistosomiasis

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Abstract

Schistosomiasis is caused by *Schistosoma mansoni* and is a public health problem in Brazil. The typical granulomatous lesion is associated with the increase in the oxidative damage by generation of free radicals. The aim of this work was to correlate some oxidative stress markers with the worm burden on carriers of schistosomiasis (n = 30) in the acute phase in comparison to healthy subjects (n = 30). The pro-oxidant parameter used was the colorimetric quantification of reactive substances to thiobarbituric acid, while the antioxidant markers used were blood content of reduced glutathione and determination of the activity of catalase. The worm burden was assessed by Kato-Katz method. The results pointed out that initially there was no difference in the catalase activity. However, there was a positive correlation between the increase in parasitic load and intensity of lipid peroxidation, and decrease in the content of reduced glutathione. Additionally, only the aspartate aminotransferase levels presented to be high, while there was a decrease in bilirubin level. Therefore, a possible association between the establishment of the oxidative stress in tissue and the parasitic load of *Schistosoma mansoni* is suggested.

Keywords

Schistosoma mansoni, oxidative stress, worm burden

Introduction

Schistosomiasis is a disease caused by helminth *Schistosoma mansoni*, which has *Biomphalaria* sp. as the intermediate host and that can evolve from asymptomatic forms to extremely serious clinical forms (Amaral and Porto 1994, Lima and Timbó 1999, Bender *et al*. 2002). The severity of the illness is related to the intensity of worm burden and the consequent release of large quantities of eggs, leading to major clinical and epidemiological implications (Motta and Silva 2002). The main injury is the granulomatous reaction, which is an inflammatory process resulting from the cellular immune response, induced by the presence of eggs in organs such as liver and gut. As a result, important metabolic alterations may occur and the physical conditions of the host may be affected. Studies indicate that this reaction can involve reactive oxygen species which are produced by granuloma, being this production essential for destruction of eggs (Feldman *et al*. 1990, Gharib *et al*. 1999, Silva *et al*. 2002, Ramos *et al*. 2004, Eboumbou *et al*. 2005, Lima 2005, Hanna *et al*. 2005).

Under physiological conditions there are several lines of defense against the action of oxidants: enzymes such as catalase (CAT) and molecules containing thiol as reduced glutathione (GSH), evaluated in this work, together with a pro-oxidant molecule as malondialdehyde (MDA) (Gaté *et al*. 1999). Catalase is the second enzyme which acts in cellular detoxification because it converts H_2O_2 into H_2O and O_2, and it is important because of its ability to tackle the chains of unsaturated fatty acids present in the membranes, and other macromolecules, such as proteins (Beutler 1984). In the cell GSH plays a role in many biological processes, including the synthesis of protein, maintenance of cellular activity, xenobiotics and reactive aldehydes detoxification (such as MDA), metabolism and cell acting protection against free radicals (Meister 1991, Jordão *et al*. 1998). MDA is the primary and most studied product of polyunsaturated fatty acid peroxidation, being therefore, the molecule that most reacts with the

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thiobarbituric acid (TBA), which is found in higher concentrations (Del Rio *et al*. 2005).

Some studies have shown that the oxidative processes which occur upon contact with *S. mansoni* eggs trapped in the liver seem to go uncontrolled, since the enzymatic activities involved in O^{-}_{2} and H_2O_2 detoxification decreased drastically. Such events may be, at least in part, responsible for the pathology associated with schistosomiasis (Gharib *et al*. 1999).

Materials and methods

Study population

Some parameters were evaluated for oxidative stress in 30 subjects without schistosomiasis and 30 with schistosomiasis and resident in the municipal district of Touros-RN, which is an endemic region for this disease. After a clinical evaluation, was determined that these individuals were in the acute phase of illness. The subjects were of both genders with ages varying between 16 and 30 years.

Blood collection and laboratory tests

For these experiments, 20 ml of blood were drawn, in the period of April of 2006 to February of 2007. After, the samples were distributed in tubes with anticoagulant ethylenediaminetetraacetic acid (EDTA 5%) (NewProv®, Pinhais, PR) and submitted to the specific procedures for each analysis.

Determination of reduced glutathione (*GSH*)

The GSH, present in the sample, reacts with 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) (Sigma, St. Louis, MO) that is a disulfide compound that is readily reduced by sulfhydryl compounds, forming a yellowish anion. The reaction is followed at 412 nm (Beutler 1984).

Catalase activity (*CAT*)

CAT catalyzes the breakdown of hydrogen peroxide (H_2O_2) and the rate of decomposition into oxygen (O_2) and water (H_2O) by CAT is measured spectrophotometrically at 230 nm ($\varepsilon = 0.071\ mM^{-1}.\ cm^{-1}$) a 37°C (Beutler 1984).

Determination of reactive substances to thiobarbituric acid (*TBARS*)

Thiobarbituric acid (TBA) (Sigma, St. Louis, MO) reacts with a series of peroxidation by products (SRATS). Most of these SRATS are represented by the malondialdehyde (MDA), in acidic medium at 100°C, forming a pink complex which is quantified spectrophotometrically at 535 nm (Dahle *et al*. 1962, Yagi 1982).

Determination of hepatic function

The determination of serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl-transferase (GGT), was performed using Labtest® kits. The readings were carried out in Analyser Chemistry RA – 50 Bayer®, Dublin, Ireland. The blood levels of ALT and AST were evaluated by means of UV-kinetic method IFCC, with the following values of reference considered normal: 10 to 37 U/L in women, and 11 to 39 U/L in men for the ALT and 13 to 49 U/L in women and from 14 to 50 U/L in men for the AST. The level of GGT was determined by the Szasz method modified with the reference values of 5 to 27 U/L for women and of 7 to 45 U/L for men, according to the kit used. The total bilirubin and fractions were determined by colorimetric test macro, where the reference values considered normal for direct bilirubin were up to 0.4 mg/dL, and the total bilirubin were up to 1.2 mg/dL, and indirect result of the fraction of bilirubin was produced by the difference and total bilirubin fraction corresponding to direct bilirubin.

Worm burden evaluation

The parasitological diagnosis of schistosomiasis was performed by using the Kato-Katz method, which allows the visualization and quantification of eggs per gram of faeces present in the parasitized subjects with the *Schistosoma mansoni* and provides a safe indicator to evaluate the infection intensity and the effectiveness of the treatment (Ruiz-Guevara *et al*. 2007).

Statistical analysis

For statistical evaluation of results was used unpaired t test for parametric samples and independent (biochemical parameters and assessment of oxidative stress). The level of significance was $p < 0.05$. Furthermore, Spearman's correlation test were use. Data were tabulated using Microsoft Excel (Microsoft Corporations Inc. 2002) for statistical analysis, Graphpad Instat 3.01 (Graphpad Software Inc. 2004) and generation of graphs through the Prism 4 for Windows (Graphpad Software Inc., in 2004).

Results

The profile of antioxidant/oxidant enzymes and molecules of subjects resident in the municipal district of Touros-RN is shown in Figs 1, 2 and 3. Regarding the antioxidant molecule (GSH), it was observed that there was a significant statistical difference between the groups, showing a decrease in GSH in subjects with schistosomiasis (group test) (11.00 ± 2.64 vs 5.59 ± 3.64 μM/dL). However, with regard to the activity of the enzyme catalase, as observed in Fig. 2, there was no significant statistical difference between the test and con-

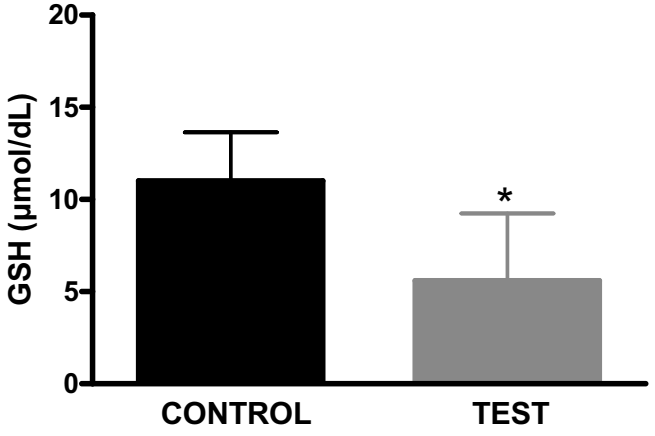

Fig. 1. Concentration of GSH (reduced glutathione) content in subjects evaluated in the municipal districts of Touros-RN and Natal-RN, in the period of April of 2006 to February of 2007. The results are expressed as mean ± SD (*p<0.05). Control: individuals without schistosomiasis. Test: individuals with schistosomiasis

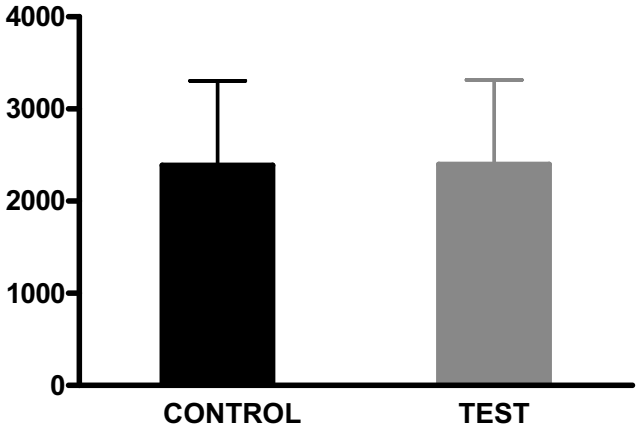

Fig. 2. CAT (catalase) activity in subjects evaluated in the municipal districts of Touros-RN and Natal-RN, in the period of April of 2006 to February of 2007. The results are expressed as mean ± SD (*p<0.05). Control: individuals without schistosomiasis. Test: individuals with schistosomiasis

trol groups studied, respectively (2,398.259 ± 915,7844 vs 2,389.674 ± 915,9039 U/mg Hb).

In Fig. 3, results with SRAT/MDA showed an increase of this oxidant molecule in the group of infected subjects, in relation to the control group (4.72 ± 0.59 vs 5.73 ± 0.91 nmol/mL).

In relation to hepatic profile, which can be seen in Fig. 4, the group of patients with schistosomiasis had higher values of AST (30.76 ± 9.55 U/L) when compared to the control group (22.11 ± 6.70 U/L). This fact was not observed when considering the ALT (26.06 ± 16.10 vs 20.18 ± 14.99 U/L) and GGT (22.58 ± 16.26 vs 25.68 ± 14.44 U/L). In subjects carrying the disease, there is also depletion in total and indirect bilirubin levels when compared to the control group.

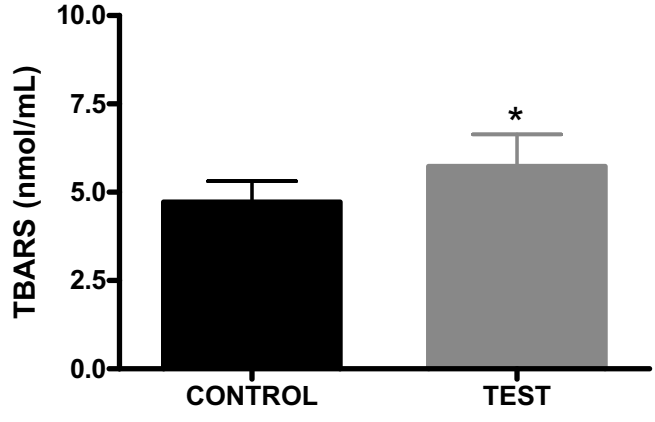

Fig. 3. Concentration of oxidative molecule MDA/SRAT (malondialdehyde/reactive substances to thiobarbituric acid) in subjects evaluated in the municipal district of Touros-RN and Natal-RN, in the period of April of 2006 to February of 2007. The results are expressed as mean ± SD (*p<0.05). Control: individuals without schistosomiasis. Test: individuals with schistosomiasis

As observed in Fig. 5 there is a relationship between the hepatic profile and SRAT/MDA (molecule used as an indicator of lipid peroxidation) and the GSH (molecule used as an antioxidant parameter). In the group of subjects with the disease, there was an increase in the levels of AST, in relation to the control group. However, changes in ALT was not observed in this work.

The relationship of SRAT/MDA with the number of eggs deposited by the parasite per gram of faeces is shown in Fig. 6. There is a tendency to an increase of SRAT/MDA, in accordance with the increase in the quantity of eggs eliminated in the faeces of infected subjects.

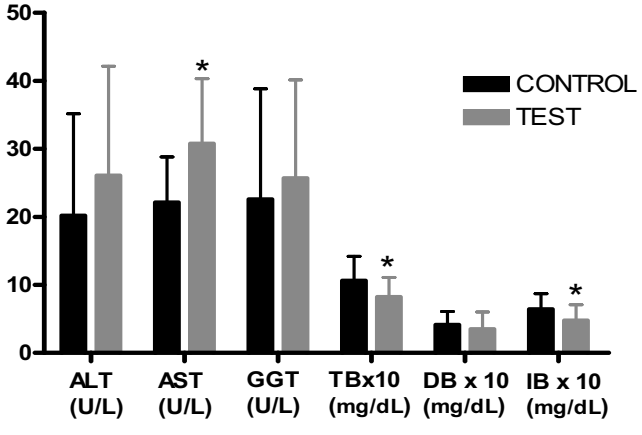

Fig. 4. Hepatic profile of schistosomiasis group of municipal district of Touros-RN and control group of Natal-RN, in the period of April of 2006 to February of 2007. The results are expressed as mean ± SD (*p<0.05). Control: individuals without schistosomiasis. Test: individuals with schistosomiasis. ALT (alanine aminotransferase), AST (aspartate aminotransferase), GGT (gama-glutamil transferase), TB (total bilirubin), DB (direct bilirubin), IB (indirect bilirubin). Values of reference: AST and ALT of 10–37 U/L for women and of 11–39 U/L for men, GGT of 7–45 U/L, TB up to 1.2 mg/dL, DB up to 0.4 mg/dL

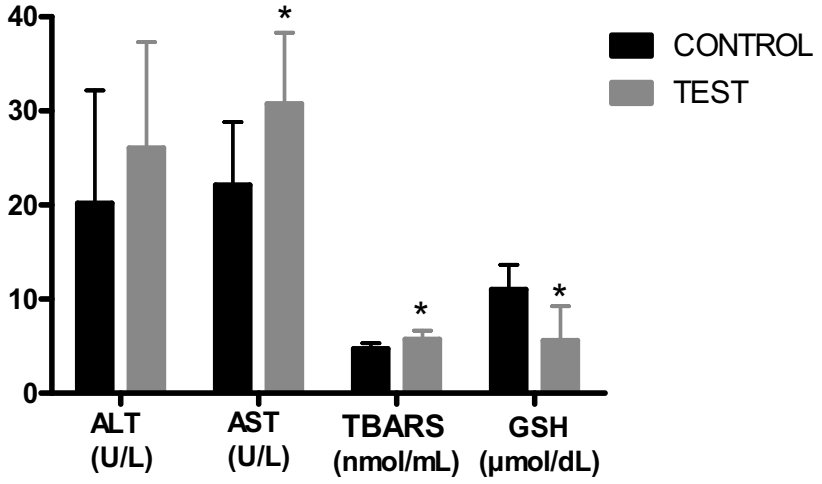

Fig. 5. Concentration of antioxidant/oxidative molecules correlated with the hepatic profile of subjects evaluated in the municipal districts of Touros-RN and Natal-RN, in the period of April of 2006 to February of 2007. The results are expressed as mean ± SD (*p<0.05). Control: individuals without schistosomiasis. Test: individuals with schistosomiasis. ALT (alanine aminotransferase), AST (aspartate aminotransferase), TBARS/MDA (reactive substances to thiobarbituric acid/malondialdehyde), GSH (reduced glutathione). Values of reference: AST and ALT of 10–37 U/L for women and of 11–39 U/L for men

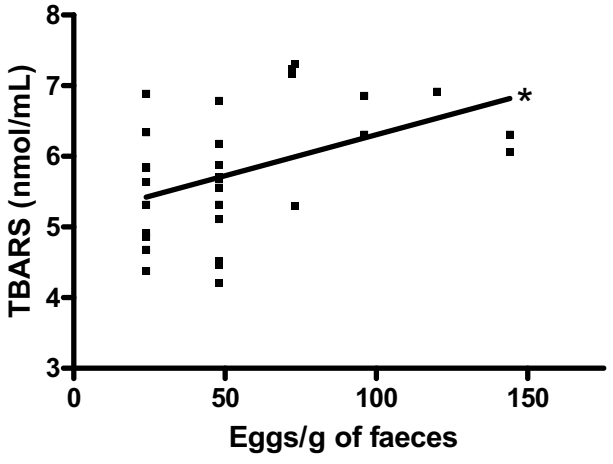

Fig. 6. Correlation between MDA (malondialdehyde) and number of eggs/g of faeces in patients resident in the municipal district of Touros-RN, in the period of April of 2006 to February of 2007

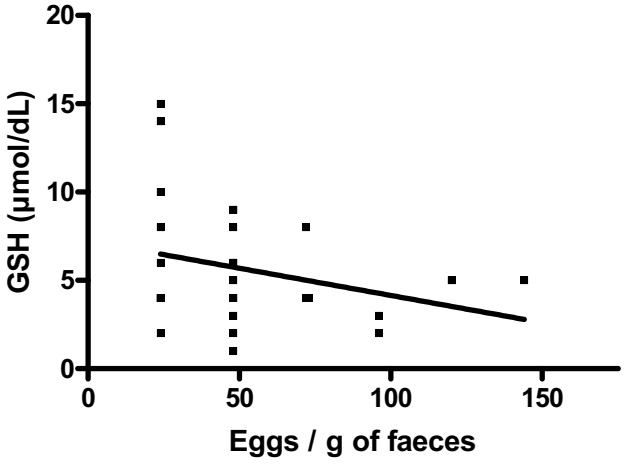

Fig. 7. Correlation between GSH (reduced glutathione) and number of eggs/g of faeces in patients resident in the municipal district of Touros-RN, in the period of April of 2006 to February of 2007

The results shown in Fig. 7 revealed the negative correlation of GSH with the number of eggs deposited by the parasite per gram of faeces. A trend of decrease in GSH levels can be seen, despite this data is not statistically significant.

Discussion

Previous studies indicate that free radicals are formed next to the eggs of *S. mansoni*, in a process of granulomatous inflammation in which eggs of the parasite are destroyed and some cells of defense, such as macrophages and eosinophils, are attracted to the inflammation site. Some antioxidant enzymes and molecules are also attracted in order to destroy the free radicals present in the region inflammatory (Hogan *et al*. 2002; Stavitsky 2004; Neves *et al*. 2006). The expression of antioxidant enzymes can be affected by many factors, such as inflammatory processes, and some diseases and immunodeficiency, so by having a imbalance between free radicals present in high quantities, and few antioxidants sufficiently formed to destroy them (Alger *et al*. 2002; Bolukbas *et al*. 2005; Barbosa *et al*. 2007).

The GSH can be considered one of the most important agents of the antioxidant defense system of the cell. It is known that one of its functions and other antioxidants is to prevent deleterious effects of oxidation by inhibiting the ini-

tiation of lipid peroxidation, scavenging free radicals, destroying them (Ferreira and Matsubara 1997; Jordão Júnior *et al*. 1998; Rodrigues *et al*. 2003). Then, an excess of oxidative agents and deficiency of GSH characterize the oxidative stress. Therefore, the depletion of GSH is a strong indicative of establishment of oxidative stress, since it is consumed in combating the high levels of the radical peroxides system glutathione peroxidase (Ferreira and Matsubara 1997; Gandra *et al*. 2004). Moreover, it has been reported that schistosomiasis causes an impairment of liver GSH content. As a consequence, the antioxidant capacity of the liver decreases, leading to the generation of lipid peroxides that may play a pivotal role in the pathology associated with schistosomiasis (El-Shenawy *et al*. 2006).

Authors as Gharib *et al*. (1999) and Abdallahi *et al*. (1999) showed that carriers in the chronic phase of the schistosomiasis had decreased activity of catalase. In this work, was not evidenced a decreasing in catalase activity because these patients were in the acute phase, confirming the findings of the authors above, as shown in Fig. 2. The fact that catalase did not suffer any change between the groups studied may have occurred probably because this enzyme is only activated or released when there is an excess in the concentration of H_2O_2, as in small quantities to glutathione peroxidase, which plays the role of transforming the peroxide hydrogen in water. This means that, in most cases, the presence of catalase is important to protect the organism, in cases of acute oxidative stress (Urbina *et al*. 2004; Andrade Júnior *et al*. 2005).

Regarding the significant increase in the TBARS/MDA in the test group, it was observed that these data were also reported in other papers, which show an association between the *Schistosoma mansoni* and high levels of reactive substances to thiobarbituric acid (TBARS) and reactive oxygen species (ROS) (Facundo *et al*. 2004; Del Rio *et al*. 2005; Eboumbou *et al*. 2005). In man, infection with *S. mansoni* is associated with increased levels of circulating TBARS and ROS adducts (Pascal *et al*. 2000).

Concerning the hepatic profile, shown in Fig. 4, it is suggested that the increase of AST is related to the presence of a possible liver damage caused by the parasite in subjects with the disease, showing that the liver of these individuals can be damaged (Alves Júnior *et al*. 2003). Amaral *et al*., in 2002, reported that GGT can be increased in subjects who present the hepatosplenic form of the disease. However, further studies are needed to understand the mechanism responsible for the increase of GGT in this clinical form of the disease.

In this work, this increase was not observed, since all subjects are in the acute phase of the disease according to clinical diagnosis obtained in the municipal district. This shows that the results are in agreement with the literature. In addition, it is suggested that the ALT may be increased because of the number of eggs deposited by the parasite and did not correlate with the increase of GGT. The decrease of bilirubin in subjects carrying the disease could be related to the antioxidant role of this molecule because the possible condition of oxidative stress caused by schistosomiasis would lead to higher consumption of bilirubin when trying to combat the free radicals. The same was not observed for direct bilirubin, which was unchanged (Gandra *et al*. 2004).

It is observed in Fig. 5, in group of subjects with the disease, an increase in the level of AST, in relation to control group. Although an increase of ALT was not observed in this work, previous studies suggest that high parasite load is one of the mechanisms for elevation of ALT in schistosomiasis (Amaral *et al*. 2002).

A high parasite load may produce TBARS/MDA and therefore it may cause a condition of oxidative stress, which could be leading to hepatic damage evidenced by the increase of AST. At the same time, the GSH may be required in order to protect the organism of free radicals formed around the eggs. Thus, glutathione is consumed, justifying the reduced levels found (El-Shenawy *et al*. 2006).

Results show a tendency for an increase in TBARS/MDA by increasing the quantity of eggs released by the individual with schistosomiasis (Fig. 6). This fact confirms the data found in recent studies, which reported that the reactive oxygen species (ROS) are produced near eggs deposited in the liver and they are responsible for destroying the eggs. In addition to the destruction of the eggs, ROS have devastating side effects since liver redox homeostasis is altered with a decrease in the antioxidant defenses of the organ (Eboumbou *et al*. 2005).

It is also known that the ROS attract some cells of defense to the site, such as macrophages and eosinophils, and molecules and enzymes antioxidants, with the same function. Meanwhile, the ROS have also deleterious effects, since the redox state of the liver is altered with a decrease in antioxidant defenses in organism (Feldman *et al*. 1990; Eboumbou *et al*. 2005).

Some studies report that the expression of antioxidant enzymes can be affected by several factors, such as inflammatory processes, some diseases and immunodeficiency, due to an imbalance between free radicals, present in high quantities and few antioxidants sufficiently formed to destroy them (Zelck and Von Janowsky 2004; Hanna *et al*. 2005).

Abdallahi *et al*. (1999) showed that inflammatory cells release ROS around the eggs, as soon as some granulocytes are present. At the same time, the activity of enzymes scavengers of H_2O_2, as catalase and glutathione peroxidase decreases drastically, as well as the reserves of reduced glutathione, in the liver.

Therefore, it is suggested that the higher the parasite load, the inflammatory reaction is intensified, consequently, there is a tendency to decrease the levels of GSH, as shown in Fig. 7.

As can be seen in Figs 6 and 7 and based on previous work, it was observed that individuals with the disease have changes in the levels of some parameters of oxidative stress (TBARS/MDA and GSH). The lipid peroxidation demonstrated by the increase of TBARS/MDA and the decrease in

GSH suggest a possible situation for oxidative stress in the group of subjects with schistosomiasis (test group). In addition, the results reinforce that there is a strong association between the parasitic load and intensity of lipid peroxidation in these subjects.

In conclusion, our findings point out that the oxidative stress may play an important role in the pathology associated to *S. mansoni*, as observed changes in levels of some parameters of enzymes and molecules that evaluate oxidative stress (MDA and GSH). Furthermore, it is important to study other indices that evaluate oxidative stress, such as determining the activity of superoxide dismutase and glutathione peroxidase, which can enhance the results obtained.

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