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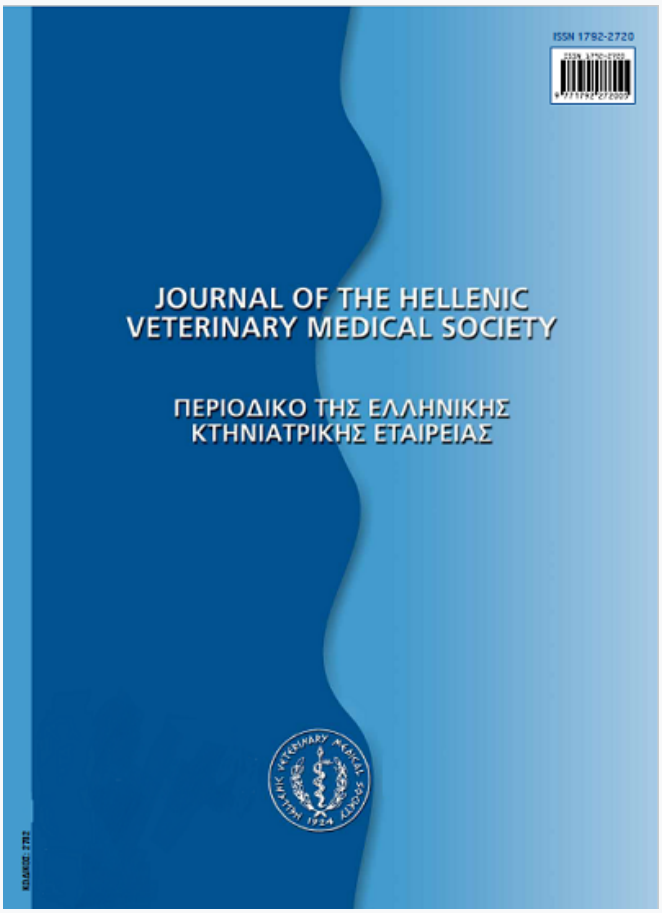

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Research article
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Significance of selected biochemical markers in predicting the outcome of schistosomiasis

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ABSTRACT. This study aimed to correlate the histopathological changes in mice liver with alterations either in liver tissue antioxidants enzymes (catalase and reduced glutathione (GSH)), oxidative stress marker (malondialdehyde (MDA)) or in serum liver function parameters (Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (T.P.) and albumin) to predict the outcome of schistosomiasis. Fourty male Swiss albino mice were used in this study and infected with *Schistosoma mansoni* for 2, 4, 6 and 8 weeks (8 mice for each group), while, the uninfected mice were used as negative control. Liver tissue antioxidants enzymes, oxidative stress marker and serum liver function parameters were determined in coincide with the liver tissue histopathological changes. All selected biochemical makers showed a strong significant positive correlation ($p < 0.05$) with liver histopathology score except serum albumin and liver tissue catalase enzyme. The last two parameters exhibited negative correlation with liver histopathology score. These results revealed that the more increase in the level of AST, ALT, T.P. and globulin in serum or liver tissue MDA and GSH indicating severs histopathological changed into the affected liver and hopeless prognosis is expected. On contrary, the increase in albumin level in serum or catalase level in liver tissue of affected patient/animal demonstrating mild liver histopathological changes. Subsequently, good prognosis and response to anti-schistosomal treatment will be predictable. This study opens the way to predict the outcome of schistosomiasis through easy and rapid biochemical test. Therefore, other studies are required to apply such correlation with other biochemical parameters especially that synthesized into the liver.

Keywords: *S. mansoni*; Mice; Oxidative stress; Histopathology.

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INTRODUCTION

Schistosomes are blood flukes, inhabit the portal blood system of many mammalian species and considered the causative agent of the second most important human parasitic disease in the world following malaria (Despommier et al., 2000).

Schistosomiasis is a serious parasitic disease causing a severe impairment in the liver functions in approximately 10 % of infected persons and affecting more than 200 million people in tropics and subtropics with 97 % of them living in Africa (Steinmann et al., 2006). It is usually characterized by an unnoticed acute phase, followed by liver fibrosis at chronic and advanced stages (Cheever et al., 2002). The chronic and debilitating nature of the disease has resulted in great losses in public health and economic productivity in developing countries (Fenwick et al., 2003). Due to the chronic nature of this disease, predicting its outcome is urgently required.

Schistosoma mansoni (*S. mansoni*) infection is characterized by the embolization of eggs from the intestine to the liver through the portal system. Next, most pathology is attributed to the host reaction to the eggs (Abdallahi et al., 1999). The toxic egg material destroys the host tissue cells and the antigenic material stimulates the development of strong inflammatory reactions around the egg. At the site of inflammation, oxidative stress occurs and leads to the generation of free radicals and the reduction of endogenous antioxidants (Abdallahi et al., 1999). Therefore, the present study was carried out to correlate the liver histopathological changes with the antioxidant enzymes, oxidative stress marker responses in *S. mansoni* infected mice. That will help in rapidly and easily predicting the outcome of this disease under field condition.

MATERIAL AND METHODS

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Animals

40 male Swiss albino mice (aged between 6-8 weeks) were bred and maintained at the experimental animal research unit of the *Schistosoma* biological supply program at Theodor Bilharz Research Institute (TBRI), Giza, Egypt). Mice were kept on a standard commercial pellet diet (El-Kahira company for oils and soap) and provided with water ad libitum in an air- conditioned animal house at 20-22°C. The animal experiments were conducted at the TBRI animal unit in accordance with international, ethical guidelines after approval of the institutional ethical committee of TBRI.

Experimental infection of mice with *S. mansoni*

Animals were infected with the Egyptian strain of *S. mansoni* (80 ± 10 cercariae/mouse) using the body immersion technique according to the method described by Liang et al. (1987).

Experimental design and blood samples collection

Mice were infected with *S. mansoni* for 2, 4, 6 and 8 weeks and the uninfected mice served as a control (eight mice for each group). Blood samples were collected from each mouse by cardiac puncture. Serum of each mouse was separated by centrifugation (1500 xg for 10 min) and kept frozen at −80°C until use. The experiment was performed twice.

Tissue homogenate

The liver was homogenized as previously described by Jatsa et al. (2015). Briefly, the liver lobe was collected from each mouse and homogenized in Tris–Hcl 50 mM buffer. Next, the homogenates were centrifuged at 3500 rpm for 25 min at 4 °C and supernatants were stored at −80 °C for the determination of oxidative stress biomarkers.

Liver function test

Total protein (T.P.) and albumin were measured spectrophotometrically using commercial test kits (Biodiagnostic, Cairo, Egypt) according to standard methods. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using commercial test kits (Biodiagnostic, Cairo, Egypt) according to Murray (1984).

Antioxidant enzymes and oxidative stress marker determination

Malondialdehyde (MDA) level, reduced gluta-

thione (GSH) and catalase enzymes activities were measured spectrophotometrically using commercial test kits (Biodiagnostic, Cairo, Egypt) according to (Beutler et al., 1963; Yoshioka et al., 1979, and Aebi, 1984), respectively.

Histopathological examination and scoring

Specimens from liver were collected and fixed in 10% buffered formalin and processed to paraffin blocks. Sections of 5-µm thickness were prepared from all specimens and stained by Haematoxylin and Eosin stain for microscopical examination (Teixeiral et al., 1996). The scoring was performed independently as fellows; no change in liver tissue, portal veins and blood vessels = 0, mild congestion in blood vessels with absence of worm, necrosis or fibrosis = 1, mild hydropic degeneration = 2, infiltration with inflammatory cells = 3, presence of *Schistosoma mansoni* worm in portal vein = 4, severe hydropic degeneration = 5, presence of coagulative necrosis = 6, presence of hepatic granuloma = 7, hepatic fibrosis = 8, hepatic fibrosis and bile duct hyperplasia = 9.

Faecal Examination

Faecal samples were collected from each mouse and examined for *S. mansoni* egg by sedimentation method as previously described (Katz et al., 1972).

Touchdown PCR

The 121-bp tandem repeats DNA sequence unit of *S. mansoni* described previously (Hamburger et al., 1991) was selected for our experiments. Primers for the touchdown PCR were 5′-CCGACCAACCGTTCTATGAA-3′ and 5′-CCCACGCTCTCGCAAATAAT-3′.The expected length of the product of the amplification was 92bp. *Schistosoma*-infected mouse sera were used directly as templates without a DNA extraction step. Human serum sample was included as negative control. Touchdown PCR was performed by using a GeneAmp PCR System 2700 (Applied Biosystems, CA, USA). A two-step cycle was applied in the touchdown PCR; i.e. a denaturing step and an annealing step. The annealing temperature (60 °C) was gradually lowered (1 °C after each cycle) to 50 °C. Fourty-cycle amplification was then performed with an annealing temperature of 50 °C. The PCR products were Acquiring to FAM fluorescence. By sequencing of the cloned amplification product, it was verified to be identical to the part of the 121-bp highly repeated DNA sequence.

Statistical analysis

Data analysis was performed using SPSS version 16.0 (SPSS). Mean values and standard deviation for each assessed variable were calculated. Statistical differences between examined groups were performed using one-way ANOVA with *post hoc* Duncan multiple comparison test. Differences between means at $p < 0.05$ were considered significant. Non-parametric correlation test (Kendall's tau-b and Spearman's rho correlation tests were used to test relation between liver histopathology score and selected biochemical variables. It assesses how well the relationship between variables can be described using a monotonic function.

RESULTS

Effect of *S. mansoni* infection on the liver function parameters, antioxidant enzymes and oxidative stress marker

In view of evaluating the impact of *S. mansoni* infection on the liver function, some parameters that known to be indicators of liver injuries were measured in the mice serum. ALT, AST activities and T.P. levels were increased significantly ($p < 0.05$) at 4, 6 and 8 weeks post-infection in comparison with control group (Table 1). On the other hand, there was a significant decrease ($p < 0.05$) in albumin level starting from 6 weeks post-infection (P.I.) (Table 1).

GSH and catalase have a great role in protecting the cells against oxidative stress. Our results revealed that GSH activity significantly increased ($p < 0.05$) in comparison with control with increasing the period of infection starting from 4 weeks P.I. (Table 2). On contrary, a significant decrease ($p < 0.05$) in the catalase activity was observed at 4, 6 and 8 weeks P.I. (Table 2).

MDA is the most important free radical that produced as sequel to lipid peroxidation process. Therefore, determination its level is an important indicator to the cellular oxidative destruction. MDA

Table 1. Effect of *S. mansoni* infection on mice serum ALT, AST activities, total proteins and albumin levels.

Groups	ALT (U/L)	AST (U/L)	T.P. (g/l)	Albumin (g/l)	Globulin (g/l)
Healthy mice	30.10 ± 2.80 ^{d}	31.60 ± 3.40 ^{d}	58 ± 1.40 ^{c}	48.50 ± 3.06 ^{a}	9.50 ± 1.7^{d}
2 weeks P.I.	29.80 ± 0.50 ^{d}	33.20 ± 1.10 ^{d}	56.90 ± 1.10 ^{c}	46.70 ± 1.49 ^{a}	10.20 ± 0.41^{d}
4 weeks P.I.	84 ± 4.10 ^{b}	140.20 ± 6.50 ^{b}	65.80 ± 3.20 ^{b}	46.40 ± 2.40 ^{a}	19.43 ± 0.88^{c}
6 weeks P.I.	94.50 ± 2.20 ^{a}	178 ± 7.80 ^{a}	69.20 ± 3.20 ^{b}	32.60 ± 2.50 ^{b}	36.69 ± 0.73^{b}
8 weeks P.I.	78.80 ± 3.30 ^{c}	128.70 ± 8.70 ^{c}	87.20 ± 2.90 ^{a}	26.80 ± 3.11 ^{c}	60.41± 0.24^{a}

a, b, c, d Variables with different superscript letters in the same column means significantly different at P < 0.05. Each value represents the mean ± S.D. for two experiments. P.I.; post infection, T.P.; Total protein, ALT; Alanine aminotransferase, AST; Aspartate aminotransferase.

Table 2. Effect of *S. mansoni* infection on the level of mice liver tissue antioxidant enzymes and oxidative stress marker.

Groups	Catalase (U/g)	GSH (mg/g)	MDA (mg/g)
Healthy mice	1.05 ± 0.10^{a}	12.30 ± 1.40 ^{d}	0.29 ± 0.10 ^{d}
2 weeks P.I.	1.06 ± 0.08 ^{a}	12.60 ± 1.07 ^{d}	0.52 ±0.04 ^{c}
4 weeks P.I.	0.48 ± 0.041 ^{b}	20.90 ± 2.10 ^{c}	0.66 ±0.06 ^{b}
6 weeks P.I.	0.38 ± 0.036 ^{c}	54.10 ± 6.04 ^{b}	1.47 ±0.19 ^{a}
8 weeks P.I.	0.26 ± 0.027^{d}	87.90 ± 6.90 ^{a}	1.82 ± 0.07 ^{a}

a, b, c, d Variables with different superscript letters in the same column means significantly different at P < 0.05. Each value represents the mean ± S.D. for two experiments. P.I.; post infection, GSH; Reduced glutathione, MDA; Malondialdehyde.

activity was increased significantly ($p < 0.05$) with increasing the period of infection (Table 2). Thus, MDA is the most sensitive biochemical parameter to the oxidative damage resulting from *S. mansoni* infection.

Effect of *S. mansoni* infection on the liver tissue histopathology

Two weeks infection by *S. mansoni* resulted in hydropic degeneration besides mild congested blood vessels in the mice hepatic tissues (Fig. 1a). Two weeks later, the immature *Schistosoma* worms were observed in portal vein (Fig. 1b). Subsequently, the portal areas became infiltrated with inflammatory cells mainly eosinophil, congestion was observed at the portal hepatic vessels and severe hydropic degeneration was seen at the hepatocytes (Fig. 1c). Two weeks later, *Schistosoma* eggs were observed in portal vein and the portal areas were infiltrated with eosinophil and round cells. Moreover, focal areas of coagulative necrosis infiltrated with eosinophils were seen adjacent to portal vein (Fig. 1d). In addition, young egg granuloma consisted from single or multiple mature or immature eggs surrounded with inflammatory cells mainly eosinophils were seen replacing the hepatic parenchyma (Fig. 1e). Two months post infection, the hepatic parenchyma were focally replaced with young or old hepatic granuloma (Fig. 1f). Caseous necrosis and fibrous tissue infiltrated with eosinophils and macrophages were surrounded this granuloma (Fig. 1g) and some egg nodules were completely replaced with mature fibrous tissue (Fig. 1h). Moreover, hyperplasia of bile ducts was seen in the portal area with congestion of hepatic blood vessels and presence of inflammatory cells (Fig. 1i).

Correlation of selected biochemical markers with liver tissue histopathological changes

All selected biochemical makers showed a strong significant positive correlation ($p < 0.05$) with liver histopathology score except serum albumin and liver tissue catalase enzyme. The last two parameters exhibited negative correlation with liver histopathology score (Tables 3 and 4). These results revealed that the more increase in the level of AST, ALT, T.P. and globulin in serum or liver tissue MDA and GSH indicating severs histopathological changed into the

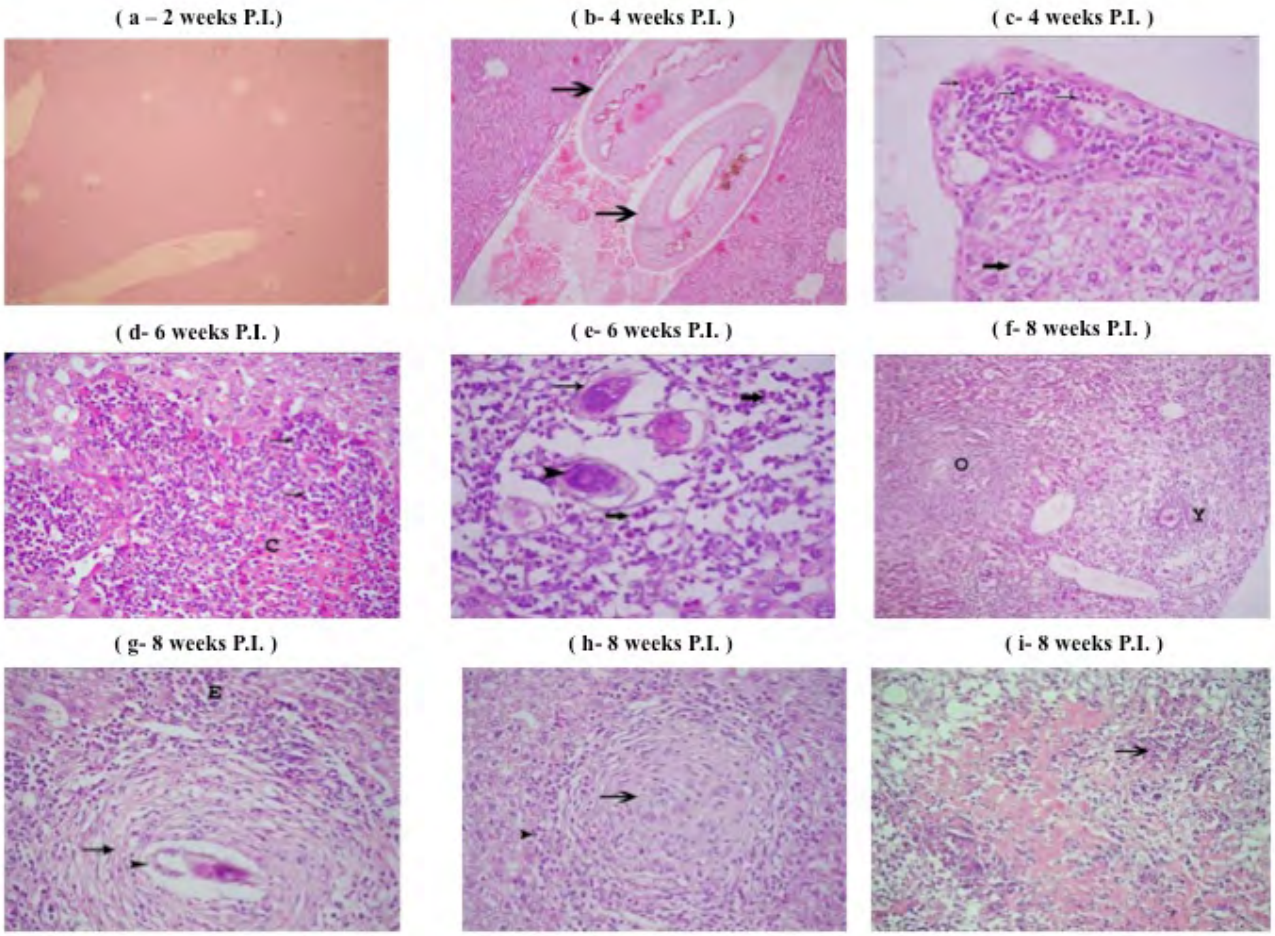


Fig 1. Histopathology of mice liver infected with *S. mansoni*. (a) Hydropic degeneration besides mild congested blood vessels were shown in the mice hepatic tissues (b) The immature *Schistosoma* worm was observed in portal vein (arrows) (c) The portal areas became infiltrated with inflammatory cells mainly eosinophil (small arrows) and severe hydropic degeneration was seen at the hepatocytes (large arrows). (d) The portal areas were infiltrated with eosinophil and round cells (arrows). Also, focal areas of coagulative necrosis infiltrated with eosinophil were seen adjacent to portal vein (C; coagulative necrosis). (e) Young egg granuloma surrounded with inflammatory cells mainly eosinophil were seen replacing the hepatic parenchyma (arrows). (f) The hepatic parenchyma was focally replaced with young or old hepatic granuloma (Y; young granuloma, O; old granuloma). (g) Caseous necrosis or fibrous tissue infiltrated with eosinophil's and macrophages were seen surrounded the old hepatic granuloma (arrow) (h) Mature fibrous tissue were shown replaced the egg nodules completely (arrows). (i) The portal areas showed hyperplasia of bile ducts besides congestion of hepatic blood vessels and inflammatory cells (arrow). P.I.; post infection.

affected liver and hopeless prognosis is expected. On contrary, the increase in albumin level in serum or catalase level in liver tissue of affected patient/animal demonstrating mild liver histopathological changes. Subsequently, good prognosis and response to anti-schistosomal treatment will be expected.

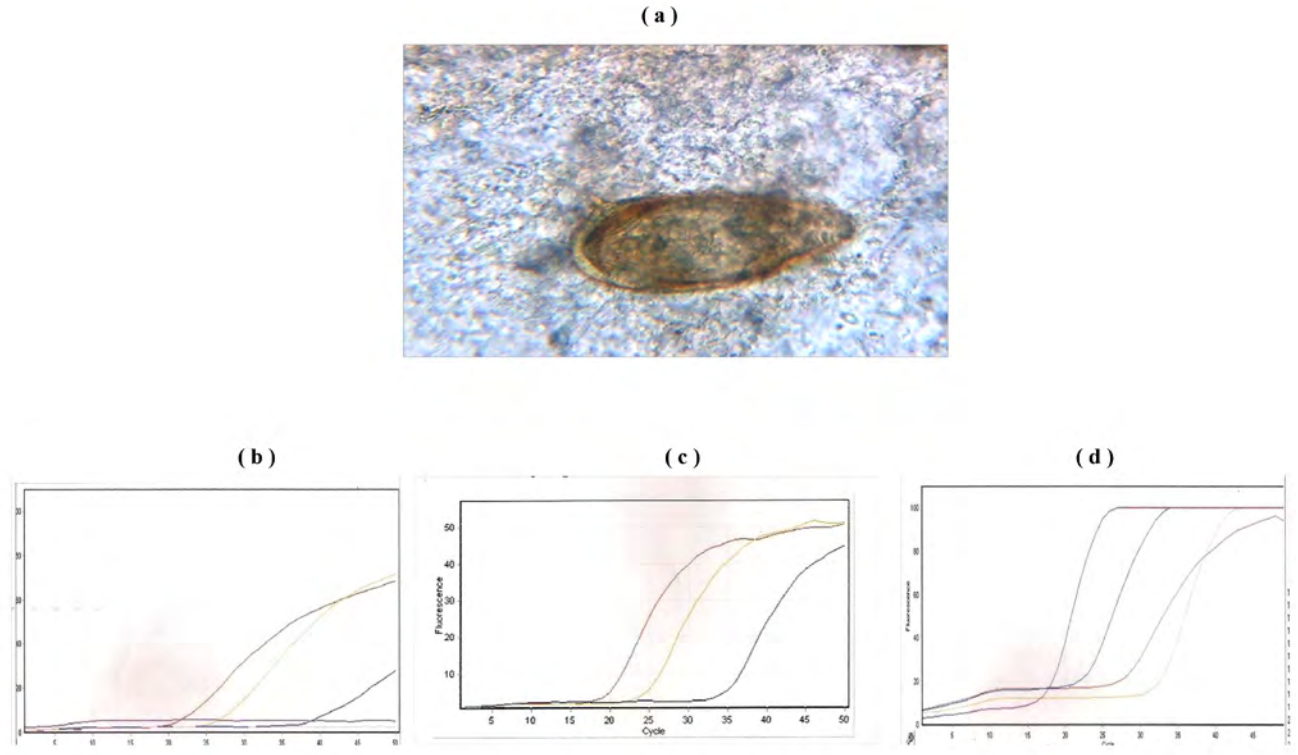
Confirmation of S. mansoni infection

In this study, 2 tests; faecal examination and touch-down PCR were used for confirmation the infection by *S. mansoni* in the infected mice. The *S. mansoni* eggs were detected in the mice faeces at 8 weeks post-infection. The detected egg was large round ovoid, non-operculated egg, containing fully mature miracidium with lateral spine **(**Supplementary figure. a**)**. On contrary, the infection was detected by touchdown PCR at 2 weeks post-infection (Ct values

≤ 29 are strong positive, Ct values 30-39 are moderate positive and Ct values ≥ 40 are weak reaction) **(**Supplementary figure. b, c and d**)**.

logical changes in liver with the antioxidant enzymes and oxidative stress markers.

The results of ALT and AST activities were in



Supplementary figure. Faecal examination and touch- down PCR for confirmation the infection by S. mansoni in mice. (a) Faecal examination. (b), (c), and (d) Touchdown PCR.bile ducts besides congestion of hepatic blood vessels and inflammatory cells (arrow). P.I.; post infection.

DISCUSSION

Schistosomiasis is a debilitating disease with high economic impact, affects many people all over the world leading to high morbidity and mortality (Curtis and Minchella, 2000). Animal models are used as tools for understanding the host-parasite relationships. Mice have been shown to be permissive to *S. mansoni* and they have been widely used to answer fundamental questions on the dynamics of *Schistosoma* infections, including diagnosis (Cheever et al., 2002 and Wang et al., 2004). Because of problems in collecting sufficient numbers of well-defined samples from human patients (from recently acquired infections), this study was conducted on *S. mansoni* infected mouse model and correlated the histopathoagreement with previous studies (Gharib et al., 1999 and EL-Sokkary et al., 2004). Such increase in transaminase enzymes activities after 4, 6 and 8 weeks P.I. might be attributed to presence of immature and mature *Schistosoma* worms in portal vein and infiltration of this area with inflammatory cells, congestion in the portal hepatic vessels and severe hydropic degeneration in the hepatocytes and hepatocytes replacement by focal areas of coagulative necrosis. All of these changes leading to decrease hepatocytes population, and increased cell membrane permeability, subsequently; transaminase enzymes were released into the circulation. Additionally, this complete destruction of hepatocytes, which are responsible for albumin synthesis might explain the decrease

Table 3. Correlation between liver histopathology score and serum ALT, AST activities, total proteins and albumin levels

Bivariate correlation	Kendall's tau-b	Spearman's rho correlation
Histopathology score * ALT	0.473 **	0.655 **
Histopathology score * AST	0.514**	0.676 **
Histopathology score * T.P	0.775**	0.901 *
Histopathology score * albumin	-0.741**	-0.851 **
Histopathology score * globulin	0.864**	0.949**

***P<0.05 a strong significant correlation between liver histopathology score and serum biochemical variable*

Table 4. Correlation between liver histopathology score and liver tissue antioxidant enzymes and oxidative stress marker

Bivariate correlation	Kendall's tau-b	Spearman's rho correlation
Histopathology score * Catalase	-0.823**	-0.927 **
Histopathology score * GSH	0.844 **	0.938**
Histopathology score * MDA	0.926**	0.982 **

***$P < 0.05$ a strong significant correlation between liver histopathology score and liver tissue antioxidant enzymes

in serum albumin level 6 weeks P.I. The decrease in albumin level was in accordance with previous studies (Gharib et al., 1999 and EL-Sokkary et al., 2004).

Liver plays an important role in protein metabolism; thereafter the hepatocytes damage will be reflected on the total protein levels (Mbuh et al., 2005). Therefore, the replacement of hepatic cells by fibrous tissue might explain the significance increase ($P< 0.05$) in T.P. levels 4, 6 and 8 weeks P.I. The increase in serum T.P. levels was in agreement with previous study (Page et al., 1972). In addition, the increase in GSH level might be interprets the increase in T.P. level, due to the critical role of GSH in proteins synthesis (Sen, 1997 and Gul et al., 2000). Moreover, the increase in globulin fraction as apart of body immunity response to the parasitic infestation (Harfoush et al., 2003) might be another theory explained the increase in serum T.P. level.

It was previously reported that in parasitic diseases there is a complex and a dynamic physiological relationship between the parasite and the antioxidant defense components of the host (Coutinho et al., 2007). Catalase enzyme has the ability to protect the cell from the accumulated H2O2 produced from dismutation of superoxide anion (Nare et al., 1990). The decrease in liver tissue catalase enzyme activity 4 weeks P.I. was in accordance with (Dessein et al., 1999 and EL-Sokkary et al., 2004). The depletion in the catalase enzyme at 4 weeks P.I. might be attributed to the rapid destruction that observed in hepatic tissue, which by its role consume the enzyme by high amount.

The results of tissue-reduced glutathione (GSH) were in accordance with Hirota et al., 1989 and Song et al., 2000. GSH is an important intracellular antioxidant and play a major role in protecting cells against reactive oxygen species (ROS) and free radicals pro-

duced even in normal metabolism (Sen, 1997 and Gul et al., 2000). Hepatic tissue represents the major GSH reservoir for extra-hepatic levels (Lew et al. 1995). Both hepatic and extra-hepatic GSH are released into the circulation by the help of stressors through an alpha –receptor mechanism (Lew et al., 1985 and Song et al., 2000). The increase in GSH level 4 weeks post-infection might be attributed to the replacement of the hepatic cells with coagulative necrosis and fibrous tissue, which stimulate the extra-hepatic reservoir to secrete high amount of GSH to overcome the shortage resulted from this damage.

Lipid peroxides were elevated by *S. mansoni* throughout the infection (Shaheen et al., 1996 and Pascal et al., 2000). Lipid peroxidation resulted in oxidative destruction of cellular membrane. Subsequently, the toxic free radicals were secreted and MDA is one of the most important free radicals (Cheeseman et al., 1993 and Paradis et al., 1997). Therefore, the elevation of MDA level was observed at 2 weeks post-infection. This finding was in accordance with previous reports (Shaheen et al., 1996 and Pascal et al., 2000).

This study aims to contribute in unveiling the correlation of different biochemical parameters either in serum or in liver tissue with hepatic tissue changes. In conclusion, liver tissue antioxidants enzymes and the oxidative stress marker are sensitive biochemical parameters to the stage of *S. mansoni* infection, and they may be useful to expect the host response to treatment in clinical case. The more increase in the level of AST, ALT, T.P. and globulin in serum or liver tissue MDA and GSH indicating severs histopathological changed into the affected liver and hopeless prognosis is expected. On contrary, the increase in albumin level in serum or catalase level in liver tissue of affected patient/animal demonstrating mild liver histopathological changes. Subsequently, good prognosis and response to anti-schistosomal treatment will be anticipated. However, we applied this preliminary correlation in mice, more studies are required to correlate the serum levels of other biochemical variables that originated from the liver as acute phase proteins with the histopathological changes in liver and other affected organs in schistosomiasis. Furthermore, other studies are required to include chronic stage of the disease on more prolonged period of the infection in mice.

CONFLICT OF INTEREST

The authors of this paper have declared that no competing interests exist.

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