Excretory-secretory product of *Fasciola hepatica* worm protects against *Schistosoma mansoni* infection in mice

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The objective of this study was to evaluate the protective immunity of excretory-secretory products of *Fasciola hepatica* (FhES) worm against *S. mansoni* infection in mice. Evaluation of FhES antigen was through measuring worm burden, ova count, granuloma size and frequency as well as the histopathological picture of the liver. The study was extended to determine the level of free radical scavengers; lipid peroxide, glutathione (GSH), vitamin C, vitamin E, catalase and superoxide dismutase (SOD). Liver function enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were also taken into consideration. Four groups of eight mice each were selected for this study. Group 1 served as control group. Group 2: normal healthy mice vaccinated with FhES product. Group 3: *S. mansoni* infected mice for 2 months and group 4: infected mice pre-vaccinated with FhES antigen. Vaccination schedule comprised of a single subcutaneous injection of FhES antigen emulsified with Freund’s complete adjuvant in a dose 0.5 mg protein/mouse, followed by intraperitoneal injections of the same antigen without adjuvant in 3 doses/week for 3 successive weeks. The total antigen inoculation was 5 mg protein/mouse. The present results revealed a drastic change in all the measured parameters after *S. mansoni* infection and a noticeable improved level after vaccination with FhES antigen. It can be concluded that FhES antigen succeeded to protect mice against schistosomiasis by a significant reduction in worm burden, ova count, granuloma size and number, improvement in the histopathological architecture of the liver as well as amelioration in the antioxidant levels under investigation.

**Keywords**: Antioxidant, Enzymes, Excretory-secretary antigen, *Fasciola hepatica*, *Schistosoma mansoni*

Schistosomiasis, caused by parasitic trematode worms (schistosomes), is a major threat to rural population in the developing world. The parasitic worms are not even identified by the self-defense system of the host because they get coated with host antigen in such a way that they are not recognized as foreign bodies by the host immune system.

A common antigen or antigenic determinant in the family of the pathogenic trematodes of humans appears partially to be responsible in the development of acquired resistance to challenge infection with homologous or closely related genera, as fascioliasis. The use of *Fasciola* as a source of antigen is because of fascioliasis worm has been demonstrated to have common or cross-reacting antigens with schistosomes, associated with high eosinophil levels, the killer cells of schistosomes as well as its capability in induction of a specific immunologic mechanism of defense against schistosomiasis. Moreover, *Fasciola hepatica*, due to its larger size, easier and safer maintenance experimentally, could provide a good source of antigen. Therefore, vaccines that can reduce, schistosomiasis morbidity and mortality by lowering intensity of infection or by modifying immune response to parasite derived antigen should be adopted for use, even if they are not effective in complete elimination of the parasites.

In this study, we have evaluated the excretory-secretory product (metabolic antigen) of *Fasciola hepatica* for inducing a protective immunity against *S. mansoni* challenge infection in mice. Worm recovery, egg count, granuloma size and frequency, liver histopathology, free radical scavengers; lipid peroxide, glutathione (GSH), vitamin C and E, catalase, superoxide dismutase (SOD) as well as liver function enzymes; [aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP)] were the parameters used for evaluation of the degree of resistance.

**Materials and Methods**

**Chemicals**— All chemicals used were of high analytical grade, products of Sigma (US), Merk (Germany) and BDH (England).
**Animals**—Male Swiss albino mice CDI strain weighting 18–22 g were obtained from Theodor Bilharz Research Institute, Cairo, Egypt and maintained on stock commercial pellet diet (El-Kahira Company for Oil and Soap) and provided water ad libitum.

**Antigen preparation**—The excretion-secretion antigen was prepared according to the method of Hillyer. Groups of 10 live worms were washed with 0.9% saline and put in petri dish containing phosphate buffer (0.01 M) at room temperature for 2 h. Worms were then removed, the remaining fluid was centrifuged, collected the supernatant and stored at −20°C till further use.

**Antigen administration regimens**—The protein content of the antigen was determined by the method of Bradford and the final quantity was then emulsified with an equal volume of Freund’s complete adjuvant. The immunization schedule was followed according to the method of Tendler et al.

Each mice was sensitized with a single dose of (0.5 mg protein) injection (subcutaneous; sc) of the emulsified antigen in the first week followed by intraperitoneal (ip) injection of the same antigen concentration without adjuvant, 3 doses a week for 3 weeks. Therefore, each mice received a total dose of 5 mg protein of antigen in 4 weeks.

**Experimental design**—The animals were divided into four groups of eight mice each comprising Group 1- normal healthy control mice; Group 2- normal healthy mice injected with 5 mg of excretory-secretory antigen by the same administration regimens described above, left for two months and sacrificed; Group 3- *Schistosoma mansoni* infected mice with 80 cercariae of Egyptian strain by tail-immersion technique and sacrificed after 2 months and Group 4- *Schistosoma mansoni* infected mice pre-vaccinated with 5 mg of excretory-secretory antigen and sacrificed two months post-infection.

**Preparation of tissue homogenate**—Liver tissue was homogenized in double distilled water and 20% of liver homogenate was prepared for estimation of lipid peroxide, glutathione, vitamin C and E, catalase and superoxide dismutase. Further, dilution of liver homogenate to10% was prepared for determination of AST, ALT and ALP.

**Assay of different Parameters**—Protein was estimated by the method of Bradford, where bovine serum albumin was used as a standard protein and the colour developed was read colourimetrically at 595 nm.

Lipid peroxide was determined as malondialdehyde. Its concentration was calculated using the extinction coefficient value 1.56 < 10³ M⁻¹ cm⁻¹ and read at 535 nm by the method of Buege and Aust.

Glutathione was estimated by the method of Moron et al. using pithiobis-2-nitrobenzoic acid (DTNB) in phosphate buffer. The reaction colour was read at 412 nm. The method adapted by Jogata and Dani was used for estimation of vitamin C using Folin reagent and the developed colour was read at 760 nm.

Vitamin E was measured by the colourimetric assay of Angustim et al. The method has been based on the oxidation of xylene-extracted tocopherols of the liver homogenate by ferric chloride and the pink complex of ferrous ions, bathophenanthroline, was measured at 536 nm.

Catalase activity was assayed spectrophotometrically following decrease in absorbance at 230 nm using molar extinction coefficient of hydrogen peroxide (62.4) according to Nelson and Kiesow.

Superoxide dismutase was estimated by the method of Nishikimi et al. The method depends on following increase in absorbance at 560 nm using molar extinction coefficient of NADH (6.22 < 10³).

Alanine (ALT) and aspartate aminotransferases (AST)—were measured by the method of Reitman and Frankel. The colorimetric determination depends on determining amounts of oxaloacetate and pyruvate formed from 2,4-dinitrophenyl hydrazine of oxaloacetate and pyruvate, the developed colour was read at 520 nm.

Alkaline phosphatase was estimated by the method of King and King. The values are represented as liberated phenol at 510 nm.

**Worm counting**—Worms were recovered by liver perfusion as described by Smithers and Terry. The per cent of reduction in worm burden after challenge was calculated by the method of Tendler et al. as follows-

\[ P = \frac{C-V}{C} \times 100 \]

where, P- protection (%); C- mean number of parasite recovered from infected animals; and V- mean number of parasite recovered from vaccinated animals.

**Oogram determination**—All viable and dead eggs were counted microscopically and classified according to the method of Pellegrino et al.
Histopathology — Representative slices from liver tissue were taken from the eviscerated animals and fixed in buffer formalin (10%). Paraffin embedded sections (4μm thick) were taken after fixation and slides were stained using haematoxylin and eosin (H&E) by the method of Hirsch *et al.*

Granuloma count — Granuloma count was carried out in five successive fields (10 × 10) of serial tissue section of more than 25μm apart. Granuloma dimensions were measured using an ocular micrometer for the lobular granuloma with central ova.

Statistical analysis — Data in the present study has been expressed as mean ± SD of eight mice in each group. The statistically significant difference between control and other groups was determined using independent *t*-test.

**Results**

Vaccination of mice with excretory-secretory antigen of *Fasciola hepatica* recorded significant reduction in total, male and female worms, amounting to 56.36, 44.09 and 72.08%, respectively. The Oogram pattern showed significant reduction in total and living ova by a ratio 43.16 and 70.22%, respectively, while number of dead ova revealed significant increase (36.81%) as compared to *S. mansoni* infected mice (Table 1). Granuloma count in vaccinated mice also showed a significant reduction (61.47%) accompanied by significant reduction in its diameter (59.67%) and total area of infection (84.46%) as seen under the microscope in low power field (Table 2).

Histopathological studied proved the earlier results, where the bilharzial liver showed multiple granulomatous lesions, and focal areas of necrosis. The granulomatous reaction results from periportal cellular infiltration around mature ova and extend towards similar lesions neighbouring portal tract. It reached its maximum size surrounding ovum at the end period of infection. The brownish black bilharzial pigmentation was also observed in Kupffer cells of the infected liver sections (Fig. 1 a, b) as compared to the normal liver section (Fig. 2a).

The liver from vaccinated animals showed improvement represented by fewer granuloma count and size with a minimal degenerative change in ova (Fig. 1 c, d), as compared with liver sections of *S. mansoni* infected mice (Fig.1a, b). However, antigen administration did not appreciably change the type of granulomatous reaction (Fig.1d). Liver of normal healthy mice vaccinated with excretory-secretory antigen showed more or less normal hepatic architecture (Fig.2b).

Infected mice recorded significant decrease in glutathione, vitamin C, vitamin E, catalase, AST and ALT levels, while significant increase in lipid peroxide, superoxide dismutase and ALP were observed (Tables 3, 4).

Vaccination of *S. mansoni* infected mice showed amelioration level in all antioxidant parameters under investigation amounting to 38.75, 26.81, 31.77, 43.00, 26.75 and 21.92% for lipid peroxide, glutathione, vitamin C, vitamin E, catalase and superoxide dismutase, respectively. Liver function enzymes recorded amelioration level to 29.33, 25.45 and 49.82% for AST, ALT and ALP, respectively (Tables 3, 4).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Infected</th>
<th>Vaccinated</th>
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<tbody>
<tr>
<td>Granuloma diam. (μm)</td>
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<tr>
<td></td>
<td>124.65 ± 4.50*</td>
<td>50.27 ± 2.11*</td>
<td>59.67</td>
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<tr>
<td>Granuloma count/LPF</td>
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<td>9.63 ± 0.31</td>
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<td>Total area of infection (μm)/LPF</td>
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<td></td>
<td>1200.37 ± 20.14</td>
<td>186.50 ± 6.32*</td>
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*significant as compared to infected group.

Table 2 — Granuloma diameter and count in infected and vaccinated mice
[Values are mean ± SD of 8 mice in each group]

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LPF -Low power field of the microscope.

a Total area of infection= number of granulomata × size of one granuloma

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Values in brackets indicate percentage change over respective control.
Insignificant change in all biochemical parameters were observed after vaccination of normal healthy mice (Tables 3, 4).

Discussion

Early studies have been focused on the area of cross-reactivity between parasitic trematodes and its relation of cross protection with emphasis on Fasciola/Schistosoma model. Fasciola/Schistosoma model is the one showing the most consistent induction of high levels of immunity to challenge with Schistosoma cercariae by sharing common and/or cross-antigens. Thus, it has been suggested that F. hepatia antigens inducing the protection are being excreted by the worm as metabolic antigens. This metabolic antigen appears to be an ideal molecule for inducing and eliciting T-lymphocytes dependent cell-mediated immunity against schistosomes. Thus, induction of lymphocytes-mediated immune responses draining to the site of injection and showing abundant IFN-α that induced resistance to re-infection and activate macrophages to kill schistosomula.

The protective immunity of Fasciola metabolic antigen against schistosomiasis has been seen in the present study as indicated by reduction in worm burden and ova count to 56.36 and 43.16%, respectively. Cheever et al. have reported that

Fig. 1—Liver sections showing (a, c) granulomatous frequency and (b, d) size in Schistosoma mansoni infected and vaccinated mice [stained H &E; 10×]
absolute number of eggs in an organ and the concentration of eggs per gram tissue depend upon duration of infection, intensity of infection, rate of laying eggs, proportion of eggs passed in the faeces, rate of being destroyed in the tissue and distribution of eggs in different organs. It was observed in the present study that there was no relationship between egg count and worm pair, this may be due to death of some worms or they are lost from the portal system, hence the number of eggs found might include eggs laid by worms not recovered by perfusion. Therefore, eggs count is not dependent on worm burden but on the fecundity status of worm.

As mentioned above, number of worm burden and ova count increase the degree of liver fibrosis and granulomatous reaction. This is in agreement with the present histopathological findings of bilharzial liver through increased number and size of granuloma, liver miracidia and extensive fibrous tissue accumulation. Vaccination of infected mice with excretory-secretory antigen of Fasciola hepatica showed diminution in number and size of granulomas, diminution of its fibrotic and collagen content accompanied with a reduction in total area of infection and destruction of ova. This was also confirmed by increase in mortality rate of female than male worm. This gave an additional support that the antigenic material in excretory-secretory product of Fasciola worm affected the fecundity state of Schistosoma worm.

It is evident that Schistosoma toxins elaborated by worms activated H$_2$O$_2$/myeloperoxidase system, the defense mechanism associated with inflammation, is activated in close contact with parasite egg. Although the process contributes to egg killing in vivo it causes accumulation of H$_2$O$_2$, superoxide anion and hydroxyl radicals in the host’s tissue. This observation was confirmed by our results through measuring different free radical scavengers: lipid peroxide, glutathione, vitamin C, E, catalase and superoxide dismutase. The present data revealed significant increase in lipid peroxide and superoxide dismutase, while significant decrease in the other parameters indicating that infection with S. mansoni impairs the antioxidant system, since the level of glutathione depletion is used as an index of oxidative stress and a sign that hepatic cells are utilizing more antioxidant defenses. Gharib et al. have attributed the decrease in glutathione level to increased cytoxicity by H$_2$O$_2$ which is produced as a result of inhibition of glutathione reductase that keeps glutathione in its reduced form.

Pascal et al. and Soliman et al. have reported that oxidative stress due to schistosomiasis causes an elevation in lipid peroxide, since the complex mechanism of lipid peroxidation is known to require the participation of highly reactive oxygen and other reactive oxygen metabolites in the chain of biochemical reactions, thus whenever these free radicals are involved, lipid peroxides in turn increase.

![Liver sections of (a) normal healthy control mice; and (b) normal healthy vaccinated mice](stained H & E ; 10×)
HAMED: EXCRETORY-SECRETORY PRODUCT OF *FASCIOLA HEPATICA* WORM PROTECTS

With regard to vitamin C, and coinciding with the present results, Frei *et al.*\(^40\) have reported that peroxyl radicals are trapped by ascorbate and thus, the level of the enzyme decreases during the free radical scavenging process. Also, the reduction of vitamin E after schistosomal infection occurs since the vitamin acted as a soluble antioxidant to protect biological membranes against oxidative stress which led to distribution of cell function. In a related study, Sokal *et al.*\(^41\) have reported that vitamin E protects hepatocytes against lipid peroxidation and toxic injury.

During oxidative stress, such as in case of schistosomal infection, peroxide dismutation yields \(\text{H}_2\text{O}_2\), which is detoxified by catalase and thus, results in decline in its activity\(^37, 42\). Superoxide dismutase detoxifies the cytotoxic \(\text{O}_2^-\) and is, thus, generally considered as a potent antioxidant \(^43\). The present data revealed significant increase in SOD after *S.mansoni* infection in mice, which has been confirmed by Shaheen *et al.*\(^44\) and Rizk\(^45\) who have reported the same results and attributed this increase to increase in peroxidative stress in liver. Sanz *et al.*\(^46\) have studied the antioxidant defense in rats against oxidative stress and reported cell defense mechanisms against oxygen toxicity increased in the liver to suppress oxidative imbalance, thus SOD increases and glutathione decreases.

With respect to transaminases, a significant reduction was observed in both AST and ALT activities following schistosomal infection. As mentioned earlier, the free radicals are enhanced by schistosomal infection and this may cause irreversible damage to mitochondrial membrane which may lead to discharge of its enzyme contents. In this regard, Ozares *et al.*\(^47\) have stated that these enzymes decrease lower liver protein content because of their release to the blood stream or decrease synthesis. Since aminotransferases are marker enzymes for cell toxicity, this gives an additional support to the liver injury induced by infection. This hepatocellular damage results from egg deposition resulting in cell fibrosis and/or increase in cell permeability leading to enzyme discharge to the blood stream\(^48\). Also, the activities of transaminases can serve as index of metabolic aerobity degree, since AST can provide Krebs cycle intermediates, while ALT can be correlated with lactate production. These observations indicate

### Table 3—Effect of vaccination with excretory-secretory antigen on different antioxidant levels in mice liver

[Values are mean ± SD of 8 mice in each group]

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Control vaccinated</th>
<th>Infected</th>
<th>Vaccinated infected</th>
<th>% Improvement (Vaccinated infected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid peroxide</td>
<td>0.80 ± 0.05</td>
<td>0.82 ± 0.04 (+2.5)</td>
<td>1.21 ± 0.03(^*) (+51.25)</td>
<td>0.90 ± 0.02(^*) (+12.5)</td>
<td>38.75</td>
</tr>
<tr>
<td>Glutathione</td>
<td>30.24 ± 0.61</td>
<td>29.88 ± 0.60 (-1.19)</td>
<td>18.98 ± 0.43(^*) (-37.23)</td>
<td>27.09 ± 0.52(^*) (-10.41)</td>
<td>26.81</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>9.63 ± 0.31</td>
<td>9.38 ± 0.27 (-2.59)</td>
<td>5.32 ± 0.14(^*) (-44.75)</td>
<td>8.38 ± 0.15(^*) (-12.98)</td>
<td>31.77</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>7.51 ± 0.16</td>
<td>7.17 ± 0.17 (-4.52)</td>
<td>3.24 ± 0.10(^*) (-56.85)</td>
<td>6.47 ± 0.14(^*) (-13.84)</td>
<td>43.00</td>
</tr>
<tr>
<td>Catalase</td>
<td>60.17 ± 1.55</td>
<td>58.74 ± 1.43 (-2.37)</td>
<td>36.56 ± 2.23(^*) (-64.57)</td>
<td>52.66 ± 1.59(^*) (-12.48)</td>
<td>26.75</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>158.26 ± 2.44</td>
<td>160.16 ± 2.12 (+1.00)</td>
<td>197.14 ± 2.50(^*) (+24.56)</td>
<td>162.44 ± 1.86(^*) (+2.64)</td>
<td>21.92</td>
</tr>
</tbody>
</table>

Lipid peroxide is expressed as nmol/mg protein. Glutathione and vitamin C & E are expressed as μg/mg protein. Catalase and superoxide dismutase are expressed as μmol/mg protein.

\(^*\)significant as compared to control group.

Values in brackets indicate % change over control.

### Table 4—Effect of vaccination with excretory-secretory antigen on liver function enzymes in mice liver

[Values are mean ± SD of 8 mice in each group]

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Control vaccinated</th>
<th>Infected</th>
<th>Vaccinated infected</th>
<th>% Improvement (vaccinated infected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate aminotransferase (AST)</td>
<td>45.23 ± 2.13</td>
<td>43.98 ± 2.18(^*) (-2.76)</td>
<td>24.20 ± 1.96(^*) (-46.49)</td>
<td>37.47 ± 1.68(^*) (-17.15)</td>
<td>29.33</td>
</tr>
<tr>
<td>Alanine aminotransferase (ALT)</td>
<td>22.71 ± 1.54</td>
<td>21.58 ± 1.26(^*) (-4.97)</td>
<td>13.66 ± 1.13(^*) (-39.85)</td>
<td>19.44 ± 1.32(^*) (-14.53)</td>
<td>25.45</td>
</tr>
<tr>
<td>Alkaline phosphatase (ALP)</td>
<td>5.66 ± 0.34</td>
<td>6.00 ± 0.48(^*) (+6.00)</td>
<td>9.93 ± 0.21(^*) (+75.44)</td>
<td>7.11 ± 0.56(^*) (+25.61)</td>
<td>49.82</td>
</tr>
</tbody>
</table>

All values are expressed as μmol/min/mg protein.

\(^*\)significant as compared to control group.

Values in brackets indicate % change over respective control.
about the regulatory role of AST in oxidative metabolism and ALT in anaerobic glycolysis.

In the present study, alkaline phosphatase enzyme activity shows a significant increase in infected mice as mentioned earlier by El-Aasar et al.\(^4\) They have attributed the increase in enzyme activity to irritation of liver cells by toxins or metabolic products of growing schistosomules, adult worms and eggs, while Mansy et al.\(^5\) have attributed this increase to proliferation of bile ductules and bile canaliculi as a result of schistosomiasis. These results have confirmed the observation of Kaplan\(^6\) who has suggested that the response of liver to any form of biliary tree obstruction is to synthesize more ALP.

Vaccination of mice with the metabolic antigen of Fasciola hepatica worm enhanced the levels of glutathione, vitamin C, E, catalase, AST and ALT, whereas lipid peroxide, superoxide dismutase and ALP decreased, but not reached the normal values.

In conclusion, metabolic antigen of Fasciola hepatica succeeded to protect mice against S. mansoni infection by reduction in worm burden, ova count, granuloma diameter and its number as well as decreased the levels of toxins enhanced by them. This proved the role of metabolic antigen in eliminating the product of oxidative stress and assistance in immune-mediated destruction of egg that ameliorate liver cell toxicity and preserve its function.

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