Garlic Treatment Reduces Granuloma and P53 expression in Experimental Schistosomiasis

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Abstract
Schistosomiasis is one of the major human parasitic diseases in many developing countries and is one of the causes of morbidity and mortality in the human population. The present work aimed to study the effects of garlic on granuloma sizes and liver fibrosis. Also; the alternation in p53 expression of in mouse liver tissues in different groups under study were observed. Forty adult male mice were divided into four groups (10 animals each). First and second groups were the control and garlic groups respectively while the third group was the infected group in which mouse liver were infected with Schistosoma mansoni live cercariae for 6 weeks. The fourth group was the treated group in which mice were infected with Schistosoma mansoni live cercariae and then treated with garlic after schistosoma infection. Treatment with garlic led to a significant reduction in granuloma area in treated infected mice (301.2±10.3 nm) compared to non-treated infected mice (382.3±9.7 nm). Significant increase in GGT, ALT and AST levels in infected group with schistosoma when compared with control group. On the other hand; mice that received garlic showed a significant decrease (P<0.01) in serum GGT, ALT and AST induced by S. mansoni infection. Significant increase in the apoptotic protein p53 in infected liver with schistosoma when compared with control group. P53 expression and the incidence of apoptotic cells were very low in control and garlic liver sections groups, where it was 0.4±0.23 and 0.47±0.83. P53 positive cells in infected group were 3.87±0.53. A significant decreased in p53 positive cells in treated infected liver with garlic (1.12±0.65) when compared with infected group. Treatment of infected mice with garlic reduces the incidence of apoptotic cells when compared with that in infected liver.

Key words: Schistosomiasis, Garlic, Granuloma, Liver functions, Fibrosis, P53 expression.

Introduction
IN 1851, Theodor Bilharz described a parasitic infection (bilharzia) that would later be termed schistosomiasis. Currently, 200 million people in 74 countries have this disease; 120 million of them have symptoms, 20 million have severe illness and about 650 million are at the risk (Amy and Behrman, 2008; Farias et al., 2010). Schistosomiasis is a parasitic infection caused by trematodes of the genus Schistosoma, which causing chronic ill health in humans. Hepatic schistosomiasis caused by S. mansoni is due to a host immune reaction to the ova deposited in the liver through the blood stream. Although morbidity is frequently associated with inflammatory granulomatous reaction and the subsequent liver fibrosis, drugs used to treat this disease are generally unable to reduce these pathological sequelae. The fibrosis evolves to portal hypertension and sometimes fatal bleeding. Therefore, the search for new strategies able to reduce granuloma formation and fibrosis or its sequelae is quite relevant for treatment of this disease (Warren, 1979; Dittmar et al., 2006).

An integrated approach for schistosomiasis control should be through chemotherapy, health education and snail control. In the absence of an effective and safe vaccination, treatment with chemotherapy will continue to play a central role in schistosomiasis control programs (Helmy et al., 2009). The antischistosomal drug of choice is praziquantel, however, a considerable tolerance or resistance of S. mansoni to praziquantel may be developed by this parasite (Davis, 2003; Abdul-Ghani et al., 2009). Therefore, the increasing use of praziquantel makes the requirements for antischistosomal drugs very pressing. Some plant species were proposed as sources for schistosomal drugs.

Garlic, Allium sativum, have a medical importance, and is a member of the lily family that has been cultivated by humans as a food plant for over 10,000 years. Ancient Egyptian records mentioned that use of garlic as a remedy for a variety of diseases (Block, 1985). Recently, it has been found that the sulfur-containing compounds of garlic have anti-mutagenesis and anti-carcinogenesis effects. In vivo studies show that garlic and its associated sulfur components suppress the incidence of tumors in rodent models (Song and Milner, 1999). Epidemiological findings also demonstrated an inverse relationship between garlic consumption and the incidence of stomach, colorectal and prostate cancer (Hsing et al., 2002).

Apoptosis is the process of programmed cell death that may occur in multicellular organisms. Biochemical events lead to characteristic cell changes (morphology) and death. P53 tumor suppressor protein is a transcription factor that regulates the transcription rate of several genes involved in the regulation of cells cycle, DNA repair and apoptosis (Wang et al., 2005; Tousson et al., 2012). When the cells expose to the external damage stimuli they activate the regulation of expression of these genes.
such, expressed p53 negatively delays cell cycle progression that leads to the apoptosis if the damage cannot be repaired (Haupt et al., 2003). P53 acts as a protector of genomic activity by inducing either cell cycle arrest (at G1 and/or G2 phase) or apoptosis (Jin and Levine, 2001). The present work has been planned to study the effects of garlic on granuloma sizes and liver fibrosis induced by schistosoma experimental infection. Also; the alternation in p53 expression of in mouse liver tissues in different groups under study were showed.

Material and Methods

**Mice and parasite infection:** Male albino mice of MC1 strain, weighing 20±2 gm were obtained originally from Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TMBRI) Giza, Egypt and maintained under laboratory conditions at 21ºC with water and food supplies ad libitum.

_Schistosoma mansoni_ cercariae were collected after shedding from laboratory-bred infected Biomphalaria alexandrina snails. Mice were infected with 60±5 cercaria of _Schistosoma mansoni_ by immersion of its tails into a suspension of cercariae in specific tubes for 30 minutes (Peters and Warren, 1969). This cercaria were developed according to their life cycles inside animal body into larva, which converted into oocyst which progressed into adult worm then the deposition of ova in the liver (Warren, 1979).

**Preparation of Garlic Extract:** Garlic extract was prepared by homogenizing the required amount of dehusked cloves of garlic in an appropriate volume of distilled water to prepare a concentration of 20 mg/ml [(Balasenthil et al., 1999). The homogenate was centrifuged at 3000 x g for 10 minutes to remove particulate matter and the supernatant fraction was used for the experiment. Garlic extract is composed of different beneficial molecules, especially antioxidants and disulfide.

Forty male mice were equally divided into Four groups; first (G1) and second (G2) groups were the control and garlic groups respectively while third group (G3) was the infected group which mice were infected with _Schistosoma mansoni_ cercariae. The fourth group (G4) was treated group in which mice were infected with _Schistosoma mansoni_ cercariae and then treated orally with garlic for six weeks (Yi-Fang et al., 2008).

**Blood and tissue sampling:** At the end of treatment period, mice in each group were sacrificed, where the blood and the liver of each animal were collected immediately and labeled. Blood was incubated at room temperature for 10 minutes and left to clot then centrifuged at 6000 rpm. Serum and liver samples were kept at -21ºC for biochemical investigation. Gamma glutamyl transferase (GGT) activity in serum was estimated according to Szasz and Persijn Persijn (1974). Estimation of alanine aminotransferase ALT and aspartate aminotransferase (AST) activity in serum were estimated according to the method of Reitman and Frankel (1957).

**Granuloma measurement and liver histopathology:** A specimen of liver from each mouse was fixed in 10% neutral buffered formalin and processed to make paraffin blocks. The blocks were cut into 5μm sections then were stained with Ehrlich’s haematoxylin and counter stained with eosin (Banerof and Cook, 1994). An Ocular micrometer was used for measuring the diameters of granulomas surrounding a single _Schistosoma_ egg. Micrographs were taken under a Olympus microscope and images were captured by a digital camera (Cannon 620). The mean granuloma diameter was calculated from 40-50 granulomas.

**P53 Expression:** Expression of p53 was detected using avidin Biotin Complex (ABC) method (Sternberger, 1979). Dewaxed and rehydrated sections were washed in distilled water for 5 min, rinsed in PBST for 10 min and incubated with 10% normal goat serum for 15 min to reduce non-specific background staining. Then, the sections were incubated with anti-mouse p53 monoclonal antibody (Dako, 1:800 and 1:100, respectively) for 2 hours at room temperature. The sections after 5 baths in PBST were incubated with biotinylated goat antirabbit immunglobulin (Nichirei, Tokyo, Japan). The sections after 5 baths in PBST were further incubated with Avidin Biotin Complex (ABC; Nichirei, Tokyo, Japan) for 1 hour at RT. The reaction was developed by using 20 mg 3-3’-diaminobenzidine tetrahydrochloride (DAB, Wako pure chemical industries, Ltd) in 40 ml PBST, pH 7.2 containing 10 ml of hydrogen peroxide (H2O2) for 7-9 min at a dark room followed by distilled water then dehydrated and mounted. The criterion for a positive reaction confirming the presence of p53 is a dark, brownish, intracytoplasmic precipitate. For the negative control, the primary antibody was omitted to guard against any false positive results which might develop from a non-specific reaction. Negative control sections were done by substituting p53 primary antibodies by normal serum of goat. Sections of aged liver immunohistochemically proven to be p53 positive were used a positive control with each run. The prepared sections were examined by mean of a research microscope. Brightness, contrast were adjusted using Adobe Photoshop software. Image analysis was adjusted using PAX-it image analysis software. The data was statistically analyzed using SPSS.

**Results**

Table (1) showed that; GGT activity was highly significant increased in infected group with _S. mansoni_ (G3) when compare with control (G1) and garlic (G2) groups. Treatment of infected mice with _S. mansoni_ by garlic (G3) reduced the GGT activity when compared with infected mice (G1). On the other hand, serum ALT and AST levels were significantly increased in infected mice with _S. mansoni_ (G3) when compared with the control and garlic (G2) groups. On the other hand, treatment of infected mice with...
Livers sections in control (G1) and garlic (G2) groups showing radially arranged cords of hepatocytes which extend from a central vein to the periphery of the hepatic lobules at which the portal tracts appears (Figs. 1A & 1B respectively). The hepatocytes are polygonal in shape with eosinophilic granular cytoplasm and vesicular basophilic nuclei. Some hepatocytes are binucleated. A variety of major histopathological lesions were observed in the liver sections of infected mice with schistosoma compared with control mice. The major histopathological lesions were variable numbers of periportal granulomas, diffuse infiltration of inflammatory cells, mainly eosinophils and small mononuclear cells, and fibrosis of portal areas and interlobular septa (Figs. 1C & 1D). Each granuloma has Schistosoma mansoni-ova surrounded by dense inflammatory cells and fibrotic tissues. Liver sections in treated infected mice with garlic showed normal hepatocytes with few inflammations in the portal tract (Figs. 1C & 1D). Treatment with garlic led to a significant reduction in granuloma area and hepatic fibrosis in treated infected mice compared to non-treated infected mice (Figs. 1E & 1F). The results in table (1) indicated a decrease in the mean diameter of hepatic granuloma in garlic treated group (316.2±10.5 nm) as compared with infected control (332.3±9.7 nm).

P53 expression and the incidence of apoptotic cells were very low in control (G1) and garlic (G2) liver sections groups, where it was 0.44±0.23 and 0.47±0.83 (Figs. 2A & 2B respectively). The numbers of p53 positive cells in infected group (G3) was 3.87±0.55 (Fig. 2C & 2D), on the other hand a significant decrease in the numbers of p53 positive cells (1.12±0.65) in treated infected liver with garlic (G4) when compared with the p53 positive cells in infected group (Fig. 2E & 2F).

**Discussion**

The present study is the first to demonstrate that garlic extract inhibits liver fibrosis induced by *Schistosoma mansoni* in mice. Liver diseases remain one of the serious health problems. Liver fibrosis is participated by a variety of etiologies leading to sustained cellular injury. Liver fibrosis is one of the most prevalent chronic diseases in the world hence; the investigation for an efficient hepatoprotective drug from natural source is an urgent need. Liver fibrosis is a reaction to chronic liver injury, and it is characterized by an excessive accumulation of extracellular matrix proteins including vellagren. *Schistosoma mansoni* is an infection of trematodes that cause periportal fibrosis and liver cirrhosis due to deposition of eggs in the small portal venules (Manzella, et al., 2007). Serum AST, ALT and GGT are biomarkers in the diagnosis of hepatic damage because they are released into the circulation after cellular damage (Naik and Panda, 2007). In the present study, mice infected with *Schistosoma mansoni* showed significant increase in serum GGT, ALT and AST levels compared with their control group. The elevation of serum ALT and AST of infected mice seems to be a consequence of the damage of hepatic cells and/or impaired permeability of cell membranes, or may be due to heavy schistosoma egg deposition. Liver functions were also improved apparently, treatment of infected mice with garlic revealed significant decrease in the serum GGT, ALT and AST when compared with their respective infected group. Due to its ability to reduce free radical-induced oxidative damage in the liver (Gedik et al., 2005), garlic extract has been shown to decrease liver enzymes in serum and prevent liver damage of rats with liver fibrosis (Nakagawat et al., 1989). These results are in accordance with Mahmoud and Gawish (2005), who found that mice infected with *Schistosoma mansoni* live cercariae showed elevation of serum ALT and AST levels compared with the normal control group and treatment of infected mice with Praziquantel revealed reduction in the serum ALT and AST nearly to the normal values at 16 weeks post infection.

In the liver, the initial pathologic response to eggs is an immunologic reaction to antigens secreted by the organism inside the eggs, and this response appears as a granulomatous reaction (Warren, 1979). Herein, we demonstrated that treatment with garlic in experimental schistosomiasis led to reduction of granuloma area and hepatic fibrosis. Our experiments showed that treatment of infected mice with garlic, ameliorated hepatocytes degeneration, necrosis and infiltration of inflammatory cells and reduced the scores of necroinflammation significantly compared with model group.

Apoptosis of individual cells may present a protective mechanism against neoplastic development in the organism by eliminating genetically damaged cells (Wang et al., 2005). In the current study, the immunohistochemical observations of the liver tissues showed a significant increase of the apoptotic protein p53 after infection with the cercariae of schistosoma, comparing with the control one. On the other hand, a significant decrease in the expression of the cytoplasmic p53 was observed after garlic treatment. Apoptosis, or programmed cell death, is a crucial cellular activity in the behavior of mammalian cells in a wide range of pathophysiological conditions.

Apoptosis may be initiated in any phase of the cell cycle, but the majority of cells undergo apoptosis in the G1 phase. P53 acts on the G1/S checkpoint (Appella, 2001). The wildtype p53 protein causes cell cycle arrest and can also stimulate apoptosis when DNA damage is irreparable (Nagatu, 1997; Thierry, 2000). The present results of the liver tissues showed a significant increase of the apoptotic protein p53 after infection with the cercariae of schistosoma, comparing with the control one. The expression of the cytoplasmic p53 was very low in the control liver sections. A significant decrease in the expression of the cytoplasmic p53 was observed after garlic treatment. So, the increasing of p53 apoptotic cells in the present study reveal the possibility of apoptosis occurrence after Schistosoma infection and the decreasing after treatment with garlic.
Table 1: Changes in granuloma diameters and serum GGT, ALT and AST levels (mean±SD) in different groups under study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Granuloma diameter (nm)</th>
<th>GGT (U/l)</th>
<th>ALT (U/l)</th>
<th>AST (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₁</td>
<td>0</td>
<td>4.76±0.23</td>
<td>26.65±0.25</td>
<td>40.87±0.22</td>
</tr>
<tr>
<td>G₂</td>
<td>0</td>
<td>4.45±0.56</td>
<td>25.43±0.24</td>
<td>42.33±0.37</td>
</tr>
<tr>
<td>G₃</td>
<td>332.3±9.7</td>
<td>8.77±0.85</td>
<td>40.82±1.41*</td>
<td>76.11±0.86*</td>
</tr>
<tr>
<td>G₄</td>
<td>316.2±10.5</td>
<td>7.53±0.41*</td>
<td>29.37±0.94</td>
<td>63.05±0.71</td>
</tr>
</tbody>
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Figure 1: Photomicrographs of Hematoxylin-eosin-stained liver sections. A&B: Liver sections in control and garlic groups showing central vein (CV) and hepatocytes (Ht) HE X 100. C&D: Variable numbers of periportal granulomas surrounded by dense inflammatory cells and fibrotic tissues, diffuse infiltration of inflammatory cells. (arrow, granuloma; arrow head, fibrotic tissues) HE X 100. E&F: Reduction in granuloma area in treated infected mice with garlic in addition to, normal hepatocytes with little congestion in portal tract HE X 400.
Figure 2: Photomicrographs of P53-ir in the mice liver sections. **A&B:** Control and garlic groups (G1 and G2) showing very weak reaction for P53 (X 200). **C&D:** Liver sections in infected with Schistosoma mansoni group (G3) showing strong positive reaction for P53 around granuloma (arrow head) and in the fibrotic tissues (arrow) (X 100). **E&F:** Liver sections in treated infected mice with garlic (G4) showing significant decrease in P53 in fibrotic tissues, positive cytoplasmic hepatocytes and granuloma (X 100).

References

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