EDITORIAL

Preventive Effects of Resveratrol against *Schistosoma mansoni*-Induced Liver Fibrosis in Mice

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Abstract:

Background: In Schistosomiasis, hepatocyte injury and Kupffer's cell activation can result in reactive oxygen species generation, pro-inflammatory and profibrogenic mediators release. This can result in stellate cells activation and consequently, liver fibrosis. Resveratrol, a natural polyphenol, has been shown to possess antioxidant and anti-inflammatory properties. However, studies into its protective effects against *Schistosoma mansoni*-induced liver fibrosis are limited. **Aims:** The present study was designed to examine the preventive effects of resveratrol on *Schistosoma mansoni*-induced liver fibrosis in mice.**Methods**: Sixty male albino mice were divided into four groups of 15 mice as follows: normal resveratrol-untreated, normal resveratrol-treated, *Schistosoma mansoni*-infected resveratrol-untreated and *schistosoma mansoni*-infected resveratrol-treated. At the end of the experimental period, blood samples were collected to measure serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and TNFα. Liver tissue was collected for malondialdehyde (MDA) measurement and histopathological examination.**Results**: AST, ALT and TNF-α, and MDA levels were significantly increased in the infected resveratrol-untreated group compared to normal resveratrol-untreated group (all, $P< 0.05$). However, their levels were significantly decreased in the infected resveratrol-treated group compared to infected resveratrol-untreated group (all, $P< 0.05$).In addition resveratrol treatment improved *S. mansoni*-induced liver damage in IT group as it reduced the fibrosis compared to IU group.

Conclusion: Results of the study indicate that resveratrol can prevent *S. mansoni*-induced liver fibrosis via mechanisms involving its anti-oxidant and anti-inflammatory anti-fibrotic properties.

Keywords: Liver fibrosis, *Schistosoma mansoni*, Resveratrol, TNFα, lipid peroxidation.

Introduction:

Schistosomiasis, a disease caused by parasitic worms of the genus Schistosoma, is one of the major causes of morbidity and mortality in the developing world.Worldwide, more than 207 million individuals are chronically infected with *Schistosoma mansoni*, and 20 million people are suffering from its complications including liver fibrosis(1).Liver fibrosis, resulting from *S. mansoni* infection, is of clinical importance among other chronic liver diseases(2).Liver fibrosis is initiated by a periportal granulomatous inflammation around parasite eggs, which reach the liver through the portal circulation(3).Presence of the parasite eggs in the liver and their aggregation in hepatic portal venules can result in a strong inflammatory response and consequently, periovular granuloma formation. Subsequently, a large amount of fibronectin is produced by macrophage composing a large part of the extracellular matrix (ECM)(4).This is followed by the deposition of a number of fibrogenic proteins including but not limited to the proteoglycans dermatan sulfate, the interstitial collagen types (I, III), and fibrotic tissue(5).

There is strong evidence to indicate the involvement of hepatic stellate cells (HSCs) in the

pathogenesis of schistosomiasis. It has been demonstrated in mice that activated HSCs take part in the formation of the periovular granulomas [6]. In addition, in human, liver myofibroblasts have been identified in the portal fibrous tissue [7]. In addition, oxidative stress has been shown to play a crucial role in liver fibrosis [8,9].Induction of ROS and subsequent HSCs stimulation can result in increased collagen production and fibronectin expression[10,12].
Resveratrol (3, 4′, 5- trihydroxy-trans-stilbene), a natural polyphenol found mainly in grapes and peanuts, has been reported to have a wide range of biological properties and multiple molecular targets[13].It possesses potent antioxidant, anti-proliferative anti-inflammatory properties[14,15].

In one study, resveratrol has been found to supress the development of *S. mansoni*- induced liver fibrosis. However, studies investigating the protective effects of resveratrol against Schistosomiasis- induced liver fibrosis and the possible mechanisms are scarce. Therefore, the aims of the present study were to investigate the protective effects of resveratrol against liver fibrosis induced by *S. mansoni* and to elucidate possible mechanisms.

Materials and Methods:

Experimental animals

This study was carried out on 60 Male CD1Swiss albino mice weighing 18-22 gm each, were purchased from the Schistosoma Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. Mice were fed on standard diet containing 24% protein, 4% fat and about 4-5% fiber and water ad libitum. Animals were maintained under standard conditions of temperature about 22-250C with regular 12 h light/dark cycle. All procedures involving the animals were conducted in accordance with the protocol approved by the Ethics Committee, Faculty of Medicine, and Alexandria University.

Experimental Design

The potential preventive effects of reveratrol on *S. mansoni*-induced liver fibrosis.
Mice were divided randomly into four main groups as follows: Normal resveratrol-untreated (NU): Consisted of 15 mice, received distilled water as placebo infection and treatment. Normal resveratrol-treated (NT): Consisted of 15 mice injected with placebo infection and then treated with resveratrol (Sigma-Aldrich Chemie; Steinheim, Germany). Resveratrol was injected intraperitoneal (I.P) in a dose of 20 mg/kg body weight, twice/week. The treatment started after 4 weeks from beginning of the experiment and continued for 10 weeks.Infected resveratrol-untreated (IU) group: Consisted of 15 mice, infected with an Egyptian strain of *S. mansoni* cericarae. The strain was maintained by a laboratory passage in an Egyptian strain of *Biomphalaria alexandrina* snails. Infection was done by subcutaneous injection of each mouse with ± 80 *S. mansoni* cercariae suspended in 0.2 ml dechlorinated water. Mice were not treated till the end of the study. Infected resveratrol-treated (IT): It consisted of 15 mice. Each mouse was infected with *Schistosoma mansoni* cercariae as previously mentioned in Group III prior to treatment with Resveratrol I.P at

dose of 20 mg/kg, twice/week. The treatment was started after 4 weeks from infection and continued till the end of the10th week (end of the study) [16].

Measurement of biochemical parameters:

Measurement of serum ALT, AST and TNF-α.

Blood samples were collected from the retro-orbital venous plexus of the mouse by a capillary haematocrit tube under light ether anesthesia [17]. Blood was collected into a clean dry non-heparinized Wassermann tubes for separation of serum. The serum was separated by centrifugation at 3000 rpm for 15 minutes and was aliquoted into 2 samples and stored at -80 ºC until assayed for biochemical estimation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) colorimetrically (NS Biotec), and TNF-α.(RayBio®, Mouse TNF-α ELISA Enzyme Immunoassay).

Measurement of liver MDA content

The mice were sacrificed with decapitation, the anterior abdomen were incised to expose the liver. The liver from each mouse was excised immediately after perfusion and rinsing with ice-cold saline solution.For hepatic MDA determination, 25 mg of tissue was weighed and 250 µl of RIPA buffer with protease inhibitors were added. The mixture was sonicated for 15 seconds at 40 V over ice and centrifuged at 1.600 × g for 10 minutes at 4°C. The supernatant also used for analysis. MDA was quantified using the thiobarbituric acid reaction as described by Ohkawa et al, and levels were expressed in µM/g tissue [18].

Liver histology

The ventral median lobe of the liver was fixed in 10% neutral buffered formalin for histopathological study to confirm liver fibrosis. After fixation, liver samples were embedded in paraffin wax, sectioned and stained with hematoxylin and eosin (HandE) and/or Masson's trichrome. At least two different sections were examined from each mouse. The tissues were examined under microscope for histopathological assessment. Photomicrographs were taken to compare between the groups.

Statistical analysis

The obtained data was expressed as mean ± standard deviation (SD). Statistical comparisons between groups were performed using analysis of variance (ANOVA) and unpaired Student's t test. $P< 0.05$ was considered statistically significant. Statistical analyses were carried out using Graphpad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

Results:

Resveratrol attenuates Schistosoma mansoni-induced liver enzymes elevation

Serum AST levels increased significantly in infected untreated (IU) group compared to both normal untreated (NU) and normal treated (NT), (34.24 ± 3.95 vs.7.73 ± 1.39 and 8.90 ± 2.30 IU/L, respectively, $p< 0.001$; Figure 1).Resveratrol treatment significantly decreased serum AST levels in the infected treated (IT) compared to infected untreated (IU) group (34.24 ± 3.95 vs. 10.55 ± 1.73 IU/L, $p < 0.001$. Figure 1).Similarly, ALT levels were significantly increased in serum

of ALT in IU group compared to NT and IT groups (40.88 ± 4.09 vs.16.05 ± 5.71, 15.99 ± 6.82 and 15.95 ± 6.41 IU/L, respectively, *P*<0.001; Figure 2).In contrast, resveratrol treatment resulted in a significant decrease in serum ALT in IT group compared to IU group (15.95 ± 6.41 vs. 40.88 ± 4.09 IU/ L *p*< 0.001; Figure 2).

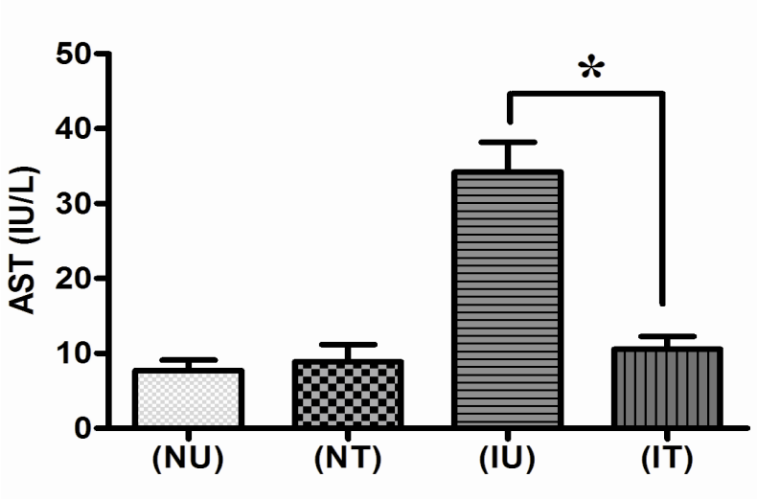

Figure (1): The effect of resveratrol on serum AST in *S. mansoni*-infected mice. NU= normal resveratrol-untreated, NT= normal resveratrol-treated, IU= *S. mansoni*-infected resveratrol-untreated and IT= *S. mansoni*-infected resveratrol-treated. * *P* < 0.001.

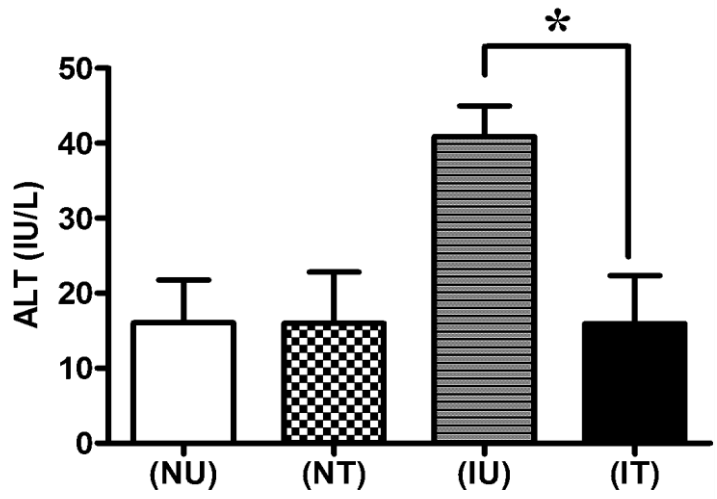

Figure (2): The effect of resveratrol on serum ALT in S. mansoni-infected mice. NU= normal resveratrol-untreated, NT= normal resveratrol-treated, IU= S. mansoni-infected resveratrol-untreated and IT= S. mansoni-infected resveratrol-treated. * P < 0.05.

Resveratrol attenuates Schistosoma mansoni-induced increase in serum TNF-α level

To investigate the anti- inflammatory effect of the Resveratrol, TNF-α level was quantified in

serum using ELISA. While TNF-α levels in IU group significantly increased compared to the NU group (249.76±122.65vs. 78.86 ± 10.39pg/ml; $P < 0.001$; Figure 3), resveratrol is significantly decreased serum TNF-α levels in IT group compared to the IU group (80.50 ± 32.56 vs. 249.76±122.65pg/ml; $P < 0.001$ (Figure 3).

Resveratrol attenuates S-mansoni-induced lipid peroxidation

Next, we investigated whether resveratrol could protect liver from lipid peroxidation by measuring MDA. There was significant increase in liver MDA levels in the IU group as compared to the NU group (10.08 ± 0.52 vs. 3.34 ± 0.68 μM/gm; P < 0.05, Figure 4). Treatment with resveratrol in IT group significantly decreased MDA levels compared to IU group (3.71 ± 0.56 vs. 10.08 ± 0.52; P < 0.05, Figure 4).

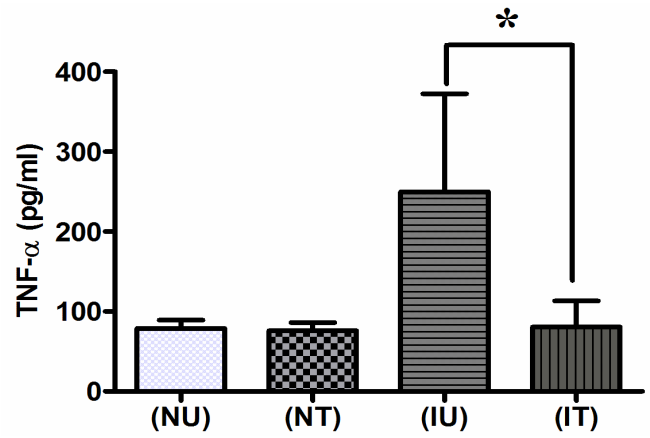

Figure (3): The effect of resveratrol on serum TNF-α level in *S. mansoni*-infected mice. **NU**= normal resveratrol-untreated, **NT**= normal resveratrol-treated, **IU**= *S. mansoni*-infected resveratrol-untreated and **IT**= *S. mansoni*-infected resveratrol-treated. * $P < 0.001$.

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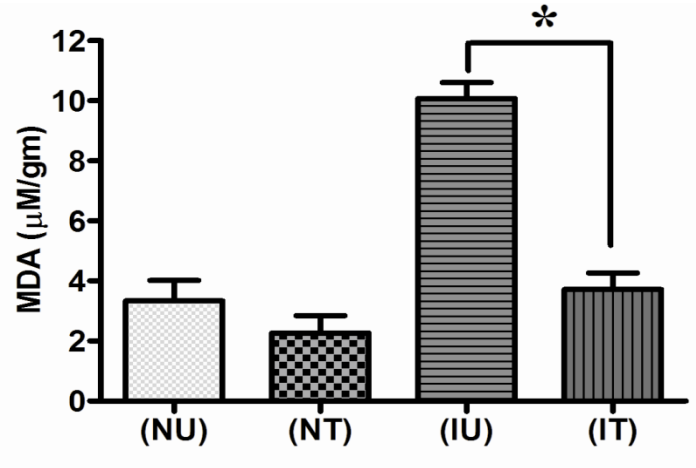

Figure (4): The effect of resveratrol on levels of malondialdehyde (MDA) in μM/gm tissue in liver of *S. mansoni*-infected mice. **NU**= normal resveratrol-untreated, **NT**= normal resveratrol-treated, **IU**= *S. mansoni*-infected resveratrol-untreated and **IT**= *S. mansoni*-infected resveratrol-treated. * $P < 0.05$.

Resveratrol attenuates S.mansoni-induced histopathological changes

Hematoxylin and Eosin (HE)-stained liver sections of the IU group showed multiple granulomas composed of central ova surrounded by laminated layers of fibrous tissue associated with inflammatory cells at the periphery with severe necrosis of the hepatic tissue (Figure 5C). However, in the IT group, granulomas were seen as a concentric focus of mononuclear and polymorphonuclear cells around the egg and the laminated layers of fibrous tissue nearly disappeared. In addition, minimal microvascular changes and no hepatocyte necrosis were noticed compared to IU group (Figure 5D). Masson's trichrome staining of liver sections of IU group showed expanded portal tracts with fibrous tissue with occasional bridging fibrosis as well as scattered periovular granulomas compared to NU group (Figure 5a and 5c). Resveratrol treatment improved *S. mansoni*-induced liver damage in IT group as it reduced the fibrosis compared to IU group (Figure 5c and 5d).

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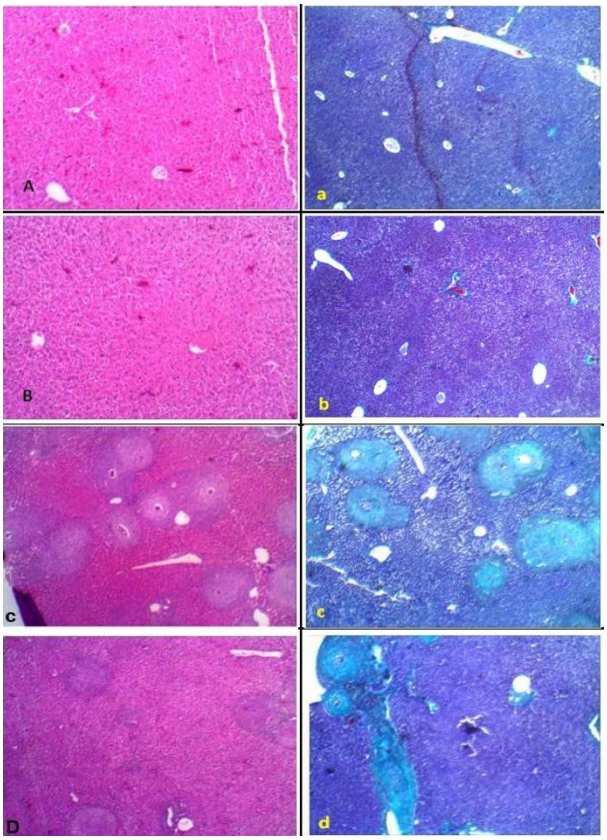

Figure (5): The effect of resveratrol on on liver histopathology in S. mansoni-infected mice. [A, a] NU= normal resveratrol-untreated, [B, b] NT= normal resveratrol-treated, [C, c] IU= S. mansoni-infected resveratrol-untreated and [D, d] IT= S. mansoni-infected resveratrol-treated. (A-D) stained with H&E staining and (a-d) stained with Masson's trichrome stain.

Disscusion:

The results of the present study demonstrate that resveratrol can attenuate *S. mansoni*-induced AST and ALT eleveation, TNF-α production, and lipid peroxidation. Furthermore, resveratrol exerts antifibrotic effects by down-regulating fibronectin gene expression, and thereby prevent the development of liver fibrosis. Hepatic fibrosis represents the response of the liver to different chronic insults and is associated with significant morbidity and mortality [(20)]. A variety of adverse stimuli such as parasitic infections, toxins, viruses, bile stasis and hypoxia can trigger fibrogenesis, In murine models of S.mansoni infection, eggs are trapped in hepatic sinusoids resulting in granulomatous response, accumulation of ECM and finally, fibrosis [(20)] .The present study was conducted to explore the protective effects of Resveratrol against *S. mansoni* induced hepatic fibrosis in mice. Resveratrol is a natural anti-oxidant compound which belongs to the phytoalexin polyphenols and its beneficial effects are increasingly investigated [(21)]. Our data have shown elevated AST and ALT levels in infected untreated mice. This can be attributed to the disturbance in hepatic cell wall; immunological reactions, inflammation and necrosis. Intraperitoneal administration of resveratrol to the schistosomal infected mice remarkably attenuated the increased levels of liver enzymes almost towards the basal levels. These findings are in line with Guijuan et al who investigated the effects of resveratrol against carbon tetrachloride (CCL4) induced hepatic

injury and reported increased AST and ALT levels could be normalized by resveratrol [22]. In that study, a single dose of resveratrol was used pointing to its potential effects. Similarly, Roberta et al, observed an increase in AST, ALT levels induced by experimental diabetes in rats. Administration of resveratrol at 10 and 20 mg/kg was able to decrease AST and ALT levels [23]. Research has demonstrated that cytokines and granulomatous reactions which occur in Schistosomiasis induce macrophages to release cytokines that can activate HSCs which in turn trigger the expression of fibronectin and collagens [24]. One of the major hepatotoxic mediators is TNF-α, a proinflammatory cytokine has been found to be elevated in liver injury [25]. In this study, TNF-α serum levels were increased in the infected untreated mice and could be ameliorated by resveratrol administration. Our findings are comparable to previous data where resveratrol has been demonstrated to down-regulate TNF-α expression through mechanisms involving inhibition of the nuclear factor Kappa β (NF-kB) transcription factor [26]. Muriel et al. have defined more specifically that NF-kB regulates expression of an array of genes encoding cytokines such as TNFα [27]. In damaged hepatocytes and elevated TNFα can induce NF-kB activation resulting in more TNFα production which may result in more destruction in the hepatic parenchyma and impaired liver cell function. In contrast, Luis and colleagues have demonstrated in mice that resveratrol can decrease IL-1 with no effect on the high level of the TNFα induced by alcohol administration [28]. This discrepancy might be explained by the different methods that were used to induce liver fibrosis.Lipid peroxidation is considered a hallmark of oxidative stress, in which ROS interact with polyunsaturated fatty acids resulting in MDA production with subsequent cellular membrane injury and cell necrosis[29].It has been described that oxidative stress can cause fibrosis[30]. Studies have also shown that activated hepatic stellate cells (HSCs) can secrete inflammatory cytokines and up-regulate adhesion molecules that promote immune cell attraction and increase collagen synthesis [31]. In the present study, infection of mice with S.mansoni resulted in an increase in lipid peroxidation as measured by MDA level in liver homogenates. Our data is in accordance with Hanna et al., who found increased MDA level and changes in antioxidant enzyme activity in patients with liver fibrosis [32]. Similarly, Ming et al., observed that Thioacetamide (TAA)-induced liver fibrosis in rats was associated with a significant increase in hepatic MDA level, suggesting the impairment hepatic anti-oxidant capacities [33]. Our data have demonstrated that administration of resveratrol to the infected mice can decrease MDA levels. Resveratrol is known to have strong antioxidant effects, including inhibition of free radical formation and/or lipid peroxidation propagation, keeping the structural integrity of the membrane and preventing cellular damages. This data is in line with data obtained by Ara et al., who reported that resveratrol can decrease MDA levels in liver tissues induced by bile duct ligation [34]. However, it has been reported that resveratrol administration can result in an increased brain MDA levels [35]. Similar data has been reported in renal tissue [36]. The differences in organ responsiveness towards various harmful agents and resveratrol could explain such discrepancies. Interestingly, a significant decrease in hepatic MDA levels was noted in the NT mice compared with the NU pointing to resveratrol potent anti-oxidant effects. These findings are in agreement with a previous study showing that resveratrol can up-regulate antioxidant enzymes mRNA expression and consequently, lipid

peroxidation prevention [37]. Histopathological examination of HandE stained liver sections of infected untreated mice showed multiple granulomas associated with inflammatory cells infiltration and necrosis as compared to the normal liver architecture in normal control group. These hepatic changes are in agreement with the previous study of El-Agamy et al., which demonstrated that S.mansomi infection can result in enlarged fibrotic granulomas around the eggs with marked increase in the amount of collagen fibers, and focal necrosis [38].Administration of resveratrol to the infected mice reduces the concentric granulomas and the laminated layers of the fibrous tissue, pointing to its protective effects against development of liver fibrosis. These findings confirm further previous data reporting that resveratrol can reduce live fibrosis in *S.mansoni*-infected mice [38].The anti-fibrotic effects of resveratrol has been explained by its capacity to disrupt signal transduction pathway and cell cycle protein expression resutlting in HSCs inactivation [39].

In conclusion, Schistosomiasis can lead to extensive hepatocellular damage resulting in fibrosis. Resveratrol possesses potent antifibrotic activity in preventing *S.mansoni*-induced liver fibrosis. Therefore, resveratrol might be of therapeutic value in hepatic disorders characterized by excessive hepatic fibrosis. However, further clinical investigations are needed.

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