

Hepatoprotective Effects of Simvastatin in a Model of Hepatitis on Top of Schistosomiasis

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Hepatitis C infection on top of schistosomiasis is prevalent in Egypt and the available hepatoprotective drugs are not sufficient. So, this study aimed to explore hepatoprotective effect of simvastatin. Induction of hepatitis model on top of schistosomiasis was done by injection of mice with D-galactosamine after schistosome infestation. We used 40 mice, 10 were non-infected while 30 were injected with D-galactosamine after infestation with schistosomemansoni divided into; non-treated, simvastatin-treated, and combined simvastatin&praziquantel treated. Simvastatin-treated mice showed spastic appearance of oral and ventral suckers of worms. It also showed shortening of tubercles and decreased number of the spines. These findings were associated with a reduction in ova deposition of the worms in the liver and decrease of the fibrous component of the liver granuloma and mitigation of necro-inflammatory reaction in the liver. Combination of simvastatin with praziquantel produced more pronounced hepatoprotective effect. The hepatoprotective effect was associated with lowering of IL-10 levels. So simvastatin could be added to praziquantel for treatment of hepatitis on top of schistosomiasis as both drugs affect the worm viability and the ova deposition while simvastatin has an additional anti-inflammatory, antifibrotic and immunomodulatory effects through IL-10. Further clinical assessments are required.

Key Words: Schistosomamansoni, D-galactosamine, hepatitis, simvastatin, praziquantel, IL-10

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1. INTRODUCTION

Schistosomiasis is considered the commonest cause of liver disease in Egypt (Barakat, 2013). The Egyptian Ministry of Health arranged campaigns using injection with tartar emetic in period from 1950 to 1980, to eradicate schistosomiasis before discovery of praziquantel (PZQ). Unfortunately, this resulted in increased prevalence of hepatitis C virus (HCV) especially in Lower Egypt (Strickland, 2006). PZQ replaced tartar emetic as an effective oral therapy for schistosomiasis by the mid-1980s. This reduced both schistosomiasis and reduced HCV epidemic (Abruzzi, Fried, & Alikhan, 2016). Praziquantel is the standard chemotherapy for schistosomiasis but unfortunately, it achieves only from 80-97% success rate and has no role on viral hepatitis (Caffrey, 2007).

Vandewaa et al (1989) said that "Statins inhibit the enzyme, 3-hydroxy 3-methyl glutaryl-CoA reductase (HMG-CoA)". This reduces mevalonate, a precursor of cholesterol and which is important for egg disposition in schistosomes (Vandewaa, Mills, Chen, Foster, & Bennett,

1989). Statins has many pleiotropic effects as anti-schistosomal effects^{5, 6, 7, 8} and anti-viral effect^{9, 10}.

D-Galactosamine (D-GaIN) produces a diffuse type of liver injury which is resembling in function and morphology human viral hepatitis with inflammation and necrosis^{11, 12}. In this study, we aimed to investigate the role of SIM in a combined model of hepatitis and schistosomiasis simulating HCV on top of schistosomiasis in human.

2. MATERIALS AND METHODS

2.1. Drugs

1. Simvastatin (SIM) (Zocor®): obtained from MERCK (20 mg/ tablet).
2. Praziquantel (PZQ) (Distocide®, EpicoPharma, Cairo, Egypt (600mg/tablet)

2.2. Infection of animals with schistosomamansoni:

2.2.1 Cercarial shedding and animal infection

Infected *B. alexandrina* snails was obtained from Theodor Bilharz Research Institute (TBRI)- Egypt, were washed with dechlorinated water and kept in an aerated aquarium in a dark place supplied with lettuce leaves (Liang et al.,1987). To release cercariae, snails were suspended in water (1 ml/snail) and left under white fluorescent light for a period of 30–60 min. 1 ml of cercarial suspension was pipetted and placed on glass slides; a drop of iodine was added to stain the cercariae. The average number per 1 ml was calculated to estimate the injection dose. Mice were injected subcutaneously with 50 cercaria / 0.1ml dechlorinated water using sterile 1ml plastic syringe (Lewis, Karunaratne, Lewis, Freitas, & Liang, 2001).

2.3. Animals grouping:

Forty Swiss male albino mice (CD-1), with average weight (18–20g) were used in this study, 10 of them were not infected and are used as control non-treated group while 30 were infected by schistosomamansonicercaria as previously mentioned. After 40 days of parasitic infestation, the infected animals were divided into (non-treated, SIM, combined SIM and PZQ treated). SIM was given in a dose of (200 mg/kg/day) orally, suspended in (0.5%)methyl-cellulose solution as a vehicle(Alencar et al., 2016). PZQ was given in a dose of (500 mg/kg, p.o) in (2%) aqueous suspension; on 2 successive days (Araújo et al., 2008). The animals were maintained in the our laboratory under a 12-h light/dark cycle in a (75±15%) humidity, (20±2°C) temperature, and free access to food and water, in filtered laminar air flow controlled room. The research protocol has been approved by our institutional research board (IRB) on (9/4/2016)which is adherent to Declaration of Helsinki.

2.4. Serum analysis:

Two weeks later after initiation of therapy, animals were killed by decapitation. Collected sera were used for the measurement of:

- . ALT using commercial kits obtained from Abcam (United Kingdom).
- . IL10using commercial kits obtained from Abcam (United Kingdom).

2.5. Parasitological study

- Evaluation of drug efficacy was based on the following parasitological parameters:

2.5.1. Adult worms count, the perfusion technique was carried out according to Lewis et al(Lewis et al., 2001).The collected worms were counted and differentiation into male and female worms was considered. **Scanning electron microscopy (SEM)** JEOL JSM 6510 Iv, Electron

microscopy unit, Mansoura University was used for examination of worms from different groups.

2.5.2. Tissue egg counts and patterns in the liver and intestine were calculated according to Cançado et al., (Cançado, da Cunha, Carvalho DG, Carvalho, & Cambraia, 1965).

2.6.1. Histopathological assessment and measurement of granuloma size:

- Liver specimens were fixed in neutral buffered formalin (10%) and embedded in paraffin blocks then cut as 4 µm thick sections which were stained with hematoxylin and eosin (H&E) and were used for microscopic examination. The granuloma type (cellular, fibrocellular or fibrous), granuloma number/10 fields/mouse, egg type (intact or degenerated) were assessed. Calculation of mean diameter of each granuloma was done by using the measurement of two perpendicular diameters of the granuloma (LICHTENBERG, 1962).

2.6.2. Morphometric assessment of fibrosis in the granuloma:

- Masson trichrome-stained liver sections were used for examination of collagen fiber deposition. Images of total of 50 granulomas for each mouse were taken randomly with a digital camera mounted on a BX41 Olympus optical microscope Olympus Corporation, Tokyo, Japan). Photoshop software was used for extraction of collagen fibers content in the granuloma by splitting the picture using into blue and green channels and analysis were done using the NIH image software (Scion Corp., Frederick, MD). The extent of fibrosis in the granuloma was expressed as the % of the stained area in relation to the total granuloma area. The obtained % of collagen content were expressed as mean ± S.D (James, Bosch, Aronson, & Houtkooper, 1990).

2.7. Statistical analysis

SPSS program, Package 20, was used for Data analysis. Kolmogorov-Smirnov test was used for normal distributions of Data. We used one way ANOVA test followed by post hoc (tukey) analysis to detect significance between data between groups. P-values less than 0.05 were considered as a significant difference.

3. RESULTS

3.1. Effect of SIM alone or in combination with PZQ on both ALT & IL-10 levels in serum of mice having D-GAIN induced hepatitis on top of schistosomiasis:

Injection of D-GaIN to mice infected with schistosomamansoni resulted in a significant elevation of ALT and IL-10 levels in serum (P1< 0.001). Treatment

of diseased groups with either SIM alone or in combination with PZQ produced a significant reduction of ALT ($P < 0.001$). While treatment of different groups produced an increase in the IL-10 levels significantly ($P < 0.001$) (table 1).

3.2. Effect of SIM alone or in combination with PZQ on Oogram%, Liver ova, and worms (female& total) in mice having D-GAIN induced hepatitis on top of schistosomiasis:

Diseased groups showed ova and worms in the liver and intestine (fig1&4). Treatment of different diseased groups with either SIM alone or in combination with PZQ produced a significant reduction of live mature, immature and dead ova in the intestine and also of liver ova count, $P < 0.001$, table (2). Treatment of infected groups with SIM has a significant reduction of both female and total worms ($P < 0.01$) while combined therapy resulted in significant reduction of female and total worm count ($P < 0.001$), table (2).

SEM examination of worms extracted from mice treated with SIM showed spastic appearance of oral and ventral suckers and shortening of tubercles in addition to shortening and decreased number (some loss) of the spines with swelling and fusion of tegumental ridges (fig 2). While, worms from diseased mice treated by combined SIM&PZQ showed decreased number of both the tubercles and spines, continuation of tegumental

ridges with area of focal swellings and vesiculations (fig3).

3.3. Effect of SIM alone or in combination with PZQ on pathological parameters in mice having D-GAIN induced on top of schistosomiasis:

Liver sections from animals with hepatitis on top of schistosomiasis showed granuloma in the liver associated with large foci of necrosis in parenchyma and increased inflammatory cells in comparison to normal control group (fig4). Both treated groups showed significant reduction of necro-inflammatory score ($P < 0.001$). Treatment of SIM alone or in combination with PZQ produced a significant reduction of granuloma number ($P < 0.05$ & 0.001) respectively while SIM alone produced non-significant reduction of granuloma diameter ($P > 0.05$) but combined SIM& PZQ treatment produced a significant reduction of granuloma diameter ($P < 0.01$) table (3) (fig 4).

It is noted that % fibrosis and collagen deposition in granuloma was increased with SIM alone or in combination with PZQ compared with infected non-treated ($P < 0.05$ & 0.01) respectively (table3), (Fig 5). The number of degenerated ova increased more in groups treated with PZQ& SIM in relation to infected non-treated group or SIM treated group.

Table (1): Serum Levels of SIL ALT and IL-10 in animals having D-GalN induced hepatitis on top of schistosomiasis (treated with SIM or SIM &PZQ). Data are expressed as mean ± SD

Groups	ALT (Iu/L)	IL-10 (pg/ml)
Control normal	21.8±4.4	260.1±22.9
Control diseased non treated	75.0±10.4 $P < 0.001$	386.3±57.2 $P < 0.001$
Diseased SIM treated	41.8±3.5 $P < 0.001$	569.4±42.5 $P < 0.001$
Diseased PZQ&SIM treated	36.0±3.2 $P < 0.001$	570.5±46.1 $P < 0.001$

P1 represents the significance between diseased non-treated animal group and normal animal group. P2 represents the significance between diseased treated animal groups and infected non-treated animal group. SIM; simvastatin, PZQ; praziquantel

Table (2): Oogram%, Liver ova, and worms (female& total) in animals having both schistosomiasis&D-GalN induced hepatitis (treated with SIM or SIM &PZQ). Data are expressed as mean ± SD

Groups	Oogram %				Liver ova X10 ³	Female worms	Total worms
	Live mature ova %	Live immature ova %	Dead ova %	Intestinal ova			
Control diseased non treated	50.3±1.0	22.5± 2.3	27.1± 1.7	698.3±69.8	17.2± 2.8	3.3±0.8	7.2±1.5
Diseased SIM treated	16.8± 1.8 $P < 0.001$	6.2± 1.7 $P < 0.001$	77.3± 3.1 $P < 0.001$	545.3±78.6 $P < 0.001$	10.5±3.1 $P < 0.001$	2.0±0.6 $P < 0.01$	4.8±1.0 $P < 0.01$
Diseased PZQ&SIM treated	4.0 ± 0.8 $P < 0.001$	1.7± 0.7 $P < 0.001$	94.3± 1.5 $P < 0.001$	94.3±10.3 $P < 0.001$	3.0±0.7 $P < 0.001$	0.7± 0.4 $P < 0.001$	2.1±0.7 $P < 0.001$

P1 represents the significance between infected treated animal group and infected non-treated animal group. P2 represents the significance between infected treated animal group with SIM&PZQ and infected treated group with SIM alone. SIM; simvastatin, PZQ; praziquantel.

Table (3): Effect of simvastatin with or without PZQ on granuloma number, diameter, % fibrosis, necro-inflammatory score and eggs state of in different animal groups. Expression of data as (mean ± SD)

	Granuloma no	% reduction	Granuloma diameter (µm)	% reduction	%fibrosis	Necro-inflammatory score	% state of eggs	
							intact	Degenerated
Control diseased non treated	6.2±0.9		140.2±23.7		18.9±1.9	6.0±1.0	84	16
Diseased SIM treated	4.9±0.7 P1<0.05	20.9	133.7±14.2 P1>0.05	4.6	21.9±2.4 P1<0.05	3.1±0.9 P1<0.001	45	55
Diseased PZQ&SIM treated	3.1±0.9 P1<0.001 P2<0.01	36.7	128.6±14.5 P1<0.01	8.3	22.7±2.4 P1<0.01	2.6±0.7 P1<0.001	5	95

P1 represents the significance between diseased treated animal group and infected non-treated animal group. P2 represents the significance between diseased treated animal group with SIM&PZQ and diseased treated group with SIM alone. SIM; simvastatin, PZQ; praziquantel

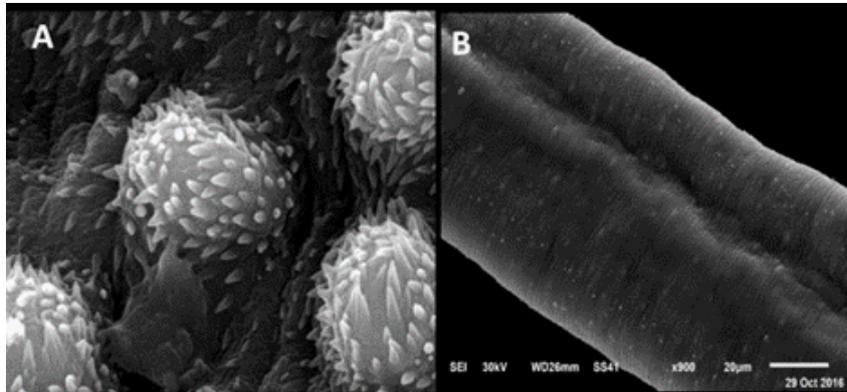


Figure (1): SEM *S.mansoni* worms from mice having D-GAIN induced hepatitis on top of schistosomiasis. A. Tegument of male worm (X500); B. Tegument of female worm (X 900).

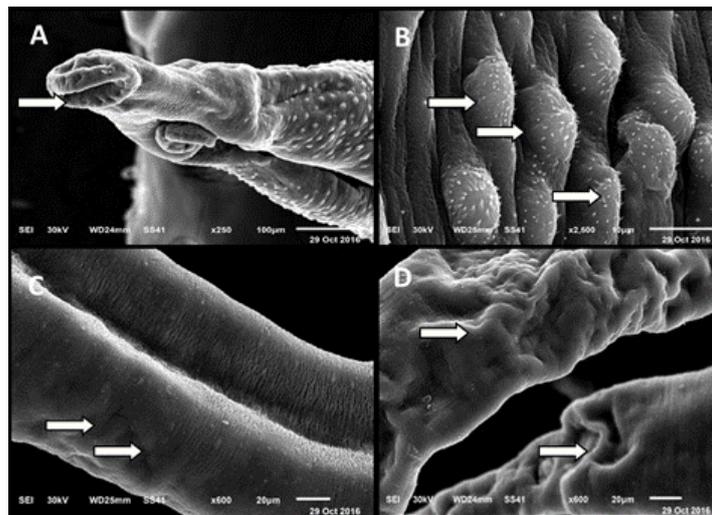


Figure (2): SEM of *S.mansoni* worms from mice having D-GAIN induced hepatitis on top of schistosomiasis treated with SIM. A. Spastic appearance of oral and ventral suckers of male (X250); B. Tegument of male worm showing shortening of tubercles, shortening and decreased number (some loss) of the spines with swelling and fusion of tegumental ridges (X2,500); C. Tegument of female worm showing swelling and fusion of tegumental ridges (X600). D. Tegument of female worm showing extensive swelling in tegument (X600).

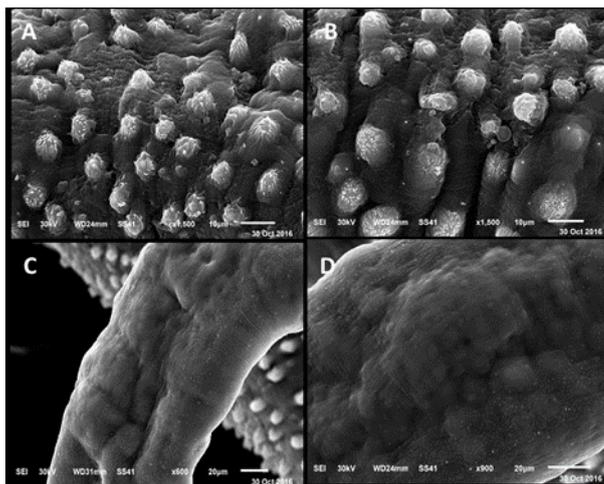


Figure (3): SEM of *S.mansoni* worms from mice having D-GaIN induced hepatitis on top of schistosomiasis treated by SIM&PZQ. A. Tegument of male worm with decreased number of both the tubercles and spines, continuation of tegumental ridges with area of focal swellings and vesiculations (X1,500). B. Tegument of male worm with destructed tubercles and area of spine loss, swellings, and vesiculations (X1,500). C, D. Tegument of female worm with extensive swelling with loss of tegumental ridges (X600), (X900).

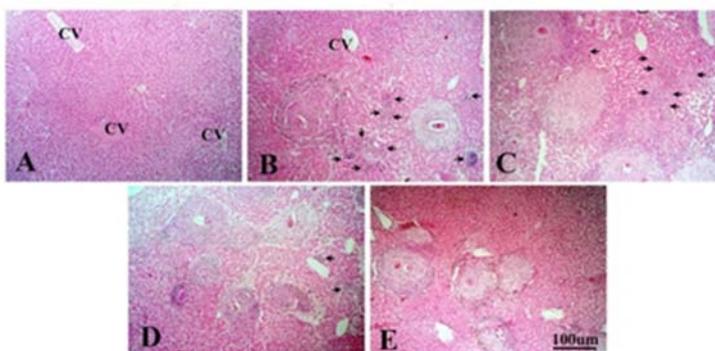


Figure (4): Effect of administration of SIM and PZQ on granuloma type and egg type (A) Normal liver with minimal inflammatory cells (B) liver from infected mice with schistosomiasis showed cellular granuloma with intact viable egg. (C) liver from mice D-GaIN induced hepatitis on top of schistosomiasis showed larger cellular granuloma with prominent parenchymal necrosis (arrows) (D) liver from diseased mice treated with SIM showed fibrocellular granuloma with reduced parenchymal necrosis (arrows) (E) liver from diseased mice treated with SIM and PZQ showed decrease granuloma size with much less cellularity and parenchymal necrosis (H&E)

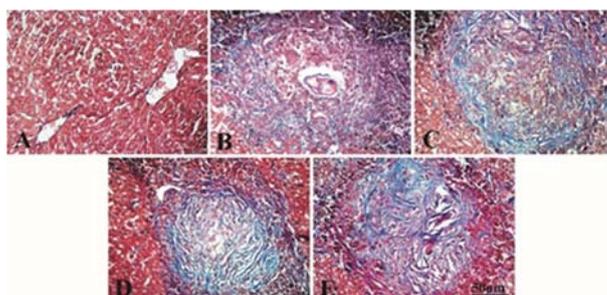


Figure (5): Effect of administration of SIM and PZQ on fibrosis of the granuloma. (A) Normal liver with few collagen fibers around central veins (blue). (B) Liver from mice with schistosomiasis (C) liver from diseased mice with D-GaIN on top of schistosomiasis shows cellular granuloma with collagen fibers deposition. (D) Liver from diseased mice treated with SIM showed less cellular granuloma with more collagen fibers deposition. (E) Liver from diseased mice treated with SIM & PZQ shows more decrease in granuloma cellularity with more collagen deposition (Masson trichrome)

4. DISCUSSION

In the current study, infected mice with schistosomiasis were injected with D-GaIN to induce a model simulating viral hepatitis on top of schistosomiasis. D-GaIN produces a liver injury of diffuse-type which resembles human viral hepatitis morphologically and functionally^{13;14}. D-GaIN causes progressive damage of cellular membranes. The elevation in serum ALT occurred, with administration of D-GaIN could be due to change in membrane permeability and/or destruction of a liver cell (Tang et al., 2004), (Wang et al., 2015). Infected mice showed a significant elevation of ALT in the serum with histopathological examination revealed a schistosomal granuloma surrounded by necrosis. Improvement of necrosis associated with decreased granuloma size and fibrosis content within the schistosomal granuloma could be explained by anti-inflammatory and antifibrotic properties of SIM in addition to its antischistosomal effects. In this study, images taken by EM showed changes in the muscular integument organization, and bundles of muscle cells disorganization. These findings suggest that statins may have a role in the reduction of worm motility and inhibition of sucker-mediated attachment, as it was reported for PZQ (Chai, 2013). Furthermore, SEM showed erosion and swelling of the tegument, in addition to flattening or loss of tubercles with disappearance of spines of the tubercles.

The tegument plays important roles in the absorption of nutrient and secretion, motility and in the protection from the host's immune system and acts as a target for antischistosomal drugs (J Utzinger, Xiao, N'Goran, Bergquist, & Tanner, 2001). So affection of tegument decreases worms' viability in addition to destruction of suckers which leads to removal from intestine.

The mevalonate pathway is important for the essential function of the parasites. Mevalonate is a precursor of cholesterol. Rojo-Arreola et al reported that "3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is the rate-limiting enzyme in the pathway and is targeted by the ubiquitous statin drugs to treat hypercholesterolemia" (Rojo-Arreola et al., 2014). Different studies confirmed the cidal effect of simvastatin against schistosome worms and these effects are possibly due to inhibition of HMGR responsible for cholesterol synthesis which is important for viability of schistosome (Rojo-Arreola et al., 2014). Alencar et al., (2009) showed that an environment rich in cholesterol provides favorable conditions for reproductive success of adult worms (de Barros Alencar et al., 2009).

In-vitro study carried by Rojo-Arreola et al., (2014) showed that SIM kill immature and adult *S.mansoni*. Worm death was reduced by addition of excess mevalonate, the product of HMGR. In addition, RNA interference (RNAi) of the enzyme (HMGR) kills the somules in vitro and this effect is blocked by addition of excess mevalonate. The activation of apoptotic caspase was associated with death of worms (Rojo-Arreola et al., 2014).

We reported that simvastatin has antischistosomal, antifibrotic effect in a previous study conducted in our lab. It affected egg deposition and worm count. It also produced morphological changes to the tegument and decreased egg production (Elmasry et al., 2017)

Administration of SIM reduced ALT in addition histopathological examination showed a reduction in necrosis and inflammation. Histopathological examination confirmed SIM's hepatoprotective and membrane stabilizing effects as it decreased confluent and focal necrosis around schistosomal granuloma in the liver induced by D-GaIN administration on top of schistosomiasis.

Introduction of newer approaches for therapy of HCV are needed. The lipid metabolism of host cell is very important for infectivity of HCV and viral replication in patients with chronic infection (Syed, Amako, & Siddiqui, 2010).

The HCV virus circulates in the plasma of infected patients as a lipoviral particle. It resembles very low-density lipoprotein which is rich in cholesterol esters and contains ApoE and ApoB lipoproteins. These apolipoproteins are important for entry of virus, acting as ligands for scavenger receptor (B type I) and the LDL receptor. Infection with HCV affects lipid metabolism through up-regulation of the sterol response element binding protein. These interactions activate genes that are included in lipid metabolism at the onset of viremia. In addition, HCV infection inhibits fatty acid β -oxidation through inhibiting the expression of PPAR- α transcription factor. Furthermore, adenosine monophosphate-activated protein kinase, which augments fatty acid oxidation, appears to be inactivated in HCV-infected cells. So inhibition of cholesterol and other genes involved in synthesis and transport of fatty acids block HCV.

The use of statins in HCV patients produced an improvement of virological response (VR) rates to the standard antiviral therapy and reduction of progression of fibrosis in liver and incidence of HCC. These data support the use of statins in patients with HCV^{8,19,20}.

Statins reduce fibrosis progression in the liver of infected patients. This effect may be due to the antiviral and immunomodulatory effects of statin (Sun & Singh,

2009). Statins decrease portal hypertension through increasing splanchnic nitric oxide. (Zafra et al., 2004) in addition they up-regulate transcription factors which produce vasoprotective effects in the liver and suppress stellate cells, and potentially mitigate fibrosis (Marrone et al., 2013).

It was noted that mitigation effects of SIM on necroinflammatory reactions and granuloma reaction in the liver is associated with increased IL-10 levels. In schistosomiasis, chronic egg deposition induced inflammation in the liver which could lead to several bad consequences like fibrosis, portal hypertension, bleeding, and even death. IL-10 and other cytokine released by Th-2 cells in response to response of egg deposition. It seems that IL-10 has a role in ameliorating granulomatous inflammation and fibrosis which leads to decrease morbidity and mortality. IL-10 mutant or knockout mice show severe liver damage with increased morbidity and mortality. In our previous work, IL-10 potentiates the antischistosomal effect of SIM and mitigates fibrosis in schistosomal granuloma (Elmasry et al., 2017).

IL-10 controls the immunological response to viral hepatitis thus preventing damage caused by proinflammatory cytokines and promote clearance of virus^{24,25}. It has the ability to attenuate the inflammatory response, deregulate cytokine production and T cell proliferation. Inadequate production of IL-10 lead to persistent infections (Couper, Blount, & Riley, 2008). If the body do not properly regulate the pathogen-triggered immune responses there will be significant damage to host tissues with persistent infection (Rojas, Avia, Martín, & Sevilla, 2017). It seems that IL-10 mediates the effect of SIM on necro-inflammatory reaction of hepatitis and granulomatous reaction of schistosomiasis.

5. CONCLUSION

Praziquantel (PZQ) is the standard therapy for treatment of all species of *Schistosoma* (Jurg Utzinger & Keiser, 2004). It reduces the morbidity of infected mice with *Schistosoma mansoni*, by killing both adult worms and mature eggs and cercariae trapped in the host tissues (Doenhoff, Cioli, & Utzinger, 2008). The addition of SIM to PZQ has a dual effect on schistosomiasis and hepatitis in addition to anti-inflammatory, antifibrotic and immunomodulatory effects. So this combination could be used for treatment of patients with HCV infection on top of schistosomiasis as add-on therapy. Additional studies are mandatory for further assessment.

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