Melittin ameliorates schistosomiasis-induced liver fibrosis by suppressing signal transducer and activator of transcription 3 and nuclear factor kappa B

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Received: 20 August 2024
Revised: 5 October 2024
Accepted: 13 October 2024
Published: 24 December 2024

Journal of The Arab Society for Medical Research 2024, 19:200–210

Background/aim
Liver fibrosis is a persistent inflammatory liver disorder that contributes to a wide variety of conditions, including schistosomiasis. There is no approved therapy for liver fibrosis to date; therefore, finding effective therapeutic targets is a crucial need. There are several studies on natural products, such as bee venom and its bioactive substances like melittin (MEL), for the treatment of inflammatory disorders. The therapeutic effect of MEL in a BALB/c mouse model of *Schistosoma mansoni*-induced liver fibrosis was studied in this research.

Materials and methods
Forty-eight male BALB/c mice were classified into six groups (eight mice each): a healthy control group and five groups infected subcutaneously with cercariae of *S. mansoni*. The infected groups were classified into the infected control group, the Praziquantel (PZQ)-treated group, and three MEL-treated groups that received three different doses (0.1, 0.2, and 0.3 mg/kg, respectively) for 14 days. Hepatic granuloma index (GI) was measured in each experimental mouse, and serum tumor necrosis factor-α (TNF-α), interleukin-17 (IL-17), interleukin-10 (IL-10), and immunoglobulin E were measured by ELISA techniques. Additionally, expressions of signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa B (NF-κB) were assessed in splenocytes. Moreover, histopathology of the liver and spleen were also investigated.

Results
S. mansoni-infected mice showed significant ($P<0.05$) increases in the pro-inflammatory mediators and upregulate expression of STAT3, and NF-κB compared with the healthy group. MEL exhibited potent anti-inflammatory effects, as evidenced by significant ($P<0.05$) inhibition of the elevated pro-inflammatory cytokines, including TNF-α and IL-17, as well as immunoglobulin E levels and hepatic GI, while the anti-inflammatory IL-10 was significantly ($P<0.05$) increased. In addition, MEL treatment significantly ($P<0.05$) inhibited the expression of STAT3 and NF-κB in splenocytes compared with healthy mice. The most positive effects were associated with MEL were observed at the maximum dose.

Conclusion
According to the findings of this study, MEL alleviates the degree of hepatic inflammation in a mouse model of *S. mansoni*-induced liver fibrosis by modulating inflammation through suppression of STAT3 and NF-κB.

Keywords:
anti-inflammatory, liver fibrosis, melittin, nuclear factor kappa B, signal transducer and activator of transcription 3

J Arab Soc Med Res 19:200–210

1687-4293

Introduction

Schistosomiasis is an endemic and serious disease caused by *Schistosoma* parasites that infect over 200 million people annually in about 80 countries [1]. The main pathological aspect of chronic schistosomiasis is granulomatous inflammation that is induced by the presence of schistosome eggs that can reach the liver via the portal circulation and aggregate the inflammatory cells around it, leading to hepatic granulomas and fibrosis [2,3].

Liver fibrosis caused by *Schistosoma mansoni*-infection is the most relevant chronic hepatopathologies worldwide [4]. Chronic liver diseases resulting from liver fibrosis have caused marked morbidity and mortality [5]. Praziquantel (PZQ), the only efficient medication for schistosome infection, has failed in recent years due to resistance and incomplete effectiveness in addition to its side effects. Therefore, it is significant for developing alternative

and efficient treatments with antischistosomal and anti-fibrotic properties [6–9].

For centuries, bee venom (BV) has been applied to acute and chronic human diseases [10]. BV consists of active peptides, enzymes, and nonpeptide amines [11]. The principal bioactive compound of the honey BV (*Apis mellifera*) is melittin (MEL), which constitutes about 50% of its dry weight [12]. Despite having cytolytic and antimicrobial features, MEL and BV have been reported to exhibit significant effects in inflammation [13]. Several studies indicated that MEL reduces the expression of inflammatory proteins in inflammatory disorders [14,15].

This work aimed to investigate the therapeutic effects of MEL on *S. mansoni*-induced murine liver fibrosis and explore its potential mechanisms.

Materials and methods

Materials

MEL extracted from the venom of *Apis mellifera* was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) as a lyophilized endotoxin-free product. PZQ (Biltricide) was purchased from Alexandria Company for Pharmaceuticals and Chemical Industries.

Experimental animals

Total 48 male BALB/c mice (8 weeks old, 20–30 g) were purchased from Theodor Bilharz Research Institute (TBRI, Giza, Egypt). The mice were individually housed in polycarbonate animal cages and maintained under a controlled temperature (25 ±2°C) and 12 h light/12 h dark cycles, with free access to water and standard mice food pellets. Animals were allowed 7 days to acclimatize to the laboratory conditions before the experiment.

Ethical approval

All the experiments were done in commitment to the Public Health Guide for the Care and Use of Laboratory Animals. The present study was approved by the Animal Ethics Committee of the Zoology Department, Faculty of Science, Helwan University with approval no. HU-IACUC/Z/OR1006-47.

Experimental design

The mice were grouped into the following (n=8): A healthy control group and five groups were and injected subcutaneously with cercariae of *S. mansoni* (~40 cercariae) for 45 days for induction of liver fibrosis before receiving treatments [16]. *S. mansoni*-infected mice were divided as follows:

The infected control group was injected intraperitoneally (IP) with PBS for 14 consecutive days.

(a) The PZQ-treated group received a single dose of 600 mg/kg PZQ (divided into two equal doses of 300 mg/kg at intervals of 8 h) by oral gavage [17].
(b) 0.1 mg/kg MEL-treated group.
(c) 0.2 mg/kg MEL-treated group.
(d) 0.3 mg/kg MEL-treated group.

MEL-treated groups received IP injections of MEL (each dose was divided into 8 doses, twice a week for 4 weeks). The doses of MEL used here were based on a previous study [18].

Blood and tissues sampling

The experiment lasted for 28 days, then 6 h after the last treatment, the animals were sacrificed, and blood samples were collected for serum preparation. Following coagulation, serum was extracted from the blood samples by centrifuging them at 500 ×g for 15 min at 4°C. Sera were stored at −80°C for further measurements. The animals' livers and spleens were excised immediately after killing and weighed. The liver samples were cut and fixed in 10% neutral formalin for histological study. The spleen samples were excised under aseptic conditions for splenocyte suspension preparation by squishing spleen tissue in 5 ml RPMI 1640 medium (Lonza, BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, UK), 1% L-glutamine, and 1% penicillin-streptomycin. The cells were centrifuged at 500×*g* for 10 min at 20°C, then red blood cells were destroyed by Ammonium-Chloride-Potassium lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA; Sigma, St. Louis, MO). The pellets were washed and re-suspended in supplemented RPMI 1640 medium. Apart from isolated splenocytes (2×10^6 cells) were cryopreserved in 90% FBS and 10% DMSO and stored at −80°C for measuring nuclear factor kappa B (NF-κB) using a commercial ELISA kit. The second part, the isolated cells, was resuspended in PBS and kept frozen at −80 °C for the molecular techniques.

Histopathological study

The liver samples were fixed in 10% neutral formalin, dehydrated in ascending grades of alcohol, then embedded in wax. Paraffin sections were cut (5 μm thick), and hematoxylin and eosin (H and E) staining was carried out at room temperature [19]. The staining results were examined by an optical microscope (*Leica*).

Measurement of hepatic granulomas size and number

In each section, 10 granulomas were randomly selected with central eggs. Using the Image J program (version 1.x; Image J Software, U.S.), the diameters of granulomas were measured at a magnification of 10×. To find the mean diameter of each granuloma, two maximal diameters that were perpendicular to each other were measured. The number and area of granuloma were measured in each animal of the experimental groups, and the average was calculated for statistical comparison [20].

Measurement of cytokines, immunoglobulin E (IgE), and nuclear factor kappa B by ELISA

TNF-α, interleukin-17 (IL-17), IL-10, and immunoglobulin E (IgE) were measured in serum samples, while NF-κB was measured in isolated splenocytes according to the manufacturer's instructions using the Sandwich-ELISA method (Mouse ELISA kit, Sun Long Biotech, China, Catalog no. SL0547Mo, SL0314Mo, SL0310Mo, SL0597Mo, and SL0723Mo, respectively).

Gene expression of STAT3 and NF-κB by RT-PCR

Total RNA was isolated from frozen splenocytes by the TRIzol (ThermoFisher Scientific, USA). The purity and concentration of RNAs were detected by nanodrop (Agilent 2100 Technologies). cDNA was prepared from isolated RNA using first-stander reverse transcription kit (ThermoFisher Scientific, Lithuania). Applied Biosystems 7500 was used for performing all PCR reactions using QuantiTeh SYBR Green PCR Master Mix Kit (QIAGEN). The primers of STAT3 and NF-κB were purchased from Invitrogen Thermo Fisher (USA) and were shown in Table 1. GAPDH acts as an internal control for the NF-κB and STAT3 primers. Fold changes of STAT3 and NF-κB mRNAs were expressed and calculated as $2^{-\Delta\Delta CT}$ according to Livak and Schmittgen method [21].

Statistical Analysis

Statistical analysis was performed using Graphpad Prism software (version 8.4.3), USA. All data were presented as mean±SD (stander deviation) and percentage of change. One-way analysis of variance (ANOVA) was used to analyze the data, followed by Tukey's multiple comparisons test. Using the Excel program, the relative weights of liver and spleen and the percentage of changes were calculated. The relative weights were calculated as follows: The weight of the organ was divided by the weight of the mouse, and then the resulted values were multiplied by 100. The percentage of changes was calculated as follows: The experimental and control group values were subtracted as (treated – control), then the resulted values were divided by the control value and multiplied by 100. Statistical significance was set at P less than 0.05.

Table 1 Primer sequences used for real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Gene	5′–3′	Primer Sequence
STAT3	**Sense**	GCCGCCGTAGTGACAGAGAA
	Anti-sense	GGCAGCAACATCCCCAGAGT
NF-κB	**Sense**	CAGCGAGGCTTCAGATTTCG
	Anti-sense	CACCTGGCAAACCTCCATG
GAPDH	**Sense**	TGTGTCCGTCGTGGATCTGA
	Anti-sense	TTGCTGTTGAAGTCGCAGGAG

Table 2 The relative weights of liver and spleen of different experimental groups

Mice groups (n=8)	Weight of mice (g) (%)	Relative liver weight (%)	Relative spleen weight (%)
Healthy control	24.6±1.7 [ab]	4.8±0.4 [a]	0.5±0.1 [a]
Infected control	19.1±1.9 [a]	7.0±0.9 [b]	1.3±0.3 [b]
% change*	(–22.4)	(45.8)	(160)
PZQ-treated	26.1±7.8 [b]	5.7±1.1 [a]	0.8±0.2 [a]
% change*	(6.1)	(18.8)	(60)
% change#	(36.6)	(–18.6)	(–38.5)
MEL-treated			
0.1 mg/kg	20.5±2.4 [a]	6.4±0.5 [b]	1.0±0.3 [b]
% change*	(–16.7)	(33.3)	(100)
% change#	(7.3)	(–8.6)	(–23.0)
0.2 mg/kg	21.1±1.6 [a]	5.6±0.6 [a]	0.7±0.1 [a]
% change*	(–14.2)	(16.7)	(40)
% change#	(10.5)	(–20.0)	(–46.2)
0.3 mg/kg	23.2±4.1 [a]	5.2±0.4 [a]	0.7±0.2 [a]
% change*	(–5.7)	(8.3)	(40)
% change#	(21.5)	(–25.7)	(–46.2)

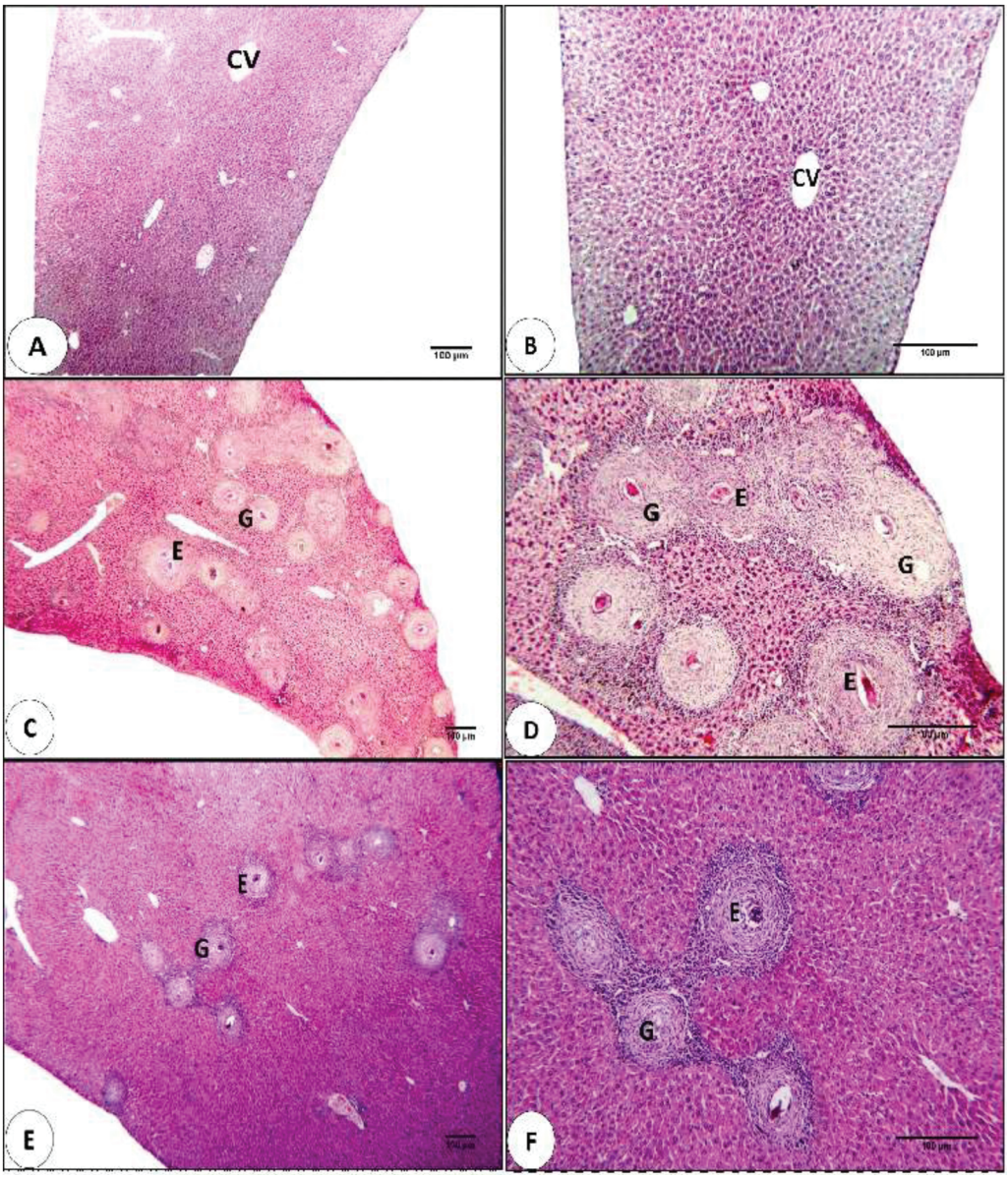
Data are represented as mean±SD and percent change. *: % change than Healthy control group. #: % change than infected control group. All data with different superscript letter (a, b) in the same column were significantly changed at P value less than 0.05, using ANOVA test.

Results

The relative weights of liver of *S. mansoni*-infected mice treated with 0.2 mg/kg MEL (16.7%) and 0.3 mg/kg MEL (8.3%) showed a non-significant increase as compared with that of healthy mice. Conversely, a significant ($P<0.05$) drop in the relative weight of livers was observed after treatment of *S. mansoni*-infected mice with 0.2 mg/kg MEL (-20.0%) or 0.3 mg/kg MEL (–25.7%) doses as compared with infected mice (Table 2). Similarly, treatment with 0.1 mg/kg MEL (100%) caused a significant ($P<0.05$) increase in the spleen' relative weights of the treated mice as compared with the healthy mice. Nevertheless, nonsignificant increments in the spleen's relative weight were recorded in 0.2 mg/kg of MEL (40%) and 0.3 mg/kg of MEL (40%). In contrast, the relative weight of spleen decreased significantly ($P<0.05$) in both 0.2 and 0.3 mg/kg doses of MEL as compared with nontreated *S. mansoni*-infected mice (Table 2).

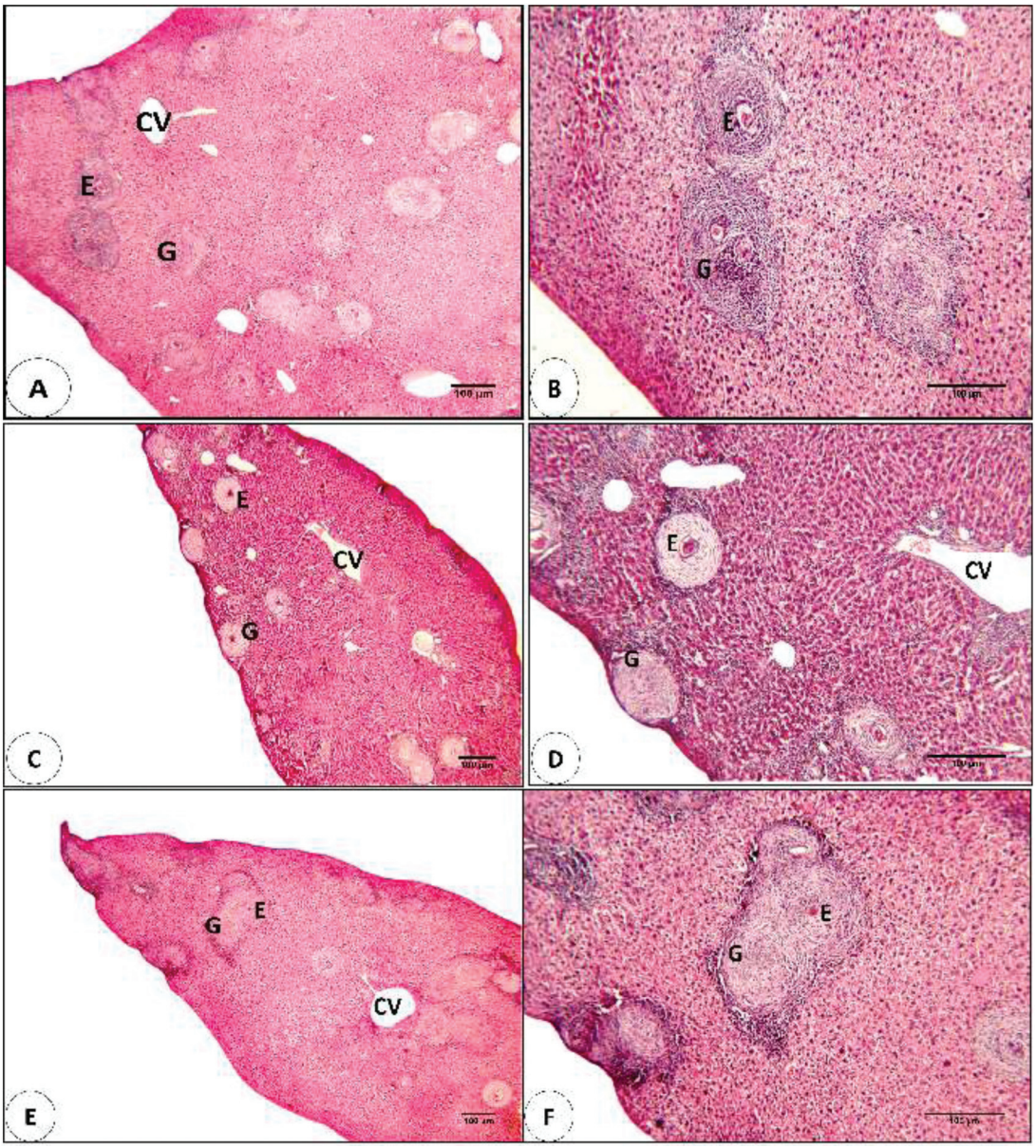
As shown in Figs. 1 and 2 Hepatic lobules and hepatic strands around a central vein were characteristic of the liver's typical structure in the healthy mice group. However, in the liver sections of *S. mansoni*-infected and treated mice, granulomas can be observed with

Figure 1



Photomicrographs of liver sections stained with H and E. A and B) Healthy control group with normal liver structure showed hepatic strands surrounding the central veins (CV). C and D) *S. mansoni*-infected group showed several granuloma (G) with Schistosome eggs (E). E and F) PZQ-treated group showed a decrease in the size of granulomas. All liver sections shown are formalin-fixed and paraffin-embedded. Magnification 4× (A, C, E,) and 10× (B, D, F). Scale bar = 100 μm.

Figure 2



Photomicrographs of liver sections stained with H and E. A and B) 0.1 mg/kg MEL-treated group. C and D) 0.2 mg/kg MEL-treated group. E and F) 0.3 mg/kg MEL-treated group. MEL-treated groups showed a reduction in the size of granulomas in a dose-dependent manner. Granuloma (G) Schistosome eggs (E) central vein (CV). All liver sections shown are formalin-fixed and paraffin-embedded. Magnification 4× (A, C, E,) and 10× (B, D, F). Scale bar = 100 μm.

variations in their diameters. The granulomas appear as organized fibrosis with collagen depositions and aggregates of activated epithelioid cells, macrophages, and other immune cells in an encircling fibrin ring with central eggs. The diameters of the hepatic granulomas in the sections of the Mel-treated mice were smaller than those of the infected and PZQ-treated animals. When compared with infected mice, the hepatic GI of *S. mansoni*-infected mice treated with MEL showed a nonsignificant inhibition at dosages of 0.1 mg/kg (–5.5%) and 0.2 mg/kg (–8.1%). On the other hand, a significant ($P<0.05$) inhibition was observed at a dosage of 0.3 mg/kg (–14.2%) as compared with infected mice. Furthermore, the GI was significantly ($P<0.05$) suppressed in the livers of infected mice treated with 0.3 mg/kg of MEL (–10.4%) compared with PZQ-treated mice (Table 3).

Sera IgE levels were significantly elevated ($P<0.05$) in *S. mansoni*-infected mice (110.2%) and PZQ-treated mice (29.3%) compared with healthy mice. Conversely, infected mice treated with MEL exhibited a significant ($P<0.05$) reduction in the serum IgE levels as compared with the healthy mice in a dose-dependent manner. Furthermore, when comparing

Table 3 Hepatic granuloma index in *S. mansoni*-infected mice and mice treated with Praziquantel (PZQ) or with melittin (MEL) groups

Mice groups (n=8)	Hepatic granuloma index
Infected control	144.1±13.3 [a]
PZQ-treated	138.1±7.3 [a]
% change*	(–4.2)
01 mg/kg MEL-treated	136.2±9.1 [a]
% change*	(–5.5)
% change#	(–1.4)
02 mg/kg MEL-treated	132.4±6.9 [a]
% change*	(–8.1%)
% change#	(–4.1)
03 mg/kg MEL-treated	123.7±11.2 [b]
% change*	(–14.2)
% change#	(–10.4)

Data are represented as mean±S.D. and percent of change. *: % change than infected control group. #: % change than PZQ-treated group. All data with different superscript letter (a, b) in the same column were significantly changed at P value less than 0.05, using ANOVA test.

the serum IgE levels of PZQ- or MEL-treated mice with infected mice, significant (P<0.05) decreases were observed. The reduction in serum IgE levels in MEL-treated mice compared with infected mice showed dosage dependence, as the reductions recorded –57.4%, –69.9%, and –76.8% at doses 0.1 mg/kg, 0.2 mg/kg, and 0.3 mg/kg of MEL, respectively (Table 4).

In comparison to healthy mice, a significant (P<0.05) increase in serum levels of TNF-α was recorded in *S. mansoni*-infected mice (24.4%) and 0.1 mg/kg MEL-treated mice (15.9%). Additionally, a nonsignificant increase in TNF-α serum levels in PZQ-treated mice (8.8%) compared with healthy mice. On the contrary, a significant decrease was reported in mice treated with 0.3 mg/kg MEL (–42.3%) as opposed to healthy mice. Furthermore, compared with *S. mansoni*-infected mice, there was a significant (P<0.05) decline in levels of TNF-α in mice treated with PZQ (–12.6%), 0.2 mg/kg MEL-treated mice (–15.2%), and 0.3 mg/kg MEL-treated mice (–53.6%). However, TNF-α levels were decreased nonsignificantly in 0.1 mg/kg MEL-treated mice (–6.8%) in comparison to infected mice. A significant (P<0.05) decrease was observed in 0.3 mg/kg MEL-treated mice compared with infected mice treated with PZQ, but at 0.1 mg/kg and 0.2 mg/kg dosages of MEL, non-significant changes were observed in comparison to PZQ-treated mice (Table 5).

In comparison to healthy mice, *S. mansoni*-infected mice showed a non-significant increase in serum level of IL-17 with a percent change of 2.8%. While a

Table 4 Immunoglobulin E levels in serum of different experimental groups

Mice groups (n=8)	IgE level (pg/ml)
Healthy control	1091.6±221.8 [a]
Infected control	2295±70.1 [b]
% change*	(110.2%)
PZQ-treated	1411.6±195.5 [c]
% change*	(29.3%)
% change#	(–38.4%)
0.1 mg/kg MEL-treated	978.4±98.2 [a]
% change*	(–10.4)
% change#	(–57.4)
0.2 mg/kg MEL-treated	691.6±156.6 [d]
% change*	(–36.6)
% change#	(–69.9)
0.3 mg/kg MEL-treated	532.7±139.5 [d]
% change*	(–51.2)
% change#	(–76.8)

Data are represented as mean±S.D. and percent change. *: % change than Healthy control group. #: % change than infected control group. All data with different superscript letter (a, b, c, d) in the same column were significantly changed at P value less than 0.05, using ANOVA test.

Table 5 Levels of TNF-α, interleukin-17 and interleukin-10 in sera of different experimental groups

	Mice groups (n=8)					
				MEL-treated		
Cytokines	Healthy control	Infected control	PZQ-treated	0.1 mg/kg	0.2 mg/kg	0.3 mg/kg
TNF-α (pg/ml)	811.4±31.7 [a]	1009.2±112.5 [b]	882.4±43.7 [a]	940.7±76.3 [b]	855.4±42.1 [c]	467.9±31.6 [e]
% change*		(24.4)	(8.8)	(15.9)	(5.4)	(–42.3)
% change#			(–12.6)	(–6.8)	(–15.2)	(–53.6)
IL-17 (pg/ml)	1837.9±27.5 [a]	1889.6±39.0 [a]	2479.6±32.7 [b]	2016.3±63.1 [c]	1941.1±20.9 [a]	1488.4±51.1 [e]
% change*		(2.8)	(34.9)	(9.7)	(5.6)	(–19.0)
% change#			(31.2)	(6.7)	(2.7)	(–21.2)
IL-10 (pg/ml)	467.2±15.7 [a]	581.5±33.7 [b]	549.3±38.1 [b]	612.8±51.1 [b]	689.1±21.2 [c]	775.5±33.5 [e]
% change*		(24.5)	(17.6)	(31.2)	(47.5)	(66.0)
% change#			(–5.5)	(5.4)	(18.6)	(33.4)

Data are represented as mean±S.D. and percent change. *: % change than Healthy control group. #: % change than infected control group. All data with different superscript letter (a, b, c, d, e) in the same raw were significantly changed at Pvalue less than 0.05, using ANOVA test.

significant ($P<0.05$) raise of sera IL-17 levels was revealed in infected mice treated with PZQ (34.9%), 0.1 mg/kg of MEL (9.7%), and 0.2 mg/kg of MEL (5.6%) compared with healthy mice. On the other hand, IL-17 levels were decreased significantly ($P<0.05$) in mice treated with 0.3 mg/kg MEL dosage (–19.0%) as compared with healthy mice. Furthermore, levels of serum IL-17 exhibited a significant ($P<0.05$) raise in infected mice treated with PZQ (31.2%) and 0.1 mg/kg of MEL (6.7%) and a non-significant increase in 0.2 mg/kg MEL-treated mice (2.7%) compared with *S. mansoni*-mice. In contrast, infected mice treated with 0.3 mg/kg MEL showed a significant ($P<0.05$) depletion in serum's IL-17 levels compared with infected mice (–21.2%). In comparison to PZQ-treated mice, a significant ($P<0.05$) increase was found in IL-17 levels at 0.1 and 0.2 mg/kg MEL-treated mice; however, a significant ($P<0.05$) inhibition was reported at 0.3 mg/kg dosage of MEL (Table 5).

A significant increase ($P<0.05$) in the serum level of IL-10 was found in *S. mansoni*-infected (24.5%), PZQ-treated (17.6%), 0.1 mg/kg MEL-treated (31.2%), 0.2 mg/kg MEL-treated (47.5%), and 0.3 mg/kg MEL-treated (66.0%) mice as opposed to healthy mice. In contrast, the IL-10 serum level of mice treated with PZQ showed a nonsignificant reduction (-5.5%) compared with that in infected mice. On the contrary, a nonsignificant raise was detected in treatment with 0.1 mg/kg by a percentage change of 5.4% compared with infected mice. Furthermore, infected mice treated with 0.2 mg/kg of MEL showed a significant increase in IL-10 level by 18.6%. The highest dosage of MEL exhibited a significant increase in IL-10 (33.4%) as opposed to infected mice. Likewise, compared with PZQ-treated mice, there was a significant ($P<0.05$) increase in serum levels of IL-10 in all MEL-treated mice in a dose-dependent manner (Table 5).

The splenocytes of mice infected with *S. mansoni* and those of mice treated with 0.1 mg/kg MEL showed a significant ($P<0.05$) increase in NF-κB levels measured by ELISA technique by 31.7% and 21.4%, respectively, in comparison to healthy mice. Despite this, there was a nonsignificant rise in NF-κB levels in the splenocytes of PZQ-treated mice (11.7%) when compared with healthy mice. Alternatively, NF-κB levels in infected mice treated with MEL at a dosage of 0.2 mg/kg (–12.7%) demonstrated a nonsignificant decrease, while at 0.3 mg/kg (–30.1%) demonstrated a significant ($P<0.05$) decrease compared with healthy mice. Otherwise, a significant ($P<0.05$) decrease was detected in the 0.2 mg/kg (–33.7%) and 0.3 mg/kg (46.9%) MEL-treated mice when compared with those in infected mice. Nevertheless, at 0.1 mg/kg MEL treatment, the decrement was not statistically significant (-7.8%). Furthermore, a significant reduction in NF-κB levels was observed in mice injected with 0.2 mg/kg and 0.3 mg/kg MEL, contrary to those treated with PZQ (Table 6).

Table 7 illustrates that the levels of STAT3 mRNA expressed in splenocytes showed a significant ($P<0.05$) upregulation in *S. mansoni*-infected mice (8.6± 1.8) compared with healthy mice. In comparison to infected mice, STAT3 expression levels were significantly ($P<0.05$) downregulated in mice treated with 0.1 mg/kg (1.7± 0.9), 0.2 mg/kg (-3.2± 2.7), and 0.3 mg/kg (-4.8±1.6) of MEL. However, a nonsignificant downregulation was observed in the mice treated with PZQ (3.3± 2.1) compared with the infected group. As compared with PZQ-treated mice, a significant down expression in STAT3 levels was detected in MEL-treated mice at 0.2 mg/kg and 0.3 mg/kg dosages, while the downregulation in levels of STAT3 was not statistically significant at 0.1 mg/kg MEL treatment.

NF-κB expression levels measured by qRT-PCR showed a significant upregulation ($P<0.05$) in the infected mice as compared with those in healthy mice. After treatment of *S. mansoni*-infected mice with PZQ, 0.2 mg/kg MEL, and 0.3 mg/kg MEL, a

Table 6 NF-κB levels in splenocytes of different experimental groups using ELISA technique

Mice groups (n=8)	NF-κB (pg/ml)
Healthy control	839.2±38.8 [a]
Infected control	1105.6±101.3 [b]
% change*	(31.7)
PZQ treated	937.8±87.6 [a]
% change*	(11.7)
% change#	(–15.2)
0.1 mg/kg MEL treated	1019.1±59.2 [b]
% change*	(21.4)
% change#	(–7.8)
0.2 mg/kg MEL treated	732.7±91.1 [a]
% change*	(–12.7)
% change#	(–33.7)
0.3 mg/kg MEL treated	587.1±115.9 [c]
% change*	(–30.1)
% change#	(–46.9)

Data are represented as mean±S.D. and percent change. *: % change than Healthy control group. #: % change than infected control group. All data with different superscript letter (a, b, c) in the same column were significantly changed at P value less than 0.05, using ANOVA test.

Table 7 Expression of mRNAs of STAT3 and NF-κB in splenocytes of different experimental groups using qRT-PCR

Mice groups (n=8)	STAT3	NF-κB
Healthy control	1.0±0.0 [a]	1.0±0.0 [a]
Infected control	8.6±1.8 [b]	5.6±1.3 [b]
% change*	(780)	(460)
PZQ-treated	3.3±2.1 [a]	1.8±0.4 [c]
% change*	(230)	(80)
% change#	(–61.6)	(–87.9)
MEL-treated		
0.1 mg/kg	1.7±0.9 [a]	1.1±0.5 [a]
% change*	(70)	(10)
% change#	(–80.2)	(–80.4)
0.2 mg/kg	–3.2±2.7 [e]	–1.9±2.4 [c]
% change*	(–420)	(–290)
% change#	(–137.2)	(–133.9)
0.3 mg/kg	–4.8±1.6 [e]	–3.3±2.0 [d]
% change*	(–580)	(–430)
% change#	(–152.3)	(–158.9)

Data are represented as mean±SD. *: % change than Healthy control group. #: % change than infected control group. All data with different superscript letter (a, b, c, d, e) in the same column were significantly changed at P value less than 0.05, using ANOVA test. Expressions of mRNAs were obtained by qRT-PCR. Fold change was presented as $2^{-\Delta\Delta CT}$ that was calculated manually in excel sheet using the comparative CT method. Upregulation (fold change > 1.5); downregulation (fold change < 0.5); unchanged expression (fold change in range of 0.5–1.5).

significant (P<0.05) downregulation of NF-κB expression levels in splenocytes was detected (1.8 ± 0.4, –1.9±2.4, and –3.3±2.0, respectively) as opposed to infected mice. In contrast, a nonsignificant downregulation was reported in mice treated with 0.2 mg/kg MEL when compared with that in mice treated with PZQ (Table 7).

Discussion

Granulomatous hepatitis is an inflammatory liver disorder that contributes to the development of liver granulomas. It may be a response to a wide variety of conditions, including malignancy, drug-induced liver injury, or bacterial, viral, or parasitic infections [22,23]. Worldwide, it has been proposed that the most common cause of liver fibrosis is schistosomiasis via egg-induced hepatic granulomas [24,25]. Zhao *et al.* [26] reported that the primary cause of sickness and mortality in Schistosome infections is hepatic fibrosis. Many natural products have been utilized as therapeutic agents, such as BV and its bioactive substances like MEL and phospholipase, mainly used for treating a variety of inflammatory diseases [15,27,28]. Therefore, the present study investigated the effectiveness of administration of different doses of MEL derived from BV for treating hepatic fibrosis caused by *S. mansoni* infection.

In this study, the liver granuloma index (GI) of infected mice treated with MEL showed a significant decrease in comparison to infected mice. The reduction in the size of granulomas suggests a possible role of Mel against liver fibrosis. Dkhil [29] reported that treatment with berberine decreased the granuloma size, reflecting its antifibrotic effect. In line with this result. The decline in GI after treatment with recombinant IL-22 reveals its anti-fibrosis role in *S. mansoni*-infected mice [30]. Consistent with our results, mice with liver fibrosis treated with MEL exhibit downregulation in expression of fibrotic genes and interruption of the NF-κB signaling pathway, thus ameliorating liver inflammation and fibrosis, so MEL is effective in preventing liver fibrosis [11].

The results of this research indicated that *S. mansoni* infection led to an increase in serum IgE levels. IgE antibodies have a crucial role in immune responses against infectious diseases such as parasitic infections, such as schistosomiasis, providing anti-reinfection capabilities. Moreover, it is involved in response to allergens in the surrounding environment [30]. According to Lynch *et al.* [31] and Malnick *et al.* [32], the elevation in total serum IgE levels could be due to helminthic infection that enhances the production of IgE antibodies. Furthermore, patients with hepatic disorders such as liver fibrosis, cirrhosis, alcoholic hepatitis, and hepatitis A and B also have elevated serum IgE levels. The current result revealed a significant decrease in serum IgE levels in MEL-treated mice for 14 days when compared with those in *S. mansoni*-infected mice. As the dosage of MEL was raised, the decrease in IgE levels increased as well. In addition to that, *S. mansoni*-infected mice treated with PZQ exhibited a significant drop in serum IgE levels when compared with that in infected mice. In agreement with this result, IgE antibody responses to schistosome egg antigens were decreased or unchanged in children treated with PZQ [33]. Similarly to that, the treatment of *S. mansoni*-infected mice with anti-IgE led to a reduction in the worm load and egg production [34]. Previous studies stated that liver diseases induced by schistosomiasis infection or other causes are associated with elevated serum IgE levels as well as liver fibrosis, which are features of liver inflammation; consequently, controlling inflammation may be a target for reducing hepatic fibrosis. Thus, the drugs able to control IgE antibody synthesis help treat inflammatory diseases caused by allergies [16,33,35]. Malnick *et al.* [32] declared that after cessation of alcohol in patients with alcoholic hepatitis, serum IgE levels were decreased. All the previous studies

may explain the role of MEL's inhibitory effects on IgE in improving Schistosomiasis-induced liver fibrosis.

The current research demonstrated that MEL has anti-inflammatory effects. After treating *S. mansoni*-infected mice with different doses of MEL, the levels of pro-inflammatory cytokines (TNF-α and IL-17) in serum were significantly decreased compared with infected mice. These results are in agreement with Moon *et al.* [36], who stated that BV and its major component, MEL, exhibit anti-inflammatory effects by suppressing the pro-inflammatory TNF-α, IL-17, IL-1β, and IL-6 in lipopolysaccharide-stimulated microglia, and thus they may be promising treatments for neurodegenerative diseases via activation of microglia. The inhibition of pro-inflammatory IL-17 activity in schistosome-infected mice via treatment with anti-IL-17 antibodies is effective in reducing the size of hepatic granulomas and thus controlling hepatic fibrosis and improving liver functions, as well as reducing the levels of TNF-α, IL-6, and IL-1β [16,37]. Our results agree with data that demonstrate the positive relationship between elevated pro-inflammatory cytokines and the severity of liver disease during schistosomiasis [38,39]. In light of these studies, MEL may serve as a potential treatment for inflammatory disease due to its effect in reducing the pro-inflammatory cytokines.

The current study revealed a significant increase in the anti-inflammatory serum IL-10 levels in *S. mansoni*-infected mice treated with MEL compared with PZQ-treated or untreated mice. This cytokine may have a role in regulating inflammatory responses during schistosomiasis. This can be supported by Hoffmann *et al.* [40] and Marinho *et al.* [41], who demonstrated that IL-10 hinders the development of *S. mansoni* egg-induced morbidity; thus, it is considered the most crucial immune-regulating factor in the pathogenesis of schistosomiasis. Mice infected with *S. mansoni* and deficient in IL-10 exhibited a significant increase in size of egg-induced hepatic granulomas, increasing liver fibrosis, and a marked raise in serum levels of TNF-α and IFN-γ; furthermore, elevated liver enzymes. Thus, IL-10 might be responsible for inhibiting the production of TNF-α in schistosome infection [42]. According to Wynn *et al.* [43], there was an increased mortality rate in IL-10-deficient mice with schistosome infection. By contrast, Wynn *et al.* [44] reported that schistosoma-infected IL-10 knockout mice exhibited a reduction in the size of pulmonary egg-induced lesions. The increase in IL-10 levels could explain the reduction in sera IgE levels, which is important for modulating egg antigen-dependent allergic implications [45]. This can be supported by the fact that IL-10 decreases IgE synthesis in humans [46]. These findings may explain the relation between the immune-regulatory effects of MEL on pro- and anti-inflammatory cytokines and schistosomiasis-related pathologies.

The results of RT-PCR demonstrated that a significant upregulation in STAT3 expression was recorded in *S. mansoni*-infected mice, although its expression was downregulated significantly in MEL-treated mice at a dosage of 0.3 mg/kg compared with that of *S. mansoni*-infected and PZQ-treated mice. STAT3 is a transcription factor in the cytoplasm that plays a crucial role in regulating many growth factors and cytokines that mediate liver injury as it exhibits hepatoprotective and proliferative properties [47,48]. In agreement with our results in hamsters, *Schistosoma*-SEA and supernatants of cultured eggs were able to activate STAT3 [49]. In addition, Yang *et al.* [50] reported that *S. japonicum*-infected mice showed activation of JAK/STAT3 signaling. Mel was able to downregulate the expression of STAT3 in *Schistosoma*-infected mice; this can be supported by Kim *et al.* [51], who illustrated how MEL inhibit the phosphorylation of STAT3 and reduce the IgE response and pro-inflammatory cytokines in HaCaT cells. The downregulation of STAT3 in MEL-treated mice suggests the positive correlation between STAT3 and hepatic granuloma progression that may explain the mechanism of MEL in alleviating egg-induced liver damage during Schistosomiasis. The deficiency of p-STAT3 attenuates liver damage associated with *S. japonicum* infection [48]. STAT3 deficiency inhibits cancer-induced liver injury [52]. Furthermore, Aydin and Akcali [53] declared that p-STAT3 deficiency suppresses inflammation and inflammatory factors during Schistosomiasis-induced liver fibrosis.

In the present study, a significant upregulation in NF-κB expression was observed in *S. mansoni*-infected mice as compared with healthy mice. NF-κB expression was markedly decreased in MEL-treated mice in comparison to the infected group. NF-κB activation depends on proteolytic phosphorylation of the inhibitor of NF-κB proteins (IκBs), which bound to NF-κB in the cytosol of resting cells, preventing its nuclear translocation [54]. In response to the pathogen, IκB is phosphorylated by IkB kinase, and consequently, NF-κB is released and translocated to the nucleus to activate target gene expression [55–57]. Consistent with our results, Wan *et al.* [57] reported that

Schistosoma-infected mice exhibit activation of NF-κB signaling pathways that are closely associated with hepatic granuloma development and fibrosis, as well as decreased hepatic granuloma size after treatment of infected mice because of suppression of NF-κB signaling pathway. MEL can bind to the IkB kinase (IKK) through the P50 subunit and thiol group [58], thus the IKK loses its catalytic effect on IkB. Therefore, NF-κB nuclear translocation as well as the production of inflammatory mediators were inhibited [59,60]. Previous studies showed that MEL suppresses the pro-inflammatory cytokines and apoptosis via inhibiting NF-κB signaling pathway in mice with acute liver failure [61,62]. Further to that, Han *et al.* [63] demonstrated that MEL inhibited LPS-mediated activation of NF-kB in neuroblastoma cells. Accordingly, MEL might be a potential agent to ameliorate *S. mansoni* egg-induced liver granuloma and fibrosis by suppressing the activity of NF-κB signaling pathways. The findings of the present research suggest that how MEL exerts its beneficial effects on *S. mansoni*-induced granulomatous hepatitis can be attributed to its anti-inflammatory activity and that this action could find clinical use in the treatment of hepatic dysfunction. Hence, further studies are necessary to elucidate the exact mechanism of the modulatory effect of MEL in hepatic disorders. The detailed mechanisms of MEL involvement in STAT3 and NF-κB signaling need further studies to examine its potential therapeutic effects in more detail.

Conclusion

In conclusion, this work demonstrated that MEL administration attenuated egg-induced hepatic granulomas and alleviated hepatic inflammation in a mouse model of *S. mansoni*-induced liver fibrosis. MEL modulates the pro-/anti-inflammatory responses by inhibiting STAT3 and NF-κB expression to achieve this therapeutic effect. The present findings provide evidence for the therapeutic use of MEL in liver fibrosis.

Financial support and sponsorship

This study is part from PhD thesis titled 'A study on the anti-schistosomal effects of some fractions of Honey BV' and has been supported by the Department of Zoology and Entomology, Faculty of Science, Helwan University, Cairo, Egypt.

Conflicts of interest

There are no conflicts of interest.

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