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ORIGINAL ARTICLE

Interlukin -17 Promotes Granuloma Formation and Epithelial-Mesenchymal Transition (EMT) of Hepatic Cells in Experimental Schistosomiasis *mansoni*

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ABSTRACT

Background: *Schistosomiasis mansoni is a severe tropical disease. The most serious pathological effect is liver fibrosis. Interlukin 17 (IL-17) affects liver fibrosis.* ***Objectives:*** *This work was done to estimate the correlation between Schistosoma mansoni granuloma size and IL-17 level in the serum of the schistosomiasis infected mice , and to find its role in liver fibrosis.* ***Methodology:*** *The experiment lasted ten weeks. Sixty Swiss albino mice were used. Mice were divided into equally six groups. Control negative group G0 and infected five groups (G2,G4,G6,G8,G10) with Schistosoma mansoni cercarie. One group was sacrificed every two weeks .Two control mice were sacrificed with each group. Sera were collected for IL-17 assessment by ELISA, liver tissues were examined histopathologically by H&E stain and granulomas size were measured in the different experimental groups. Alpha smooth muscle actin (α SMA),Desmin and Glial Fibrillary Acidic Protein (GFAP) were used to assess the role of IL- 17 in liver fibrosis.* ***Results:*** *There was a highly significant increase ($p<0.001$) in IL-17 level in G6 (6 weeks post infection (wpi). A highly significant ($p<0.001$) positive correlation between the level of IL-17 in sera of the infected groups and granuloma size . Immune stains revealed that hepatocytes expressed stellate cell markers (αSMA ,desmin and GFAP). Moreover, the immunopathology was markedly correlated to IL-17 level. Where, there were a highly significant ($p < 0.001$) increase of serum IL-17 and strong positive expression of α SMA, desmin and GFAP in mice of G6.* ***Conclusion****: These results proved that IL-17 promotes granuloma formation through epithelial mesenchymal transition (EMT) of hepatocytes which play a role in liver fibrosis. Our findings suggested that EMT is a talented therapeutic goal to decrease liver fibrosis for further study.*

INTRODUCTION

Schistosomiasis is a wide spread tropical parasitic disease[1]. Inflammatory fibrosis around eggs in the livers and intestines conistitute the main pathology[2]. Granuloma formation is initiated by $CD4^+$ T cells[3]. Switching from Th1 to Th2 is important for the modulation in immunopathological response [4].IL-17 was demonstrated to be product of $CD4^+$ T-helper (Th17) cell population [2]. The disproportion between Th1/Th2 and Th17/Treg contributing granuloma formation [2,5,6]. Several studies, revealed that activated hepatic stellate cells (HSCs) act as a source of extracellular matrix causing liver fibrosis[7]. HSCs express both mesenchymal cell markers (desmin, actin, vimentin and type I collagen) as well as neuronal or glial cell markers (synaptophysin, neural cell adhesion molecule, glial fibrillary acidic protein, and nestin[8]. Extracellular matrix (ECM) components are secreted by activated HSCs[9,10,11]. Several studies pointed to epithelial- mesenchymal transition (EMT) during carcinogenesis and chronic diseases which is associated with fibrosis in different organs[12,13]. EMT criteria were present in hepatocytes and precursor cells that isolated from human and rodents embryonic livers[14,15].

The current study aimed to evaluate the role of IL-17 in the pathogenesis of liver schistosomiasis in mice serologically using ELISA for detection of IL- 17 in the serum of infected groups, histopathologically and immunohistochemically. Also to provide evidence that IL17induces mice hepatocytes to undergo epithelial mesenchymal transition (EMT) and secrete ECM that incriminated in the liver fibrosis.

METHODOLOGY

Experimental animals:

Sixty Swiss albino male mice, 5-7 weeks old and weighing 20-25 gm were used. They were obtained from the Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI),

Giza, Egypt. Mice were parasite free, laboratory bred at the SBSC, TBRI, Giza, Egypt. They were allowed to acclimatize for seven days prior to use. The experiment lasted ten weeks. Fifty mice were infected by *S. mansoni* cercariae. Mice were equally divided into six groups as follows: G0: 10 control negative group. G2: 10 infected mice sacrificed after two wpi. G4: 10 infected mice sacrificed after four wpi. G6: 10 infected mice sacrificed after six wpi.G8:10 infected mice sacrificed after 8wpi. G10: 10 infected mice were sacrificed after ten wpi Two negative control mice were sacrificed with each group. Sera of mice were collected before sacrification for IL- 17 assessment by ELISA . liver tissues were fixed in formalin for histopathological and immunohistochemical study.

Mice infection:

S. mansoni cercariae were obtained from infected *Biomphalaria alexandrina* snails in SBSC at TBRI. Each mouse was experimentally infected with 60±10 *S. mansoni* cercariae suspended in 0.3-0.5 ml saline by subcutaneous injection[16].

Enzyme-linked immunosorbent assay:

Sera were collected every two weeks and kept at -20c^0 until used for detection of IL-17 by ELISA. Platinum ELISA kit was used for quantitative detection of mouse IL-17ng/ml according to manufacturer's instructions (MouseIL-17Platinum ELISA, e Bioscience, USA, Catalog NO BMS 6001/ BMS 6001 TEN).

Histopathological analysis:

The fresh specimens of liver tissue were obtained and fixed in 10% formalin, followed by routine paraffin-embedding. The sections (5µm) were stained by hematoxylin and eosin (H&E) stain [17] and examined miceoscopally .

Assessment of granulomas size:

To assess the size of granuloma, the mean diameter (µm) was measured [18]. A small piece of liver was sliced and preserved in 10% formalin from each mouse . Liver sections were prepared and stained with H&E stain[17]. The slides were microscopically examined to determine the granuloma diameter according to the criteria of [19] .The greatest diameter of the granuloma

egg and its perpendicular diameter were measured.

The mean of both diameters was considered as the granuloma size .The mean diameter of 10 granulomas was assessed for each slid. The mean of granuloma size was calculated for each infected group .

Immunohistochemical stains:

From each case, multiple 4-µm-thickness sections were cut. The method used for immunostaining was a strept avidin-biotin–amplified system. The primary antibodies used were mouse monoclonal antihuman α SMA, clone mAbGEa (0.1ml concentrated, Thermos Fisher Scientific, USA, Catalog number MA1-744), mouse monoclonal antihuman Desmin, Clone D33 (0.5ml concentrated, Thermos Fisher Scientific, USA, Catalog number MA5-13259) and mouse monoclonal antihuman GFAP, clone ASTRO6 (0.5ml concentrated, Thermos Fisher Scientific, USA, Catalog number MA5-12023). All antibodies were diluted 1:100 using antibody diluent. Staining was done according to [20,21]. lieomyoma tissue was used as a positive control for α SMA [20] and desmin [21] while brain tissue was used for GFAP. Replacement of the primary antibody step with a blockingbuffer was included in the staining procedure as a negative control [17].

Interpretation of α SMA, Desmin and GFAP Immunostaining:

We interpret immunostaining by means of light-microscopic examination. For all antibodies, positive expression was assigned when any number of cells showed cytoplasmic staining. It was evaluated as mild, moderate and strong according to a modified semiquantitative IRS Remmele scale [21].

Statistical Analysis:

Data were analyzed and compared by student's t-test. The p-value was used to test the significant change in the experimental animals among the different groups. The data collected were presented as mean ± SD and analyzed using Social Science (SPSS) software (version 17.0 on an IBM compatible computer; SPSS Inc., Chicago, Illinois, USA). P value was set at 0.05, P>0.05 non-significant, P value<0.05 significant and P value <0.001 highly significant [22].

RESULTS

Regarding the serum level of IL – 17, the serum of mice of G6 showed a highly significant increase ($p < 0.001$) in the level of IL- 17 in comparison to its level in the other experimental groups (Table 1).The mean granulomas sizes were measured and statistically analyzed. The mean granuloma size in liver tissue was significantly high ($p < 0.001$) in **G6** in comparison to the mean size of granulomas of the other experimental groups (Graph 1). In addition, there was a highly significant ($p < 0.001$) positive correlation between the serum level of IL- 17 and the mean granuloma size in the different groups (Table 2, Graph2). Examination of the liver sections of **control negative mice (G0)** showed the normal hepatic architecture where cords of hepatocytes radiated from the central vein. Between the plates of hepatocytes, there were hepatic blood sinusoids (Fig. 1A). Liver sections of **G2** (two wpi) revealed dilated central vein containing adult *S. mansoni* and surrounded by excessive infiltrations of inflammatory cells. whereas the hepatocytes and sinusoids around the central vein, were normal (Fig. 1B). **G4** showed *schistosome* egg granuloma formation and inflammatory cell infiltration in liver tissues (Fig. 1C). At the 6thwpi **(G6),** there were large abundant formation of *schistosome* egg granuloma, inflammatory cellular infiltration, and deformation of liver cells (Fig.

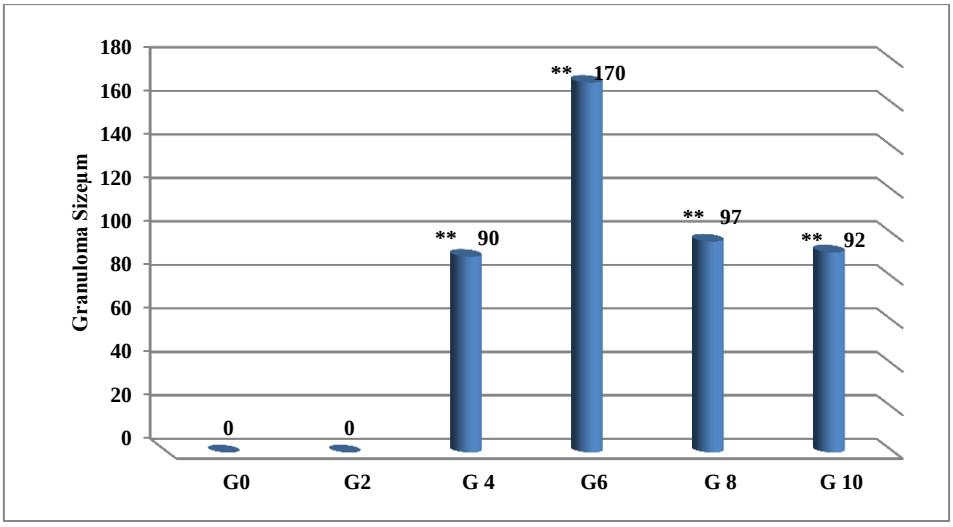
ID) At the 8thwpi **(G8),** in addition to the presence of granuloma, hyaline degeneration and necrosis of hepatocytes were present**.** (Fig. 1E). Finally, at the 10thwpi **(G10),** beside the presence of a well defined granuloma, there were complete loss of hepatic architecture, massive necrosis of hepatocytes and sever cellular infiltration of the liver tissue (Fig.1F).

Mice liver sections of both G0 and G2 showed mild positive immune reaction of hepatic blood sinusoids and negative immune reaction of hepatocytes for both α SMA and Desmin immunostaining (Fig.2A,B). However, GFAP immunostaining of the control mice liver was negative (Fig.2C). Mice liver sections of G4 showed moderate positive cytoplasmic expression of α SMA, Desmin and GFAP immunostaining of the hepatocytes (Fig.3 A, B, C).Regarding mice liver sections of G6, there were strong positive expression of hepatocytes for α SMA , desmin and GFAP immunostaining (Fig.4 A,B,C). However, at the eight wpi (G8), hepatocytes showed moderate positive immune reaction for α SMA, Desmin and GFAP immunostaining (Fig. 5 A,B, C). There were mild positive immune reaction of the hepatocytes for α SMA, Desmin and GFAP immunostaining in the liver tissue of mice of G10.

Table 1: The mean level of IL-17 in the serum of the different groups

Interleukin 17 ng/ml	**Groups (G)**	**Mean ±SD**	**Test of sig.& P value**
G 6 **Mean ±SD** (239.2±5.9)	**G0**	0.48±14.7	t =94.2 P=0.00**(≤0.001)
	G 2	91.4±5.06	t =36.7 P=0.00**(≤0.001)
	G 4	164.9±3.8	t =16.8 P=0.00**(≤0.001)
	G 8	108.5±2.3	t =39.2 P=0.00**(≤0.001)
	G 10	61.6±10.6	Mann Whitney=56.3 P=0.00**(≤0.001)

SD= the standard deviation. ** Highly significant (p<0.001).



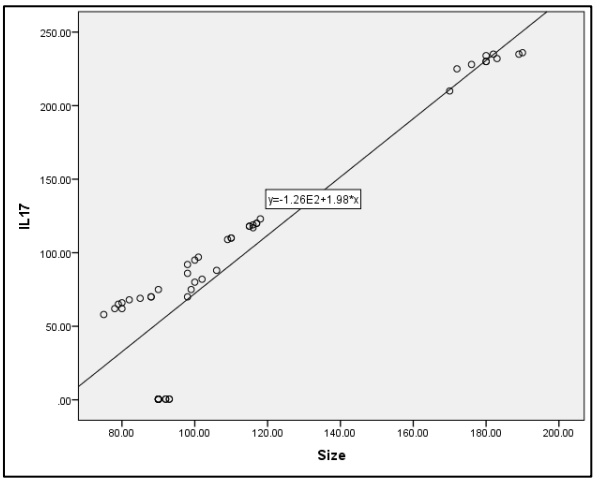
Graph 1: The mean of Granuloma size in different groups

** Highly significant (p<0.001).

Table (2): Correlation between serum IL-17 level and granuloma size

Parameter	Size	
	r	P value
IL-17	**0.921**	**0.00****

** Highly significant (p<0.001).



Graph 2: Correlation between serum IL-17 level and granuloma size

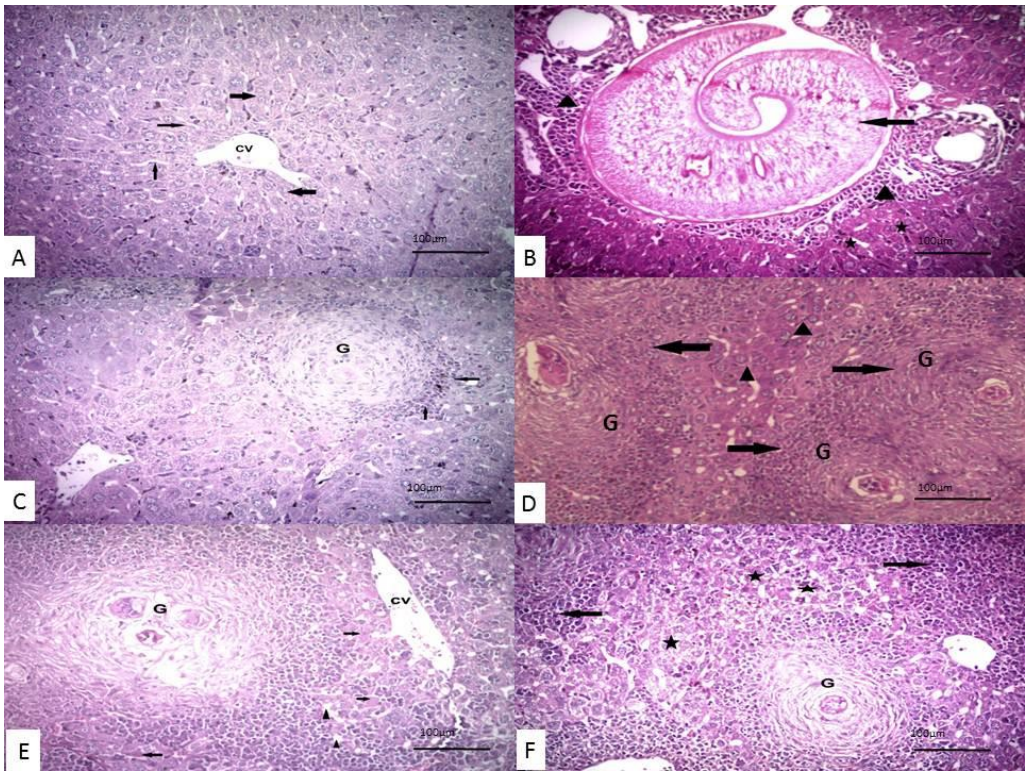


Fig. 1: H&E stained section of a mice liver: **A-** G0 (control negative group) showing normal hepatic architecture **B-** G2 showing dilated central vein containing adult *Schistosoma mansoni* (arrow). Massive cellular infiltration (arrow head). Normal hepatocytes and blood sinusoids (stars). **C-** G4 showing schistosome granuloma egg (G) and inflammatory cell infiltration (arrows). **D-** G6 showing multiple granulomas (G), inflammatory cellular infiltration (arrow), and deformation of liver cells (arrow head). **E-** G8 showing schistosoma granuloma (G), hyaline degeneration and necrosis of hepatocytes (arrows),Dilated central vein (CV) and dilated hepatic blood sinusoids (arrow heads). **F-** G10 showing well defined granuloma (G) ,loss of hepatic architecture, massive necrosis of hepatocytes (stars) and cellular infiltration (arrows). **(Hx&E x 200)**

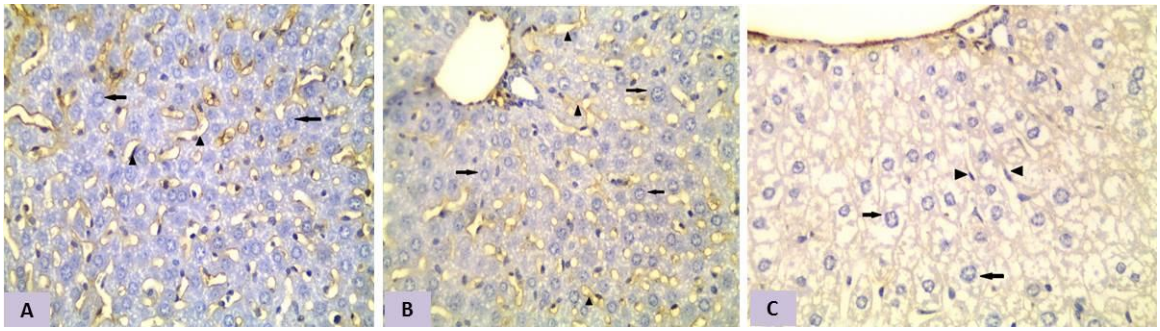


Fig. 2: Sections in mice liver of both G0 and G2 showing: Mild Positive immune reaction for α SMA, Desmin and GFAP immunostaining of hepatic blood sinusoids (arrow heads) and negative immunereaction for hepatocytes (arrows) (A, B, C respectively) **(IHC x 400)**

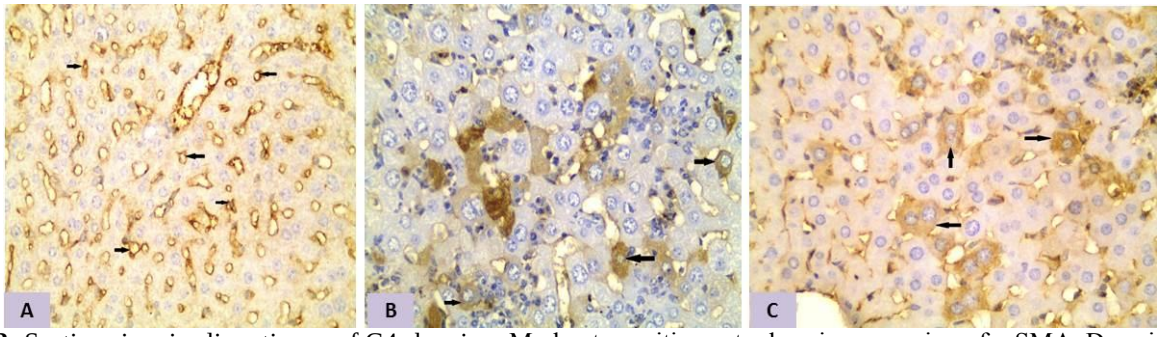


Fig. 3: Sections in mice liver tissue of G4 showing: Moderate positive cytoplasmic expression of α SMA, Desmin and GFAP immunostaining of the hepatocytes (arrows)(A, B, C respectively). **(IHC x 400)**

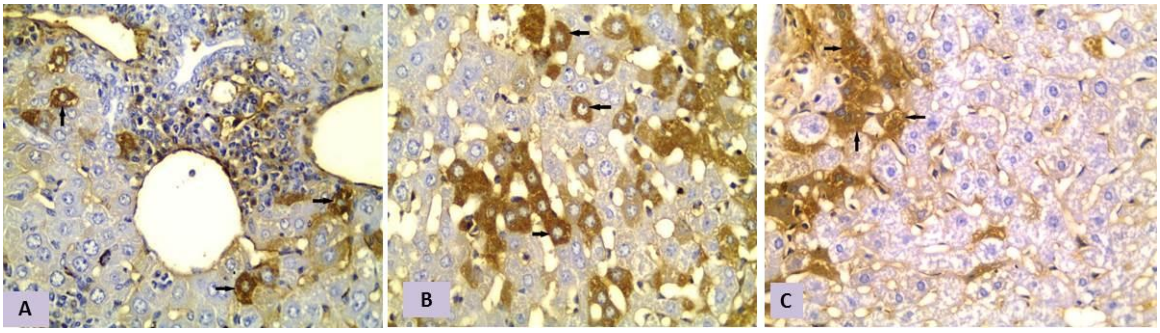


Fig. 4: Sections in mice liver tissue of G6 showing: Strong positive cytoplasmic expression of hepatocytes (arrows) for α SMA Desmin & GFAP immunostaining (A, B, C respectively). **(IHC x 400)**

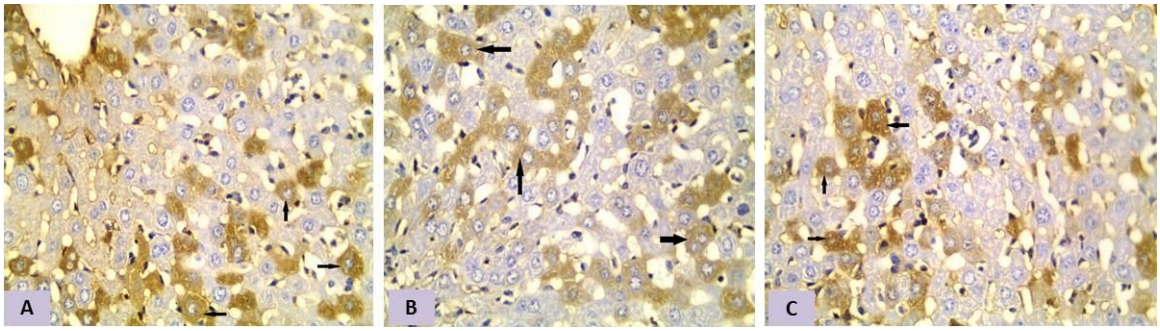


Fig. 5: Sections in mice liver tissue of G8 showing: Moderate positive cytoplasmic expression of hepatocytes (arrows) for α SMA , Desmin and GFAP immunostaining (A, B, C respectively)**. (IHCX400)**

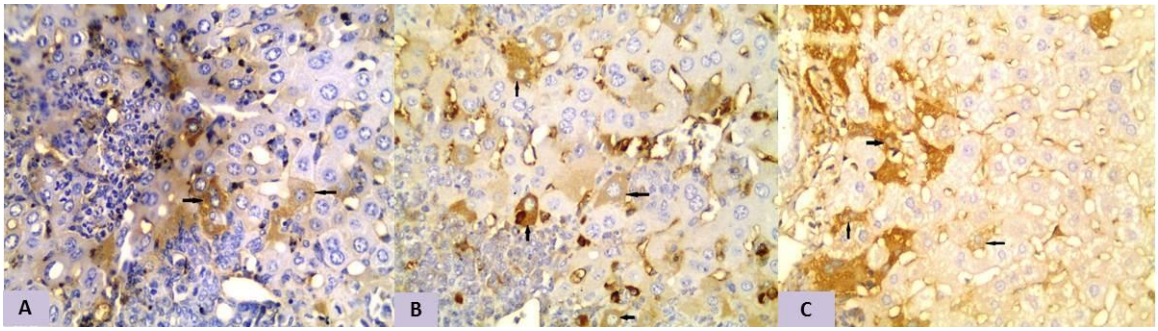


Fig. 6: Sections in mice liver tissue of G10 showing: Mild positive cytoplasmic expression of hepatocytes (arrows) for α SMA, Desmin and GFAP immunostaining (A, B, C respectively). **(IHC x 400)**

DISCUSSION

Granuloma formation around the egg is the main pathology in schistosomiasis *mansoni*.The lesions are influenced by CD4 T cells specific for egg antigen [23].T cell mediated immune response conistitute a protective immunity and the pathological response to *Schistosoma* infected mice. It was found that Th17and T.regulatory cells (Treg.) play an important role in the immune response in schistosomiasis and it is incriminated in *Schistosoma* egg granuloma and liver fibrosis [2]. IL-17A is produced by specific CD4+ Th cells (Th17) with pro-inflammatory properties that differ from other T helper cells in development and function. In our study we used *S. mansoni* infected mice as a model for schistosomiasis to find a correlation between IL-17 level in the serum of infected experimental animals and the pathological changes in their liver. Our results showed that IL-17 level was gradually increased in sera of infected mice with a high peak at 6thwpi.This was in accordance with Smith et al.[24] and Wen et al. [25] they concluded that IL-17 expression by lymphocytes is related to the degree of severity in schistosomiasis. Also Chen et al. [1] found that there was a significant increase in Th17 in hepatic and splenic tissues of infected mice with 2 peaks at 8th and 16thweek. while Rutitzky [3] found the development of pathological lesions at 7thweek.The variation may be as a result of the difference in species of *schistosoma* and the mouse strains that used in these experiments [24]. As [3] used *S. mansoni and C57BL/6 mice* while [1] used *S. japonicum* and *BalB/C* mice but we examined *S.mansoni* infected *Swiss albino* mice. In our study, the high peak was at the 6thweek , owing to parasite oviposition. This was due to sever immune response that stimulated by soluble egg antigen (SEA)[2]. Rutitzky and Stadecker [3] determined that Interleukin-17 has a major effect in liver pathology in schistosomiasis. Several studies revealed that lowering of IL-17 levels favor the host's protective responses against *Schistosoma* infection [25, 26].

Our results showed a highly significant positive correlation between the granuloma size and IL-17 level. These findings were in agreement with Chen et al. [1], Smith et al.[24], Rutitzky et al.[27], Lundy and Lukacs [28] they found that severe immunopathology was related to an increase of the pro-inflammatory cytokine IL-17, and it is more likely to be the marker of severe disease. Paquissi[29] demonstrated that IL-17 had a strong profibrogenic effect through different mechanisms: one of them was stimulation of KC to express inflammatory cytokines IL-6,IL-1, and TNF, in addition to the major fibrogenic cytokineTGF-1. The other was by the direct stimulation of HSCs to express collagen type I and helps their activation into fibrogenic myofibroblasts.

Interleukin-17 is a pro-inflammatory cytokine that facilitates recruitment of circulating leukocytes (monocytes and neutrophils), by inducing the expression of downstream cytokines where they develop into inflammatory, angiogenic, fibrogenic macrophages. Macrophages in chronic liver disease can activate HSCs to become collagen producing macrophage by releasing several mediators like TGFβ1 [8]

Zhao et al.[30] added that hepatocytes, (HSCS) and kupffer cells have receptors for IL-17 .

In liver, activated fibroblasts, plays the main role in fibrosis. Cannito et al.[31] revealed that hepatic myofibroblasts (MFs) may originate from hepatocytes through a process of EMT as they are highly proliferative and α SMA positive cells.

In the present study, we observed that hepatocytes express stellate cell markers (α SMA, Desmin and GFAP). Moreover, the expression was related to IL-17 level in the serum. This could be explained by that, hepatocyte can undergo EMT and play role in liver fibrosis [7].

These results were in accordance with Iwano et al. [32] who reported that about 40% of all fibroblasts in kidney are derived via EMT . Cannito et al.[31], Scholten et al.[33] found that EMT was one of several methods that IL-17 has been participated in flaming liver fibrogenesis.

The involvement of EMT in liver fibrogenesis suggesting that this profibrogenic mechanism in progressive hepatic fibrosis should be considered as a major source. The predictable consequence of prolonged liver fibrosis is liver cirrhosis and hepatic cancer [31]. Therefore, preventing liver fibrosis is our goal.

CONCLUSION

From all the mentioned data, we concluded that there was a direct positive correlation between the serum level of IL-17 , immuno-pathological damage and the functional involvement of hepatocytes in liver fibrosis. So IL-17and EMT are a talented therapeutic targets for decreasing liver fibrosis for further studies .

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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