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Smad 2/3 and CD8+ distribution pattern in liver of mice vaccinated with gamma radiation-attenuated Schistosoma mansoni larvae

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► ***Original article***

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ABSTRACT

Background*:** Gamma radiation-attenuated vaccine has a very promising value in controlling schistosomiasis. The objective of this study is to investigate the effects of vaccination with gamma radiation-attenuated schistosomules regarding histopathological and immunohistochemical changes in mice model of human Schistosoma mansoni infection. ***Materials and Methods*:** The study is conducted upon 40 mice, divided into four groups of 10 each: uninfected control group A (normal control), Schistosoma mansoni infected group B (infected control), Group C subcutaneously injected with 200 Gy gamma radiation–attenuated schistosomules (vaccine control) and group D injected with the same dose of gamma radiation-attenuated schistosomules then challenged after 4 weeks by S. mansoni cercariae (vaccinated-challenged). Parts of liver tissues stained with Masson's trichrome (MT) stain for classifying the granulomas into cellular, fibrocellular or fibrous according to the amount of collagens; stained bluish green. Immunohistochemistry assay was then done on the other portion through expression of Smad2/3 protein and CD8+ in the extracellular matrix (ECM). ***Results: Compared to control infected group, mild histopathological changes with decrease of egg granulomas mainly cellular is detected in vaccinated-challenged group. This is accompanied with moderate immunohistochemical expression of Smad2/3 and CD8+ proteins in vaccine-control group and mild expression in vaccinated -challenged group. ***Conclusion:*** The The results indicate that vaccination with 200 Gy gamma-irradiated schistosomules could reduce histopathological and immunohistochemical changes induced due to infection which represents an effective strategy in disease control.

Keywords: *Schistosoma mansoni, vaccination, immunohistochemistry, histochemistry.*

INTRODUCTION

Protective vaccination against schistosomiasis intended for reducing the pathology and controlling reinfection is the most effective means of control besides etiological therapy. However; complexity of *Schistosoma* interaction with the host's immune system turns the vaccine development to be a difficult task [1-3].

It is reported that Cercariae and schistosomula attenuated with irradiation elicit high immunity against the disease. Although schistosomules protect themselves from the host immune system by confining antigenic molecules in surface membrane, they are considered to be a target of the immune system in the radiation-attenuated vaccine model and trigger an inflammatory reaction inhibiting their migration [4]. Also, irradiation modifies expression of antigens released from or exposed at the surface causing functional impairment

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although no biochemical or morphological changes has been demonstrated [5].

As a result of hepatocytes infection, the release of extra cellular matrix (ECM) produced by myofibroblasts impedes the flow of solutes to the hepatocytes which lose their microvilli thereby stopping the liver functioning effectively [6].

Smad2/3 pathways promote collagen synthesis in activated hepatic stellate cells (HSCs). SMAD3 is pro-fibrotic. Its deletion inhibits type I collagen expression by epithelial myofibroblasts. Whereas, SMAD2 is protective and its disruption up regulates type I collagen expression. Stimulation of the receptors on hepatic stellate cells occurs through Smad1/5 and Smad2/3 phosphorylation. Also, the production of connective tissue growth factor (CTGF) is closely related to the IL-13/ Smad pathway [7-9].

Involvement of $CD8^+$T cells is being increasingly appreciated in several immunopathological events during helminthic infection including schistosomiasis. They recognize the antigenic peptide in target cells and hence induce apoptosis or kill them by direct contact. Also their activity increases in chronic cases. As it reduces the growth of egg granuloma and formation new one through modulating granulomatous immunopathology associated with eggs trapped in host tissue. Several studies suggested that they play a certain role in resisting infection as they release IFN- γ that promote development of protective immunity [10-12].

The aim of this study is to detect the effect of vaccination with gamma radiation-attenuated schistosomules in experimental schistosomiasis mansoni regarding hepatic histopathological changes as well as Smad2/3 and CD8+ expression.

MATERIALS AND METHODS

Attenuation of the parasite

Schistosoma mansoni schistosomulae are obtained from *Schistosome* by the Biological Supply Program (SBSP) at Theodor Bilharz Research Institute, Imbaba - Giza, Egypt through the perfusion technique from Hamster's lung 14 days post-infection with 4000 cercariae using citrated saline. They have been attenuated by irradiation at the (NCRRT) Cairo, Egypt using Indian Cobalt-60 gamma chamber 4000 A Irradiator. Then they were exposed to 200 Gy of gamma radiation at 2.5 Krad/h at the time of experimentation. Viability of cercariae is confirmed by microscopy [13, 14].

Animal treatment

Forty male Swiss albino mice of six week-old weighing 18-20 g are, at the beginning of the study, randomly divided into four groups (ten each) including a normal control group (Group A) infected control group, (Group B) infected by subcutaneous (S. C.) injection with 100 *S. mansoni* cercariae/mouse, vaccine-control (Group C) vaccinated by (S.C.) injection with 500 schistosmules irradiated with 200 Gy gamma radiation and (Group D) vaccinated with the same dose of gamma radiation-attenuated schistosomules then challenged after 4 weeks by S.C. injection with 100 *S. mansoni* cercariae. All animals have been kept at 20-25°C in a 12-h light and 12-h dark cycle, maintained under specific pathogen-free conditions, with free access to standard laboratory water and chow according to the ethics committee of the National Research Center. They were euthanized under anesthesia. Liver tissues were then obtained and divided into two parts for histo-pathological and immunohistochemical study.

Histological examination

Liver tissues have been preserved in 10% buffered formalin then processed into paraffin blocks, serially cut into 4μm thickness and stained Masson's trichrome (MT) stain in order to classify the granulomas as cellular, fibrocellular or fibrous according to the amount of collagens, stained bluish green, represented in the granulomas [15].

Immunohistochemistry

Hydrogen peroxide 0.3% in methanol for 15

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minute was used to block endogenous peroxidase activity. Liver tissues were incubated overnight in goat polyclonal anti-phosphorylated SMAD2/3 antibody (sc-11769, Santa Cruz Biotechnology) after washing in phosphate-buffered saline (PBS). Primary antibodies were diluted at 1:100 in Tris -buffered saline and 0.05% albumin then tissues washed in PBS and incubated with biotin-streptavidin-peroxidase. The reaction was revealed by a mixture of 3,3'-diaminobenzidine with hydrogen peroxide. For CD8 staining, tissues were incubated with rabbit monoclonal antimouse antibodies against CD8+ cells (Abcam) followed by incubation with biotinylated goat antipolyvalent secondary antibody. Conjugation with streptavidin peroxidase enzyme then enzyme substrate chromogen solution was applied. Counter staining was performed using Mayers hematoxylin. Immunopositivity was detected as brown membranous or membrano cytoplasmic staining. The intensity was evaluated and classified as weak, mild, moderate and strong where stained cell were counted from 10 randomly chosen high power fields [16, 17].

Statistical analysis

Results were subjected to Student's ***t***-test using SPSS program version 8 to determine the significance. Data are expressed as mean ± standard error and values with $P < 0.05$, $P < 0.01$ and $P < 0.001$ are significant, highly significant and very highly significant respectively.

RESULTS

Mean±SE of granuloma number was 14.2 ±3.2 in the control infected (Group B). In vaccine-control (Group C), it was 7.5 ±2.1 while in vaccinated-challenged (Group D) it was 5.2± 0.9 showing high significant difference with control infected group B (P<0.001). Regarding collagen content in the control infected group B, it was 40.8±5.9. On comparing with the vaccine-control (Group C) and vaccinated-challenged (Group D), they show high significant difference (18.2±3.1) and (10.5±2.1) with P <0.001 (table 1, figure1).

Data in table 2 and figure 2 show that Smad2/3 in hepatocytes of normal control (Group A) was 106.5±10.6 while infected control (Group B) revealed strong deposition of it in portal tract and hepatic sinusoids (923.3±37.1) with statistically significant increase (P<0.001) compared to normal control group. The expression showed moderate deposition in vaccine–control (Group C) (402.5±17.2). On the other hand, vaccinated-challenged (Group D) showed week expression of (190.3±12.9) with high significant decrease compared to (Group B) infected control group.

Expression of $CD8^+$, in normal control (Group A) was 135.1±12.8, dense deposition was recorded (663.6±43.8) with significant difference (P<0.001) in (Group B) infected control group. In vaccine-control group (Group C), the positive traces were reduced (490.2±17.1) compared to (Group B). Vaccinated -challenged group (Group D) showed weak deposition of collagen (181±14.1).

Hepatopathological and immunohistochemical observations

Figure 3 represents the histopathological changes in the experimental groups. Normal control group (Group A) showed normal hepatocyte morphology where the central vein is surrounded by hepatocytes arranged in strands and separated by blood sinusoids (a). (Group B) showed dense mass of collagen fibers surrounding the egg granulomas, and spread to the space around them, or extended to neighboring lobules. (Group C) vaccine-control group showed fewer fibrocellular granulomas while vaccinated group (Group D) showed reduction in granuloma number and size with few collagen depositions.

Figure 4 shows Smad 2/3 expression in the different groups, minimal expression of Smad 2/3 in liver tissue of mice in normal control group (Group A) while infected control (Group B) showed strong positive expression. In vaccine -control mice of (Group C), the hepatic tissue exhibited moderate positive expression of Smad. Hepatocytes reacted in a weak pattern and

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showed mild expression of Smad in (Group D) vaccinated-challenged.

Regarding expression of $CD8^+$ (figure 5), there is no or minimal expression in control normal group (Group A) while mice of control infected group (Group B) showed high expression. Vaccine control (Group C) showed moderate expression and there is weak expression of CD8 in vaccinated-challenge (Group D).

Table 1. Criteria of granulomatous lesions and associated histopathological changes.

Group	Granuloma Number	Collagen content	Type of infiltration	
			Cellular	Fibro Cellular
B	14.2 ±3.2	40.8±5.9	40%	60%
C	7.5 ±2.1 **	18.2±3.1**	55%	45%
D	5.2± 0.9 **	10.5±2.1**	70%	30%

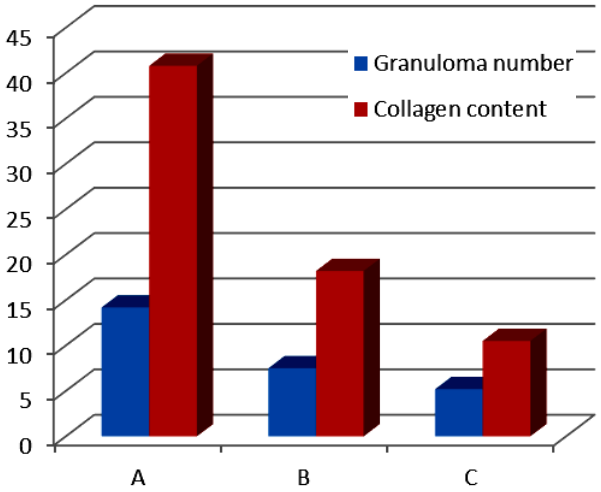


Figure 1. Granuloma number and collagen content in experimental groups.

Table 2. Effect of vaccination with 200 Gy gamma-attenuated schistosomules on hepatic expression of Smad 2/3 and CD8 in mice of experimental groups.

Parameters	Group A Normal control	Group B Infected control	Group C Vaccine control	Group D Vaccinated challenge
Smad 2/3	106.5±10.6	923.3±37.1 $^{+++}$	402.5±17.2 $^{+++***}$	190.3±12.9 $^{++***}$
$CD8^+$	135.1±12.8	663.6±43.8 $^{+++}$	490.2±17.1 $^{+++**}$	181±14.1 $^{++***}$

Data are expressed as mean ± SE. number of mice in each group is ten. P values +++ < 0.001; ++ < 0.01; + < 0.5 compared to control normal group. P values *** < 0.001; ** < 0.01; * < 0.5 compared to control infected group.

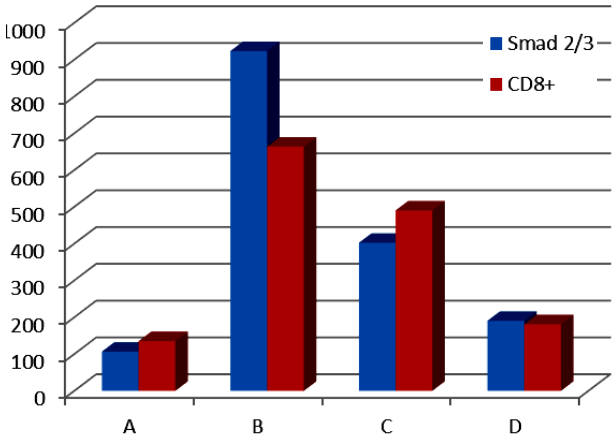


Figure 2. Expression of Smad 2/3 and CD8+ in experimental groups.

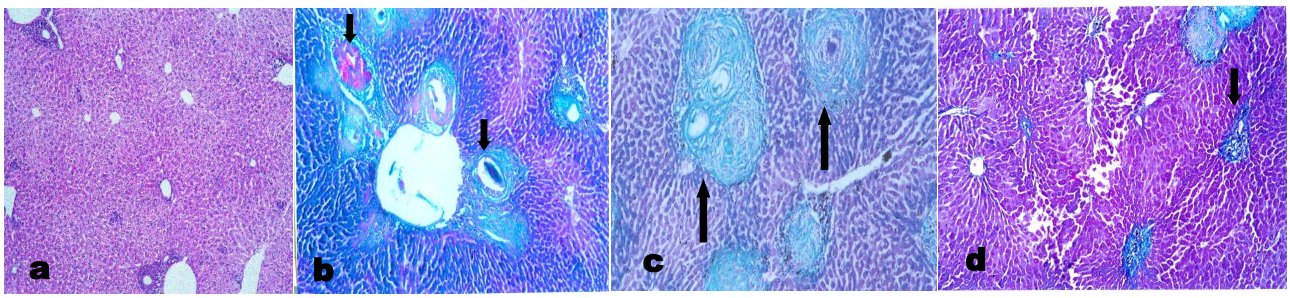


Figure 3. Liver section of mice group A stained with Masson`s trichrome showing normal hepatocyte with central vein and Portal area. Pathological observations of infected control group B mice showing multiple cellular and fibrocellular granulomas with increased collagen deposition around the egg (→). The analysis shows that liver section of group C (vaccine control) has few small granulomas with less inflammatory cellular infiltrate while section of group D (vaccinated-challenged) showing reduced collagen contents with less inflammatory cellular infiltrate (→) (Magnification = 200x for a–d).

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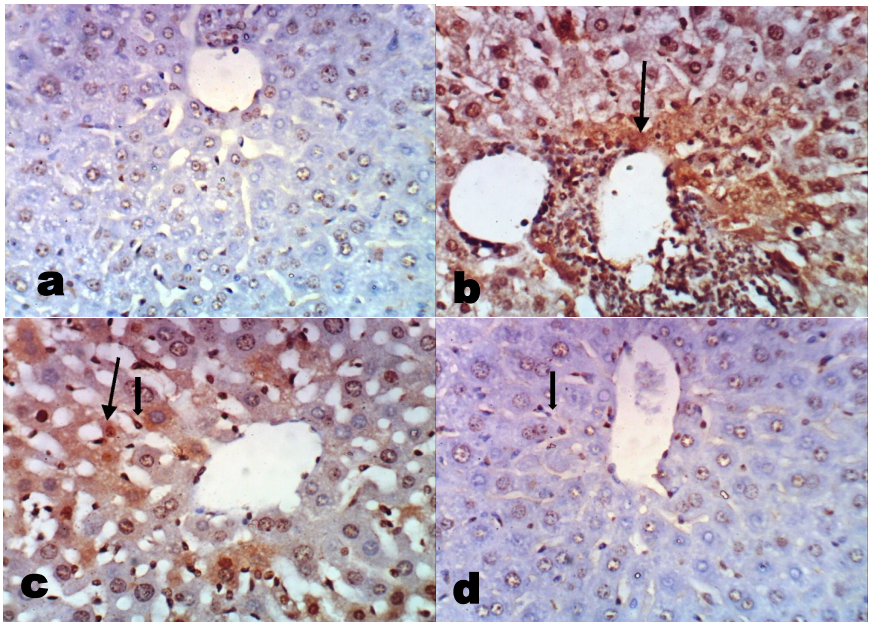


Figure 4. Expression of Smad 2/3 in liver of mice. There is no staining in normal group A, strong positive staining in control infected group b (→). Liver of mice from group C show moderate expression and weak staining in vaccinated-challenged group d (Magnification X 400 for a–d).

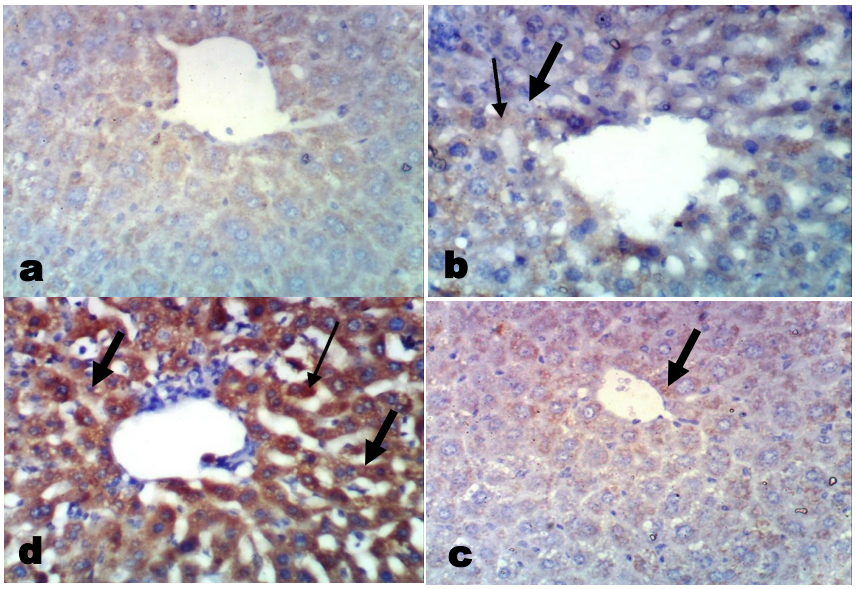


Figure 5. Immunohistochemical staining of CD8+ in liver of mice from group A showing no or minimal expression. Strong positive expression was shown in control infected group B (immunopositivity indicated by brown color) (→). Staining of CD8+ in liver of mice from group C showed moderate positive expression while group D showing weak positive expression (Magnification X 400 for a–d).

DISCUSSION

Anti-*schistosome* vaccines for use with animals have long been advocated as part of the solution to schistosomiasis control, benefitting both humans and animals and improve the local economy. Such features align with the One Health Concept synergizing human and animal health [18]. Application of live radiation-attenuated *Schistosome* larvae has received much attention and offers great promise for vaccine development. Several studies demonstrated that the optimal protection in the radiation attenuated (RA)

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vaccine model is dependent on the induction of both type-1 and type-2- associated immune responses [19-21].

The study shows severe histopathological alterations of the liver parenchyma with marked inflammatory reactions and large fibrous granulomas around the ovum in control infected group. Previous studies revealed typical hepatic lesion with extensive fibrosis around egg granuloma in chronic human *S. mansoni* pipestem. It was speculated that it may be important for attracting lymphocytes, collagen formation and blocking toxic effects of antigens released from eggs [22-24].

Vaccinated-challenged group revealed fewer granulomas which are mainly cellular with decrease in collagen content. Vaccination with 200 and 250 Gy gamma radiation-attenuated schistosomules showed significant decrease in size and number of granuloma and their collagen content with increase in the local concentration of inflammatory cells which may be related to proliferation and /or increased migration of immunologically active cells into granulomas [23]. Increasing the number of vaccinations boosts protection whereas in absence of re-stimulation the high level of protection is not sustained [25]. Several investigators exhibited high levels of protection against a challenge as immunization results in alleviated liver pathology in pigs immunized by 400 µw UV-irradiated *S. japonicum* cercariae compared with those without pre-vaccination [26]. It was reported that persistence of immunizing larvae of *S. mansoni* is an important factor in induction of resistance in mice [27].

Smads are important in regulating TGF-β/ Smad signaling pathway in schistosomiasis and expression of connective tissue growth factor is tightly linked to this pathway [28]. In the same context, it was detected that the protein expression levels of p-Smad1, p-Smad2 and p-Smad2/3 which appear to be key elements in the signal transduction pathways responsible for fibrosis were markedly increased in infected group [29].

The current work revealed minimal expression of Smad 2/3 in normal control group meanwhile infected control group showed densely-stained cells with marked expression of it surrounded and infiltrated in the portal areas and hepatic sinusoids. Similarly, low expression level of Smad2/3 in livers of the normal mice and significantly high expression in the cytoplasm and nucleus of cells around granulomas had been detected by Zhang *et al.* [30]. A high expression of pSmad2/3 compared to the normal group was detected in both acute and chronic phases of liver injury compared to the normal group. This was speculated that *Schistosoma* eggs release soluble egg antigen (SEA) which stimulates hepatic stellate cells to switch on Smad1/2 phosphorylation, leading to liver fibrosis [9, 31]. Studies of Majumdar *et al.* [32] showed that vaccination with soluble *leishmanial* antigen stimulated dendritic cells so restricted the parasites-induced phosphorylation of SMAD and regulated TGF-β in T regulatory cells.

Vaccine control group showed moderate expression of Smad2/3 while vaccinated-challenged group showed minimal expression of it. This was in accordance with Amin et al. who studied immunohistochemical changes in mice vaccinated with 200 Gy gamma irradiated schistosomules regarding expression of α-SMA, desmin, collagen type-1 and TGF-β1 and reported moderate expression in vaccine-control group and few expressions were detected in vaccinated-challenged group. Increasing the number of vaccinations boosts protection while in the absence of re-stimulation; the high level of protection is not sustained [33].

Minimal expression of $CD8^+$ T in normal control group and marked expression was detected in control infected group. Similar alterations were previously observed by different studies. Such studies revealed that $CD8^+$ T cells are implicated in several immunopathological events during helminthic infection including acute and chronic schistosomiasis [34-36].

The moderate expression of $CD8^+$ in vaccine-control group is coincided with previous studies reported that SEA is also able to stimulate an antigen-specific cytotoxic $CD8^+$ T-cell response in mice after immunization. While,

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mild expression in vaccinated-challenged group in this study conforms to previous investigators who proved that immunization of mice with antigens from different stages of *Schistosoma mansoni* decreases the expression of CD^{2+}. Also, it was reported that vaccination with tetraspanin-2 did increase a slight expression of Th1 cells, Th2 cells, $CD8^{+}$ T cells. However, this increase was not statistically significant. [37-39].

CONCLUSION

It is hereby concluded that acute schistosomiasis produce inflammatory effects which lead to severe histopathological changes and marked expression of Smad 2/3 and $CD8^{+}$. Meanwhile, the protective effect of gamma radiation-attenuated vaccine resulted in immunological responses against challenged dose and subsequently maintains the normal histopathological and immuonohistochemical characteristics of hepatic tissue.

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Conflicts of interest*: *Declared none.

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