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Interferon-A Immunotherapy, Combined with Praziquantel Downregulates VEGF Gene Expression, Causing Regression of Hepatic Fibrosis in Chronic *Schistosoma Mansoni* Experimental Infection

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ABSTRACT

Immune cells related to T helper 2 cells, supported by VEGF are accused of being the cause of the excessive fibrous granulomatous hepatic lesions in chronic schistosomiasis, resulting in mortality and morbidity within the infected cases. In this work, intraperitoneal injection of IFN- α was used, combined with praziquantel as an antagonist to the Th2 mechanism, to investigate its immunotherapeutic effect on chronic murine schistosomiasis, in comparison with a group treated with the usual dose of praziquantel. Lower values concerning granuloma number and size were attained in the IFN- α treated groups combined with praziquantel than in the non-treated group as well as the group treated with praziquantel only (P-value<0.01). Mainly cellular granulomas were seen in the IFN- α treated group, while they were mainly fibrous in the infected non-treated control group and fibro-cellular in group treated with praziquantel only. Concerning molecular testing, in the infected non-treated control group, the mean number of VEGF mRNA was 3.73 ± 0.67 . Samples from the IFN- α + Praziquantel treated group showed the lowest level of VEGF mRNA (0.88 ± 0.32), compared to the control infected group (P value<0.01). The decrease in VEGF mRNA expression level was less prominent in the Praziquantel treated group (1.65 ± 0.73), however statistically significant as compared to control. In conclusion, this study revealed immense anti-fibrotic effect for IFN- α with significant minimization of the severity of *S. mansoni* chronic infection, thus avoiding the morbid complications of end-stage hepatic disease. Its use as adjunct immunotherapy to the known specific anti-schistosomal agents is very promising, particularly in high-risk individuals, who developed exaggerated Th2 immune response, next to the chronic infection and can be diagnosed by measuring the level of VEGF as an indicator for fibrogenesis.

INTRODUCTION

Schistosomiasis mansoni affects millions of people in the developing world who continue to suffer from exacerbation of the disease and its complications, specifically, those leading to hepatic fibrosis with its serious life-threatening sequelae (Spencer *et al.*, 2017).

Schistosoma antigen was reported to directly promote hepatic cells to secrete powerful pro-fibrotic element, which is able to induce hepatic stellate cells (HSC) activation and proliferation with unusual alteration of the extracellular matrix. In addition, *Schistosoma antigens*, specifically those related to parasitic eggs were found to stimulate vascular endothelial growth factor (VEGF) considerably. Critically, VEGF augments HSC activation and proliferation plus collagen production, proposing a principal role of this growth factor in liver fibrosis (Luo *et al.*, 2017). VEGF was found to enhance Th2 mediated tissue inflammation in order to regulate tissue repair following cellular injury (Chabon *et al.*, 2014). However, the chronic expansion of the type 2 cell population over time may contribute to the establishment of pathological fibrosis (Gieseck *et al.*, 2018). In fact, mortality in chronic schistosomiasis was suggested to develop as a direct effect of Th2- type response with the development of fibrous granulomas around the eggs (DeMorais *et al.*, 2002). Based on this information and theoretical speaking, prevention of such T helper 2 response may control the process of fibrogenesis, thus reducing morbidity caused by this serious cellular process (De Morais *et al.*, 2002). Perhaps the emergence of several immunotherapeutic or biological modalities helped increase enthusiasm for this therapeutic approach.

Lately, immunotherapy has become of excessive interest to clinicians, investigators as well as pharmaceutical companies to cure several chronic diseases (Syn *et al.*, 2017). Interferons are among the widely used immunotherapeutic agents. Many forms of human interferons (IFN) are found; IFN- α , IFN- β and IFN- γ and all have been clinically approved (Aboushady *et al.*, 2019). In Egypt, there is a long history of treatment by such modality among hepatitis c patients who received interferon- α as an immunotherapeutic agent for several years in a huge campaign, with reported positive outcomes (Aboushady *et al.*, 2019).

Interferons (INF) are among T helper 1-mediated cytokines. They are of major interest in human medicine for many purposes. They own variable effects; antimicrobial, anti-tumour plus immunomodulatory properties (Ramana *et al.*, 2002).

The aim of this work was to investigate the possible impact of IFN- α in combination with the gold standard praziquantel on hepatic fibrosis, associated with chronic schistosomiasis as well as the local gene expression of VEGF, using a murine model.

MATERIALS AND METHODS

Experimental Animals and Infection:

The study comprised 28 laboratory-bred Swiss albino mice weighing 20-22 grams, kindly provided from the Theodor Bilharz Research Institute (TBRI), Giza, Egypt and maintained there until the end of the experiment. They were divided into 4 groups; Group I: infected with non-treated control, group II: infected treated with praziquantel and INF- α and group III, infected treated with praziquantel only. Infected mice received therapeutic agents at week 10 post-infection. In addition, group IV: only interferon medication without infection was used as drug control. Infection of mice was done by tail immersion within 70-80 *Schistosoma mansoni* cercariae from (TBRI).

Medication & Sacrification:

Egyferon (IFN- α 2b) lyophilized powder, delivered as vials of 3 million I.U. [Nile Company for *Pharmaceuticals & Chemical Industries*, El-Ameria, Cairo, Egypt], to be dissolved in PBS and adjusted to obtain a dose of 3000 I.U./mouse/injection, given intraperitoneally once weekly till the end of the experiment. Praziquantel [E.I.P.I.Co. *Pharmaceuticals*, Cairo, Egypt] was prepared in Cremophor-El as suspension and given orally at a dose of 250 mg/kg/day as a (S.O.D PZQ) to be repeated once again after one week. All mice were sacrificed on week 16 and for histopathology; the right lobe of the liver was preserved in 10% formalin till subsequent processing. Fresh hepatic tissue

samples were collected for molecular investigation, processed and preserved at -20°C till the time of further molecular step.

Histopathological Examination:

Paraffin sections from the liver-related the studied groups, 4µm thick, were prepared, fixed, stained with Hx & E and Masson Trichrome stains and examined to detect the number and diameters of granulomas. The stained sections with Masson Trichrome were used to classify the granulomas concerning type as cellular, fibrocellular, or fibrous according to the amount of collagen, stained bluish-green, represented in the granulomas. The number of granulomas per five-field of low power magnification, 100× were examined and the mean number was calculated. The diameter of hepatic granuloma was measured within 15 granulomas by ocular micrometer which was previously calibrated on the same microscope using low power magnification, 100× and by taking the maximum diameter of the lesion. These values, obtained in terms of ocular units were used for the calculation of the mean granuloma diameter as follows:

Gene Expression:

Total RNA was extracted from the tissue samples using commercial kits (The SV Total RNA Isolation System) [Promega Corpora Θ on · 2800 Woods Hollow Road · Madison, USA]. Samples were exposed first to liquid nitrogen and the cell lysis solution was added to the frozen powder to start the steps of spin-column nucleic acid extraction, according to the manufacturer's instructions. The purity and concentration of the extracted RNA were verified by spectrophotometry at 260 nm.

Reverse transcription was performed on freshly extracted RNA samples using M-MLV Reverse Transcriptase RNAase H- [Solis BioDyne, Tartu, Estonia]. The expression level of the VEGF gene was measured by quantitative real-time PCR (SYBR Green Microfluidic LabChip real-time PCR), using the following primer sets; 5'- CTTGCCTTGCTGCTCTACC 3' and 5'- CACACAGGATGGCTTGAAG 3'. For internal control, β-actin was used with primer

sets; 5'- GTGGGGCGCCCCAGGC ACCAG 3' and 5'-TCCTTAATGTCACG CACGAT TTC 3'. For calculation of expression level, target concentration was expressed in relation to the concentration of the housekeeping gene using Suzuki *et al.* (2000) equation; Relative mediator expression = copy no of mediator /copy no of β-actin = concentration of mediator/concentration of β-actin. The statistical tests done were the calculation of the means and their standard deviations (St. dev.), Kruskal Wallis test and Spearman correlation. P-value of 0.05 or less was considered to indicate statistical significance for all hypothesis testing.

RESULTS

Concerning the effect of IFN- α on hepatic egg granulomas, Table (1) summarised the study results; the mean number and diameter of hepatic granulomas and the predominant cell types present in the granulomas of each group. As regards the numbers of granulomas and their diameters, lower values were attained in the treated groups than in the non-treated control group (P-value<0.01). Fibrous and fibrocellular granulomas were much less denoted in the IFN- α treated group, where they were mainly cellular with the predominance of eosinophils, while they were mainly fibrous in the infected non-treated control group (Figs. 1, A to D). Concerning molecular testing, VEGF mRNA expression level was identified in the hepatic tissues of the different study groups, applying real-time PCR (Fig. 1, E). In the infected non-treated control group, the mean number of VEGF mRNA was 3.73 ±0.67. Samples from the IFN-α + Praziquantel treated group showed the lowest level of VEGF mRNA (0.88±0.32), compared to the control infected group (P value<0.01). The decrease in VEGF mRNA expression level was less prominent in the Praziquantel treated group (1.65 ± 0.73), however statistically significant as compared to the control (P-value<0.01). Liver architecture related to the group that received IFN- α without infection looked normal, but VEGF mRNA expression level was significantly low.

Table 1: Summary of the study results; mean \pm standard deviation of granuloma diameter, size and VEGF mRNA expression levels in different study groups.

Study groups	Number /5 files, 100 \times Mean \pm SD	Diameter, [μ m]/15 granulomas Mean \pm SD	Predominant cell type	VEGF expression
I: infected non-treated control	6.53 \pm 0.94	459 \pm 36	Fibrous, mainly fibrocytes,	3.73 \pm 0.67
II: IFN- α + Praziquantel treated	0.88 \pm 0.32	49 \pm 23	Cellular, mainly eosinophils	0.24 \pm 0.09
III: Praziquantel treated	2.78 \pm 1.3	113 \pm 41	Fibro cellular	1.65 \pm 0.73
IV: non infected received interferon	0	0	0	0.44 \pm 0.23

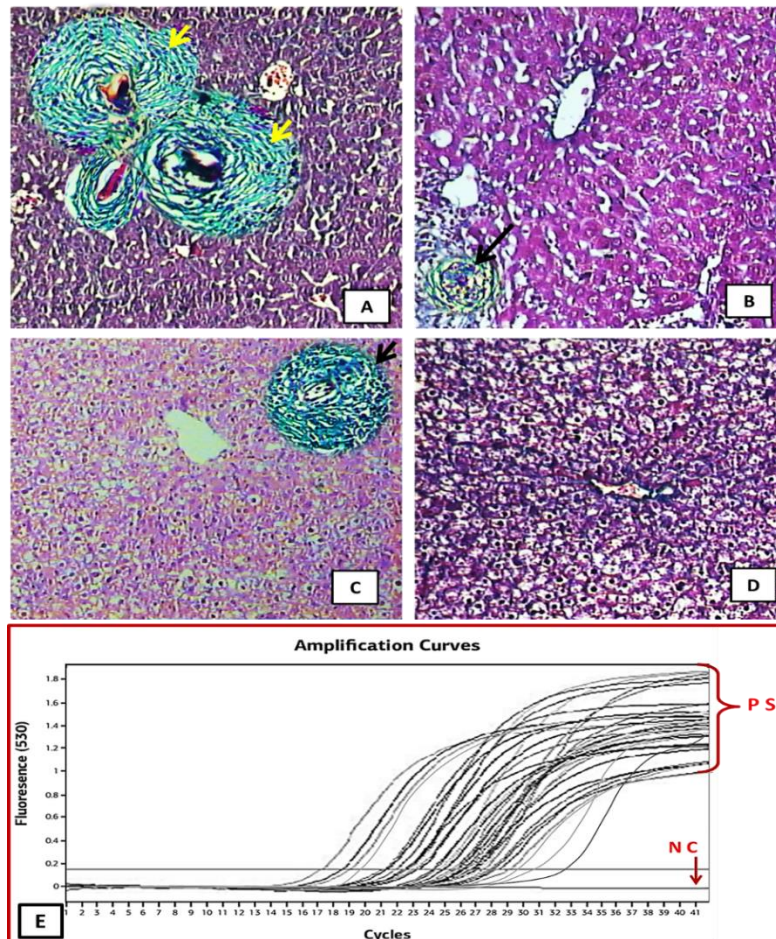


Fig. 1: Representative photographs show the variable features of the *Schistosoma* granulomatous lesions related to the study groups. A, multiple and relatively large granulomatous lesions, surrounding parasitic eggs in the infected non-treated control group (yellow arrow). B, relatively smaller granuloma (black arrow) in the group infected with IFN- α + Praziquantel. C, granuloma (black arrow) in the group infected with Praziquantel. D, normal architecture related to group non-infected and received IFN- α . E, VEGF mRNA amplification curves for positive samples (PS) and absence of signals within the negative control (NC).

DISCUSSION

Liver fibrosis with its serious fatal complications is the main cause of death in chronically infected patients with schistosomiasis *mansoni*, who are mainly under the control of T helper 2 immune cells (Spencer *et al.*, 2017). Hepatic granulomas are developed with deposited collagen fibers around the parasitic eggs, a process that ends with fibrosis. It involves many biological phases which are controlled by successive involvement of stimulatory and inhibitory signals such as immune cells, fibroblasts, cytokines and fibrogenic mediators. However, fibrogenesis is a potentially preventable process (Wynn *et al.*, 1998). Several immunotherapeutic cytokines have entered the clinical field to control, support, or modulate the immune response, in favour of the affected cases and have proved to have considerable clinical benefits (Nelson and Ballou, 2003).

Interferons (IFNs) are among the potent cytokines which have an intense effect on T-cells, being necessary for their activation, accordingly favouring the development of Th1 cells. In addition, interferons are reported to have an antifibrotic effect as they can inhibit collagen production by fibroblasts (Chadha *et al.*, 2004). For all previously mentioned properties, in this study one of these vital mediators, IFN- α was used, combined with the usual dose of praziquantel to cure schistosomiasis infection as well as the associated fibrosis.

IFN- α combined with praziquantel succeeded, not only insignificantly reducing the number and size of granulomas but also positively affecting the cellular element of the granulomas and completely eliminating the fibrous element. This antifibrotic effect, possibly via balancing the increase in Th2 is going with that reported by the works of Czaja *et al.* (1989) and Amany *et al.* (2005). In the present work, the reduction in granuloma size could be attributed to the down-regulation of the Th2 immune mechanism that is affected by the antifibrotic effect of IFN- α with a subsequent decrease in CD4+ related

cytokines which typically induce granuloma formation (Zheng *et al.*, 2020). The balance between Th1 and Th2 mediators is very important for the ineffectual progress of *S. mansoni* infection. In general, there is a positive association between the reduction of the granuloma size and the diminution in rates of portal hypertension with its subsequent morbidity (MacDonald, 2002).

The immunomodulatory properties of certain cytokines in the pathogenesis of schistosomiasis, involving granuloma formation and fibrotic activities have been widely considered. In IL-10 lacking mice, granuloma size increases drastically during the acute phase of schistosomiasis (Wynn *et al.*, 1998). Treatment with IL-4 antagonist or IFN was recorded to reduce granuloma size as well as the extent of fibrosis (Eltoum *et al.*, 1995). While IL-4 treatment causes intense perivascular fibrosis (Yamashita and Boros, 1992).

On the other hand, the responsibility of VEGF in the development of liver fibrosis in chronic schistosomiasis is still not well understood (Luo *et al.*, 2017). VEGF is reported to stimulate the fibrogenic process that reaches the highest level at 16 weeks post-*Schistosoma* infection in the chronic phase of the infection (Kresina *et al.*, 1993).

In this study, VEGF reported significantly the lowest level in the group treated with IFN- α combined with praziquantel, followed by the group treated with praziquantel alone, which was still significantly lower than the control infected non-treated group. Many authors reported a decrease in VEGF when using praziquantel as a therapeutic choice Morcos *et al.* (1985), Liang *et al.* (2011) and Luo *et al.* (2017). While Raig *et al.* (2008) reported complete inhibition for VEGF after treatment with IFN- α .

Concisely, this study revealed that IFN- α , the anti-fibrotic mediator with its powerful immunomodulatory properties, can reduce the severity of chronic schistosomiasis *mansoni* infection, avoiding the morbid complications from excessive fibrosis. Its

potential use as an adjuvant to the specific anti-bilharzial agents is particularly promising. However, the high cost of such a treatment option may be an obstacle facing its wide-scale application. Thus, this treatment modality can be limited to the high-risk group, who are suffering from exaggerated Th 2 immune response reflected in excessive fibrogenesis and who can be diagnosed by measuring serum VEGF which can aid as an indicator for fibrogenesis.

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