Original Article

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Effects of Schistosoma Bovis on Angiogenesis Factor Expression in Macrophage Cells of Rats

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ABSTRACT

BACKGROUND AND OBJECTIVE: *Schistosomiasis* is a severe disease in humans and animals caused by various *Schistosoma* in tropical areas. This study aimed to evaluate the effect of *Schistosoma bovis* adult worm antigens on the expression of angiogenesis factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF2), in rat alveolar macrophages.

METHODS: In this study, rat alveolar macrophage cell cultures were used to determine the effect of *Schistosoma bovis* adult worm antigens on the expression of genes encoding VEGF and FGF2. Rat alveolar macrophage cells were obtained through bronchoalveolar lavage and treated with different concentrations of *Schistosoma bovis* adult worm antigen (0.1, 1, 10 and 50 μg). Moreover, we determined the association between nitric oxide (NO) production and expression of the genes encoding VEGF and FGF2 in rat alveolar macrophage cells.

FINDINGS: *Schistosoma bovis* adult worm antigen at concentrations of 10 and 50 micrograms per milliliter increased the expression of the genes encoding VEGF (12 and 17 times greater than the negative control, respectively) and FGF2 in rat macrophage cells (6 and 12 times greater than the negative control, respectively). In addition, an association was observed between NO production and expression level of the aforementioned genes.

CONCLUSION: According to the results of this study, increased expression of the encoding genes of VEGF and FGF was correlated with *Schistosomiasis*, which could contribute to future studies regarding the control and prevention of this disease.

KEY WORDS: *Schistosoma bovis, Angiogenesis factors, Rat alveolar macrophages.*

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Introduction

Schistosomiasis is a neglected tropical disease caused by the parasites of the genus *Schistosoma*. To date, this disease has affected 207 million people in 76 countries across the world, and the prevalence has been reported to be higher in developing countries of Asia, Africa, Central and South America and the Middle East. Furthermore, 800 million people are at the risk of *Schistosomiasis*, while the mortality rate of this disease has been estimated at a minimum of 280,000 cases per year (1).

Schistosoma parasites are the major factor involved in human *Schistosomiasis* in Africa, the Middle East, South America and the Caribbean. In mammalian hosts, female worms lay eggs in the mesenteric artery, with the larvae passing through the intestinal tract and excreted through feces. Previous studies focusing on *Schistosoma mansoni* suggest that in vitro, parasite eggs attach to endothelial cells inducing cell migration (2). More than 50% of the *Schistosoma* eggs laid in the mesenteric artery are transported through the hepatic portal flow and stored in the liver.

In tissues, *Schistosoma* eggs secrete various soluble products known as soluble egg antigens (SEA), which augment the attachment of parasitic eggs to epithelial cells and induce T-cell-mediated granuloma formation (3, 4). As an immunologic reaction, production of granuloma protects liver parenchyma against the toxic effects of SEA. Atrophied cells in some mice and lacking thymus in other, both manifest granulomatous responses to *Schistosoma* eggs, which lead to the occurrence of hepatic necrosis (5). However, granuloma formation and fibrosis are followed by the obstruction of the hepatic portal vein. Major causes of mortality in *Schistosomiasis* are increased portal pressure, varicose veins, and hemorrhage (6). Formation of new blood vessels plays a crucialrole in embryonic development, wound healing, tumor growth, and inflammation. There are several angiogenesis inducers, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF2). Angiogenesis is directly involved in the occurrence of *Schistosomiasis*. According to the literature, *Schistosoma mansoni* egg antigens induce angiogenesis-related processes by stimulating VEGF in human endothelial cells (7). Host-parasite relationship in infected animals with *Schistosoma mansoni* activates the host immune system variably; for instance, it is associated with the release of cell mediators such as nitric oxide (NO). This study aimed to evaluate the expression of the genes encoding VEGF and FGF2 in rat alveolar macrophage cells stimulated by *Schistosoma bovis* adult worm antigens and determine the association between NO production and expression of these factors in rat macrophage cells.

Methods

Antigen preparation: In this study, *Schistosoma bovis* parasite was provided from the Laboratory of Parasitology at the University of Salamanca, Spain. To prepare *Schistosoma bovis* adult worm antigens, fresh worms (donated by Dr Antonio Moro, Professor of Parasitology at the Faculty of Pharmacy of the University of Salamanca) were washed and crushed in a porcelain mortar. Afterwards, 20 worms per milliliter were homogenized with 1% sterile phosphate-buffered saline(PBS) *solution* and 1 mM phenylmethylsulfonyl fluoride (PMSF). The obtained suspension was sonicated for one minute in three cyclesin cold conditions and centrifuged at 20000 rpm for 30 minutes at the temperature of 4°C. The protein (antigen) concentration of the supernatant was determinedusing the MicroBCA kit (Pierce, Rockford, Illinois). The antigen was aliquoted and stored at -20°C until use.

Isolation of alveolar macrophages: Alveolar Macrophage cells were collected from Wistar rats weighing 2500-300 grams using the method bronchoalveolar lavage) BAL(as previously describedby Andrade et al. (8). Initially, a catheter (VYCON Code 123.06) was slowly inserted into the trachea of rats that were anesthetized with ether. Afterwards, 3 ml of 1% PBS solution was slowly administered into the trachea using a 5-cc syringe (3-5 times) through the catheter, and the injected liquid (containing macrophage cells) was extracted immediately. The solution was filtered through sterile

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gauze and centrifuged at 400 rpm for 10 minutes at the temperature of 4°C. Precipitated macrophages were stored at -80°C after they were washed twice with 1% PBS solution.

Alveolar macrophage cell culture: To attachthe macrophages in the wells (1×10^6/well), all cells were incubated at 37°C with 5% CO_2 for two hours in culture plate (MA, Cambridge, Costar) with complete medium. 1 ml of complete medium, including DMEM (Dulbecco's Modified Eagle's Medium) supplemeted with 10% fetal calf serum, 2 mM L-glutamine (Sigma), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma). After 2 hours, non-adhering macrophages were removed by gentle washing with complete medium and 1 ml of frsh complete medium was added for further cell culture. Alveolar macrophages were incubated in different following groups:

-Negative control: Alveolar macrophages were placed in the completemedium only**;**

-Positive control: Macrophages with complete medium were incubated withlipopolysaccharide (LPS) (10 µg/ml) (*Escherichia coli* B4: 0111 serotype, Sigma);

-Macrophages treated with different concentrations of *Schistosoma bovis* adult worm antigen (10,1,0.1 and 50 µg/ml);

-Macrophages treated separately with specific inhibitors of nitric oxide synthase (NOS) and inducible isoform of nitric oxide synthase (iNOS) (Sigma (at concentration of 10 µg/ml.

After 18 h at 37°C in 5%Co_2, the supernatant was collected andcentrifuged for 10 minutes at 800 rpm. In the next stage, it was transferred to new 1.5 ml tubes and stored -80°C. To verify cellsviability in culture plate, we used the *MTT* (3-(4,5-*dimethylthiazol*-2-*yl*)-2,5-*diphenyltetrazolium bromide*) assay (8). In this method, 200 ml of MTT solution was added to each well and incubated for one hour at 37°C. Afterwards, 200 microliters of dimethyl sulfide *(DMSO)* was added to each well and incubated at room temperature for 30 minutes. In the next stage, 250 microliters were transferred to a 96-well plate, and light absorption at 550 nm was measured using the ELISA reader (Hyperion $MPR4^+$). In this stage of the study, *dimethyl sulfoxide* was used as control. Cells viability was determined using the following formula:

(treated OD/control OD ×100)

Finally, macrophage cells were collected from the plate wells, transferred to 2 ml tubes and stored at -80°C until use.

Determining the level of gene expression of VEGF, GAPDH and FGF2 in macrophage cells byRT-PCR: To extract total RNA from macrophage cells, we used the RNeasy Mini Kit (Qiagen, Germany) in accordance with the instructions of the manufacturer. Quantity of the extracted RNA was measured using the NanoDrop method. Afterwards, cDNA synthesis was performed using the *First Strand cDNA Synthesis Kit* (Roche, Switzerland) in accordance with the instructions of the manufacturer. To perform *reverse transcription* polymerase chain reaction (RT-PCR), we used 2 micrograms of the generated cDNA via the specific primers of VEGF and glyceraldehyde 3-phosphate dehydrogenase) GAPDH) to carry out PCR (table 1). Incubation consisted of a five-minute cycle (94°C) for initial cDNA denaturation, 35 repetitive cycles (94°C, 55°C, and 72°C for one minute) and a final cycle of 72°C for seven minutes. Moreover, PCR in RT-PCR was performed using 2 micrograms of FGF2 gene from the obtained cDNA with specific primers of FGF2 genes (table 1).

Table 1. Sequence of specific primers of VEGF, FGF2 and GAPDH genes

Primer	Sequence
F-VEGF	5'-CTGCTCTCTTGGGTGCACTGG-3'
R-VEGF	5'-CACCGCCTTGGCTTGTCACAT-3'
F-GAPDH	5'-GGTCGGTGTGAACGGATTTG-3'
R-GAPDH	5'-GTGAGCCCCAGCCTTCTCCAT-3'
F-FGF2	5'-GCCGGCAGCATCACTTCGCT-3'
R-FGF2	5'-CTGTCCAGGCCCCGTTTTGG-3'

Incubation program consisted of a two-minute cycle (94°C) for initial cDNA denaturation, 50 repetitive cycles (94°C for 30 seconds, 60°C for 30 seconds, and 72°C for one minute) and a final cycle of 72°C for five minutes. After the reaction, electrophoresis was performed on 5 ml of the obtained products in 2%

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agarose gel for 40 minutes (voltage: 75), and the amplified fragments were assessed at 100% markers (Fig1.A). Amplified fragments were confirmed via sequencing.

Inhibitors of NO production: Macrophage cells that were treated with the concentration of 50 mcg/µl of *Schistosoma bovis* adult worm antigens were incubated at 37°C for 18 hours with 10 micrograms of Nw-nitro-L-arginine methyl ester (L-NAME), as a specific inhibitor of NOS enzyme, and L-canavanine (Sigma), as a specific inhibitor of iNOS. After the incubation period, the supernatant was removed from the wells using a Pasteur pipette, and macrophage cells were collected from the wells and transferred to 2 ml tubes. RNA extraction and cDNA synthesis were synthesized using the instructions above. In addition, effects of the aforementioned inhibitors on the gene expression of VEGF and FGF2 were assessed using RT-PCR at the mentioned temperatures.

Results

Effect of *Schistosoma bovis* adult worm antigens on the expression of VEGF and FGF2 depends on the concentration of the antigen. According to the results of this study, VEGF gene expression at the antigen concentrations of 10 and 50 µg/ml increased 12 and 17 times more than the negative control, respectively (untreated macrophage cells with antigen) (Fig 1.A). Moreover, FGF2 gene expression at antigen concentrations of 10 and 50 µg/ml increased 6 and 12 times more than the negative control, respectively (Fig 1.B). Effects of NOS inhibitors (L-NAME and L-canavanine) on the gene expression of VEGF and FGF2 were variable, so that FGF2 gene expression level was four times lower with L-NAME, while it was 10 times lower than the positive control (macrophage cells treated with LPS) with L-canavanine. However, these inhibitors had no significant effect on the reduction of VEGF gene expression (Fig 2).

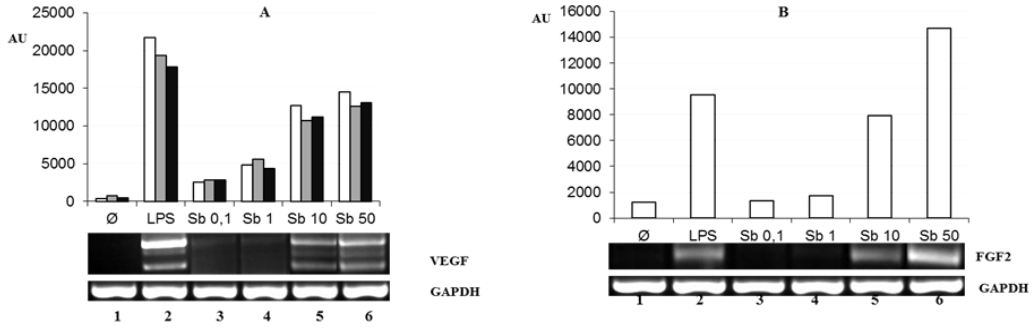


Figure 1. VEGF and FGF2 gene expression in rat alveolar macrophage cells by *Schistosoma bovis* adult worm antigen

A, B: 1) negative control; 2) positive control (cells stimulated with lipopolysaccharide); 3-6) macrophage cells stimulated with different concentrations of *Schistosoma bovis* adult worm antigen (0.1, 1, 10 and 50 µg /ml, respectively). *AU* .measurement unit by amplified fragment densitometry via PCR; 601 VEGF organic base band (white bars); 540 VEGF organic base band (gray bars); 408 VEGF organic base band (black bars); GAPDH internal control genes and 423 FGF2 organic base band (white bars).

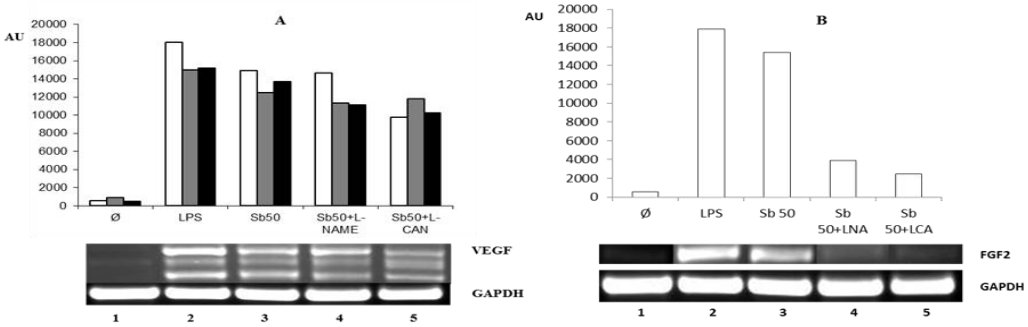


Figure 2. Effects of nitric oxide inhibitors on VEGF and FGF2 gene expression in macrophage cells stimulated by *Schistosoma bovis* adult worm antigen

A, B: GAPDH expression levels in rat alveolar macrophages used as internal control; 1) negative control; 2) positive control (macrophage cells stimulated with bacterial lipopolysaccharide); 3) macrophage cells stimulated with 50 µg/ml *Schistosoma bovis* adult worm antigen; 4) macrophage cells stimulated with 50 µg /ml *Schistosoma bovis* adult worm antigen by L-NAME; 5) macrophage cells stimulated with 50 µg /ml *Schistosoma bovis* adult worm antigen by L-canavanine.

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Discussion

According to the results of the present study, *Schistosoma bovis* adult worm antigens in vitro increase VEGF gene expression at concentrations of 10 and 50 micrograms per microliter in rat alveolar macrophage cells. This finding is in congruence with the results obtained by Shariati et al., who evaluated *Strongyloides* and *Trichinella* parasite species in this regard (9, 10).

In the mentioned study, first stage larvae of *Trichinella spiralis* and *Strongyloides venezuelensis* could increase the gene expression of VEGF and FGF2. In another study, Loeffler et al. demonstrated that SEA of *Schistosoma mansoni* increased growth factor, proliferation, and tube formation in human umbilical vein endothelial cells (7). Similarly, Van de Vijver et al. stated that through increasing the expression of growth factor encoding gene, SEA of *Schistosoma mansoni* led to the formation of new blood vessels in granulomatous inflammatory response (11). Furthermore, the study by Martin et al. suggested that overexpression of VEGF is associated with potent angiogenic effects in different tissues increasing vascular permeability and dilation (12). Findings of the current study indicated that the manifestation of the clinical symptoms of *Schistosomiasis*, such as the formation of new blood vessels due to the granuloma caused by *Schistosoma* eggs, dilation of blood vessels and inflammation, might be associated with increased expression of VEGF. In addition to VEGF, FGF2 is a significant proangiogenic factor, the expression of which was observed to increase in the current research. This is in line with the studies conducted by Shariati et al. in 2009 and 2010 (9, 10). VEGF is involved in the migration, proliferation, matrix analysis of endothelial cells, and vascular network formation, as well as NO production and its release in endothelial cells. As such, we applied NO inhibitors in the present study in order to measure their association with the changes in gene expressions. In conclusion, results of the present study indicated that NO inhibitors markedly decreased FGF2 gene expression in the macrophage cells stimulated with *Schistosoma bovis* antigen, while they had no significant effect on the gene expression of VEGF. Therefore, it seems that the routes through which *Schistosoma bovis* antigen increases the gene expression of VEGF and FGF2 are variable and require further investigation.

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