THE HEPATOPROTECTIVE EFFECT OF NITRIC OXIDE ON MURINE SCHISTOSOMIASIS MANSONI

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ABSTRACT

Background: Schistosomiasis is a major public health problem in developing countries. Currently, praziquantel (PZQ) is the drug of choice for human schistosomiasis. Isosorbide-5-mononitrate (IS-5-MN) is a nitro compound that is used as an anti-anginal remedy. It must be enzymatically metabolized to release nitric oxide (NO) to exert its pharmacologic activities. This study evaluates the vasodilator effect of the NO donor IS-5-MN on hepatic bilharzial lesions caused by *Schistosoma mansoni*, and determines whether the combined use of IS-5-MN and PZQ is synergistic or antagonistic.

Methods: Swiss albino female mice (CD 1 strain) were divided into five groups: (i) non-infected; (ii) infected non-treated; (iii) infected and treated with PZQ, 6 weeks post infection (WPI) in a dose of 500 mg/kg/day for two successive days; (iv) infected and treated with IS-5-MN from the fourth to the tenth WPI 5 days/week in a dose of 10.08 mg/kg; (v) infected and treated with IS-5-MN as group (iv) in addition to PZQ as group (iii). Parasitological, biochemical and histopathological parameters that assess disease severity and morbidity were investigated.

Results: PZQ significantly in-

MANSOURA MEDICAL JOURNAL

creased the percentage of dead eggs, decreased granuloma number but did not reduce granuloma diameter. IS-5-MN administered alone did not induce a shift in the oogram pattern, but it reduced inflammation, necrosis and granuloma diameter. The simultaneous administration of both drugs significantly increased NO level in liver homogenates and induced modulation of granuloma size. The best results were obtained in the mice group treated with IS-5-MN in addition to PZQ.

Conclusions: Our results point to IS-5-MN as a promising medication that could be used as a combined therapy with PZQ to ameliorate schistosomal liver pathology. Further studies are recommended to explore effects of IS-5-MN and PZQ co-administration in schistosomiasis advanced liver fibrosis.

Keywords: *Schistosoma mansoni*, liver fibrosis, isosorbide-5-mononitrate, praziquantel, nitric oxide, granuloma.

INTRODUCTION

Schistosomiasis is a tropical parasitic disease caused by genus *Schistosoma* [1]. In spite of the hard

control efforts, schistosomiasis remains a major health problem that ranks with malaria and tuberculosis as a major source of morbidity affecting almost 240 million people worldwide [2].

The main lesions in chronic infection are caused by a complex delayed-type hypersensitivity response to sequestered viable, dying or dead ova that are trapped in the tissues. The eggs secrete proteolytic enzymes that induce eosinophilic inflammatory and granulomatous reactions, which are progressively replaced by fibrous tissue [3].

Schistosomal liver fibrosis results from an intense accumulation of collagen fibrils in the periportal spaces, leading to pathognomonic periportal or Symmer's pipestem fibrosis with its consequences e.g. portal hypertension, splenomegaly, collateral venous circulation, portocaval shunting, and gastrointestinal varices [4]. Liver fibrosis is initiated by hepatic injury; hepatocyte damage releases cytokines such as transforming growth factor- α (TGF- α), and tumor necrosis factor- α (TNF α) [5]. In turn, the principle masters of liver fibrosis

i.e. hepatic stellate cells (HSCs) are stimulated [6] and transform into myofibroblasts [7].

Nitric oxide deficiency had been linked to liver fibrosis [8]. NO can induce apoptosis of activated HSC and inhibit their proliferation, motility, and contractility in addition to reducing excessive extracellular matrix (ECM) in fibrotic liver [9]. Besides, it impairs the release of inflammatory mediators [10], improves intrahepatic vascular response to portal blood flow [11] and leads to suppression of pro-inflammatory cytokines such as interferon- α (IFN- α) and TNF- α [12]. Consequently, NO donors can exert an anti-fibrotic action as documented in previous studies [13]. IS-5-MN is the most commonly used long-acting NO donor and one of the most frequently used drugs in the treatment of coronary artery disease characterised by well tolerance and lack of serious side effects [14].

Thus, the aim of this study was to evaluate the effect of the NO donor IS-5-MN on hepatic lesions caused by *S. mansoni* and to determine the effect of PZQ and IS-5-MN co-administration.

MATERIAL & METHODS

I. Animals, parasites and infection

All animal studies were approved by the Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University, Mansoura, Egypt, based on the institutional and national regulations for animal experimentation.

A total of 67 Swiss albino female mice of CD I strain (aged 6-8 weeks, and weighting 20-25 gm) were purchased from the Schistosome Biological Supply center (SBSC), Theodor Bilharz Research Institute (TBRI), Imbaba (Giza), Egypt. Mice were infected with *S. mansoni* cercariae Egyptian strain, freshly shed from infected *Biomphalaria alexandrina* snails, purchased from the SBSC, TBRI, after exposure to light for 30 minutes. Each mouse was subcutaneously infected with 60 ± 10 cercariae [15].

Mice were kept in an air-conditioned animal house (MERC) at 20–22 °C, with 12 h light and 12 h dark cycle, and maintained on a standard commercial pellet diet, normal drinking water ad libitum.

II. Drugs and treatment regimens

Isosorbide-5-mononitrate (Effox, Minapharm, Egypt), and praziquantel (Biltricide, Alexandria Co. for Pharmaceuticals & Chemical Industries, Egypt) were used in the study. The dose of IS-5-MN used for mice was equivalent to the highest dose, which induced improvement in rat model of carbon tetrachloride (CCl₄)-induced liver injury [13]. According to Paget and Barnes [16] drug conversion tables, IS-5-MN dose used for mice was calculated to be 10.08 mg/kg. It was administered from the fourth to the tenth WPI, 5 days/week. PZQ was ground and used as a freshly prepared aqueous suspension in 2% Cremophor EL (Sigma Chemical Co., St. Louis, MO, USA), and was given 6 WPI in a dose of 500 mg/kg/day for two successive days [17]. Drugs were administered by oral gavage using a mouse feeding needle (Kent Scientific Corporation), in a volume of 200 µl/mouse.

III. Animal groups

Mice were randomly allocated into five groups, each of 10-15 mice at the beginning of the experiment:

- Group I: normal, non-infected ($n= 10$).

- Group II: infected non-treated ($n= 14$).
- Group III: infected and treated with PZQ ($n= 13$).
- Group IV: infected and treated with IS-5-MN ($n= 15$).
- Group V: infected and treated with IS-5-MN and PZQ ($n= 15$). Mice in all groups were euthanized at the end of the study, 10 WPI.

IV. Parasitological study

After euthanasia, the peritoneal cavity was opened to obtain fragments from the small intestine and the percentages of the different egg developmental stages (oogram pattern) were examined [18].

V. Biochemical study on liver homogenates

Nitric oxide level was assessed in liver homogenates using a commercially available kit (Catalogue number NO 25 32, Biodiagnostics, Dokki, Giza, Egypt).

VI. Histopathological studies

Liver portions from euthanized mice were fixed in 10% neutral buffered formalin, and processed to paraffin blocks. Sections were cut 5 µm

thick, and then stained with haematoxylin and Eosin (H&E) to evaluate histopathological changes. Lobular inflammation was graded [19] from 0–3 based on inflammatory foci per 200^x magnification (0 = none; 1 = 1–2/200^x; 2 = up to 4/200^x; 3 = >4/200^x). Focal necrosis in liver cells around the central vein away from granuloma was scored [20] as follows: none (0%); minimal (1–10%); mild (11–30%); moderate (31–60%); and marked (> 60% of liver cells were affected). Inflammatory cellular infiltrate [21] was evaluated in five microscopic fields of highest inflammatory intensity at 40x magnification and graded as follows: minimal ($\geq$ 25% inflammatory cells); mild (26–50%); moderate (51–75%); and marked (> 75%).

Statistical analysis

Data were analyzed using statistical package for social sciences (SPSS) software (SPSS Inc., Chicago, IL, USA), version 21. Continuous variables were presented as mean $\pm$ standard deviation (SD) for parametric data, and median for non-parametric data. Analysis of variance (ANOVA) followed by Tukey's test were used to compare means of more than 2 groups (parametric

data), while Kruskal Wallis (KW) test was used to compare the median of more than two groups (non-parametric data). Comparison between categorical variables was carried out using Chi-square test. The results were considered significant when the probability of error is equal to or less than 5% ($p \leq 0.05$), and highly significant when the probability of error is equal to or less than 0.1% ($p \leq 0.001$).

RESULTS

1. Parasitological study

Treatment of mice with PZQ 6 WPI significantly decreased ($P \leq 0.01$) the percentage of mature eggs, caused complete absence of immature eggs and significantly increased ($P 0.000$) the percentage of dead eggs. IS-5-MN administered in a dose of 10.08 mg/kg for 5 weeks starting from the 4th to the 10th WPI 5 days/week caused no reduction in the percentage of dead eggs, no reduction in the percentage of immature eggs and did not increase the percentage of dead eggs. Combined administration of IS-5-MN and PZQ induced complete absence of immature eggs and significantly increased ($P < 0.05$) the percentage of dead eggs (**Table 1**).

II. Biochemical study on liver homogenates

Nitric oxide assay

Infection of mice with *S. mansoni* significantly decreased ($P < 0.05$) NO level (**Table 2**), in comparison with non-infected group. Combined administration of IS-5-MN and PZQ significantly increased ($P = 0.000$) NO level by 289.47%, when compared to infected non-treated mice.

III. Histopathological studies

Histopathological examination of the liver sections from infected non-treated mice showed preserved architecture, moderate inflammation of liver parenchyma, moderate focal necrosis, moderate inflammatory cell infiltrate, and a large number (**Figure 1A**) of irregularly outlined granuloma (**Figure2A**).

Administration of PZQ 6 WPI

caused no amelioration of the parenchymatous changes, when compared to infected non-treated group. However, it decreased the number of granuloma (**Figure 1B**) but did not reduce their size (**Figure2B**).

On the other hand, IS-5-MN led to significant amelioration of the parenchymatous changes. Liver sections exhibited mild inflammation of liver parenchyma, mild focal necrosis, significantly diminished inflammatory cell infiltrate, evident granuloma circumscription, and reduced granuloma diameter (**Figure 2C**), when compared to infected non-treated mice.

In addition, the combined administration of IS-5-MN and PZQ significantly diminished inflammatory cell infiltrate and reduced granuloma count and diameter (**Figure2D**).

Table (1): Effect of praziquantel and isosorbide-5-mononitrate alone and in combination on oogram pattern in different mice groups.

Animal groups (number of mice)	Oogram		
	Mature %	Immature %	Dead %
Infected non-treated (<i>n</i> = 13)	54.28	45.71	0
PZQ (<i>n</i> = 13)	14.28 *	0 **	85.71 **
IS-5-MN (<i>n</i> = 9)	52.17	47.82	1.61
IS-5-MN + PZQ (<i>n</i> =13)	41.67	0 **	58.33 ***

Values are expressed as medians.

* Significant difference from infected non-treated group at $p \leq 0.01$.

** Significant difference from infected non-treated group at $p 0.000$.

*** Significant difference from infected non-treated group at $p < 0.05$.

Table (2): Effect of praziquantel and isosorbide-5-mononitrate alone and in combination on nitric oxide level in liver homogenates in different mice groups.

Animal groups (number of mice)	NO level ($\mu\text{mol/L}$)
Non-infected (<i>n</i> =10)	$3.488 \pm 1.491^*$
Infected non-treated (<i>n</i> = 13)	2.146 ± 0.444
PZQ (<i>n</i> = 13)	2.522 ± 0.479 (17.52)
IS-5-MN (<i>n</i> = 9)	2.203 ± 2.07 (0.03)
IS-5-MN + PZQ (<i>n</i> =13)	8.358 ± 2.284 (289.47**) ^a

Values are expressed as means $\pm$ SD.

Values enclosed in parentheses refer to the percentage of increase compared with infected non-treated group.

* Significant difference from infected non-treated and IS-5-MN-treated groups at $p 0.05$.

** Significant difference from infected non-treated group at $p 0.000$.

^a Significant difference from non-infected, PZQ and IS-5-MN-treated groups at $p 0.000$.

Figure (1): Histopathological study of liver sections of *Schistosoma mansoni*-infected mice euthanized 10 weeks post infection (H&E *40). (A) Section from infected non-treated mice group showing large number of granuloma with irregular outline. Arrows point to eggs in granuloma. (B) Section from mice treated with PZQ (500 mg/kg/day 6 WPI for 2 successive days) showing small number of large sized granuloma with no eggs in the center. Arrows point to granuloma.

Figure (2): Histopathological study of liver sections of *Schistosoma mansoni*-infected mice euthanized 10 weeks post infection (H&E *100). (A) Section from infected non-treated mice group showing large sized granuloma with irregular outline. Arrow points to egg in granuloma. (B) Section from mice treated with PZQ (500 mg/kg/day 6 WPI for 2 successive days) showing large sized granuloma with irregular outline. Arrow points to granuloma. (C) Section from mice treated with IS-5-MN showing small sized concised granuloma and reduced inflammatory cell infiltrate. Arrow points to egg in granuloma. (D) Section from mice treated with IS-5-MN and PZQ showing small sized concised granuloma. Arrow points to egg in granuloma.

DISCUSSION

Schistosomiasis affects about 240 million people worldwide and is considered the most frequent cause of liver fibrosis [2]. In schistosomiasis, liver damage takes place 5 WPI, coincident with the onset of oviposition. Eggs that are trapped within the liver provoke a chronic granulomatous inflammatory response [22]. Inflammation and fibrosis accompanied with vascular changes underlie liver pathology in schistosomiasis from the very early scattered periportal granulomas to the advanced periportal fibrosis with its sequelae [23]. It is worth noting that anti-schistosomal drugs successfully eradicate adult parasites with minimal improvement in liver pathology that is related to parasitological cure [24]. So, other medications that can target the scarring process are urgently needed to prevent progression to irreversible cirrhosis [25].

Progressive liver fibrosis is principally maintained by chronic activation of the wound healing response and oxidative stress [26]. Among the most commonly used NO donors, organic nitrates as IS-5-MN is widely used as long acting anti-ischaemic vasodilator drug. Vasodilator proper-

ties of IS-5-MN are mediated through NO release [27]. In turn, NO is involved in each stage of wound healing process through modulating inflammation, angiogenesis, cell proliferation, matrix deposition, and remodeling [28]. In addition to the documented anti-microbial properties of NO [29], the drug can be tested in liver fibrosis model of schistosomiasis both as anti-fibrotic and as anti-schistosomal drug.

To our knowledge, this is the first study evaluating the effects of the NO donor IS-5-MN on an experimental model of *S. mansoni*.

In the current study, IS-5-MN administered alone did not cause significant alterations in the oogram pattern, indicating that the drug does not have anti-schistosomal activity; (**Table 1**), since alteration in the oogram pattern is one of the most important parameters expressing the activity of anti-schistosomal drugs [18].

Nitric oxide can have a cytoprotective or cytotoxic effect. This depends on the initial injury, the source, rate of production, the balance between NO and other inflam-

matory cytokines and mediators e.g. TNF- α , IL-1 β , IFN- γ and reactive oxygen species (ROS), and concentration in the tissues [30]. NO exerts a protective role under oxidative stress resulting from ROS. It was documented that only microM levels of NO are required to protect against these ROS [31].

Administration of IS-5-MN alone did not increase NO level because the drug did not possess anti-schistosomal activity, resulting in persistent state of oxidative stress. On the other hand, PZQ exerted anti-schistosomal effect but did not significantly alter inflammation and necrosis. Combined administration of IS-5-MN and PZQ induced a synergistic effect and significantly increased NO level, in comparison with infected non-treated group, because of the combination of anti-schistosomal activity of PZQ and the hepatoprotective effect of NO donation caused by IS-5-MN (**Table 2**).

Significant amelioration of the parenchymatous changes in IS-5-MN administered group could be visualized in the context of anti-inflammatory properties of NO. Other anti-inflammatory drugs were tested

in previous studies e.g. El-Lakkany et al. [32] who reported the effect of silymarin as an anti-inflammatory and anti-fibrotic agent alone and in combination with PZQ on schistosomiasis mansoni-infected mice.

Co-administration of PZQ and IS-5-MN diminished granuloma number and size, when compared to infected non-treated mice. Our results run in parallel with those recorded by Abdel-Hafeez et al. [33], who documented reduced granuloma number, in response to PZQ only, and diminished size and number following combined alpha lipoic acid and PZQ therapy. Also, similar findings were published by Wang et al. [34], who investigated the effect of vitamin E administration in *S. japonicum*-infected mice, and documented improvement of liver pathology following vitamin E supply, and reduced granuloma count and size.

Our results are in line with previous studies using another NO donor drug S-Nitroso-N-acetylcysteine (SNAC) as an effective anti-fibrotic agent in Sprague-Dawley rats [35]. Also, in another study, sodium nitroprusside; one of NO donors in combination with mesenchymal stem cell

(MSC) transplantation increased MSC ability to repair fibrotic liver as a consequence of NO induced HSC apoptosis [36]. Results of the present study reinforce results of a previous study from Egypt showing marked reduction of CCl₄ induced liver fibrosis after treatment with IS-5-MN alone or in combination with the anti-oxidant silymarin [13].

Conclusion

In conclusion, combined IS-5-MN and PZQ therapy attenuated *S. mansoni*-induced liver pathology in a mouse model by increasing NO generation, and decreasing granuloma size and number. Further studies are recommended to determine the actual pathways responsible for all different activities of IS-5-MN. Moreover, clinical studies should be carried out to reveal the real efficacy of this adjuvant therapy.

Acknowledgment

Our utmost gratitude to **Dr. Mohamed A. Sobh**, Director of the Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University, Egypt, for providing all facilities required during the study. We acknowledge the assistance of all staff at MERC, peculiarly the generous help of **Dr. Basma H. Othman** with animal experimentation.

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الملخص العربي

التأثير الوقائي لأكسيد النيتريك للكبد في البلهارسيا

المعوية التجريبية في الفئران

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من قسمي الطفيليات الطبية والباثولوجي** - كلية الطب - جامعة المنصورة.

تُعد البلهارسيا مشكلة صحية عامة رئيسية في الدول النامية، ويُعتبر البرازيكونتيل حالياً الاختيار الأمثل لعلاج البلهارسيا في الإنسان، و يُعد الأيزوسوربيد-٥ أحادي النترات من المركبات التي تحتوي على النيتروجين و الأكسجين و يتم تأييضه عن طريق الانزيمات لتحرير أكسيد النيتريك ليقوم بتأثيراته الدوائية.

و تُقيم هذه الدراسة تأثير مانح أكسيد النيتريك الأيزوسوربيد-٥ - أحادي النترات الموسّع للأوعية الدموية على البلهارسيا الكبدية، و تُحدّد ما إذا كان استعمال الأيزوسوربيد - ٥ - أحادي النترات مع البرازيكونتيل مؤازراً (مُقوياً) أو مضاداً.

- وقد تم تقسيم إناث الفئران البيضاء إلى خمس مجموعات:
- مجموعة ١: فئران غير مصابة (مجموعة ضابطة).
 - مجموعة ٢: فئران مصابة بالبلهارسيا المعوية (مصابة، غير معالجة).
 - مجموعة ٣: فئران مصابة و تمت معالجتها بالبرازيكونتيل بعد ٦ أسابيع من العدوى.
 - مجموعة ٤: فئران مصابة و تمت معالجتها بالأيزوسوربيد-٥ - أحادي النترات من الأسبوع الرابع حتى الأسبوع العاشر من العدوي (لمدة ٧ أسابيع).

- مجموعة ٥: فئران مصابة وتمت معالجتها بالأيزوسورييد-٥- أحادي النترات من الأسبوع الرابع حتى الأسبوع العاشر من العدوى (لمدة ٧ أسابيع)، بالإضافة إلى البرازيكونتيل بعد ٦ أسابيع من العدوى.

وقد تم تحديد تأثير العقارين عن طريق بعض الدراسات الطفيلية و الكيميائية الحيوية و النسيجية المرضية.

وقد أدى استعمال البرازيكونتيل إلى زيادة ذات دلالة إحصائية في نسبة البويضات الميتة، و انخفاض عدد الأورام الحبيبية و لكن لم يؤد إلى انخفاض قطرها، بينما لم يتسبب استخدام الأيزوسورييد-٥- أحادي النترات في تغيير نمط مراحل النمو المختلفة للبويضات و لكن أدى إلى تقليل الالتهاب و النخر و قطر الأورام الحبيبية، و أدى استعمال العقارين معاً إلى زيادة ذات دلالة إحصائية في مستوى أكسيد النيتريك في الكبد، و انخفاض في قطر الأورام الحبيبية، و قد تم الحصول على أفضل النتائج في المجموعة التي تلقت العقارين معاً.

و تُشير هذه الدراسة إلى إمكانية استعمال الأيزوسورييد-٥- أحادي النترات كعقار واعد بإضافته إلى البرازيكونتيل لتحسين التأثيرات المرضية للبلهارسيا على الكبد، و يُوصى بإجراء المزيد من الدراسات لاستكشاف تأثيرات تناول المتزامن للأيزوسورييد-٥- أحادي النترات مع البرازيكونتيل في المراحل المتقدمة من التليف الكبدي.