

In vivo antioxidant and antigenotoxic evaluation of an enaminone derivative BDHQ combined with praziquantel in uninfected and *Schistosoma mansoni* infected mice

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ABSTRACT

Chemotherapy with praziquantel is the cornerstone of schistosomiasis control, but an oxidative/nitrosative stress may occur after short-term treatment and participate in side effects. The aim of this study was to evaluate the antioxidant and antigenotoxic effects of the novel antischistosomal enaminone derivative, 4-hydroxyquinoline (BDHQ), alone or combined with PZQ, in hepatic tissues of uninfected and *Schistosoma mansoni* infected mice. Uninfected untreated control mice and infected mice were treated with 0 or 500 mg/kg PZQ, 600 mg/kg BDHQ, or PZQ (250 mg/kg) combined with BDHQ (300 mg/kg) for 2 consecutive days. The studied biomarkers, related to oxidative/nitrosative stress and DNA damage were significantly improved in infected mice treated with BDHQ combined with PZQ as compared to either drug alone. This amelioration was accompanied with reduction in hepatic granuloma size and histopathologic lesions. Furthermore, we documented a novel PZQ-induced mutation of hepatic mitochondrial genome in uninfected animals.

INTRODUCTION

Schistosomiasis, is a serious parasitic disease with an estimated 700 million people at risk of infection in 76 countries where the disease is considered endemic. The current mainstay of schistosomiasis control in highly endemic areas is morbidity control, and praziquantel (PZQ) has become the only available drug used in control programs for this purpose (Doenhoff *et al.*, 2008). Moreover, PZQ is considered as the drug of choice for treating all schistosome species in preventive chemotherapy which is the global strategy for schistosomiasis control (WHO, 2002). However, the extensive use of PZQ, in non-infected and non-diagnosed individuals for prevention, in higher doses in conjunction with new findings about its metabolism and genotoxic properties, necessitates further evaluation of the genotoxic and mutagenic effects of this drug. Furthermore, there is a pressing

need to develop new safe and effective drugs acting alone, or in combination with PZQ to combat the growing threat of drug-resistant parasites (El Ridi and Tallima, 2013).

The main cause of mortality and morbidity in schistosomiasis is hepatic fibrosis at chronic and advanced stages (Friedman, 2003), which develops as a result of inflammatory granulomas around deposited parasite eggs. However, PZQ alone failed to improve hepatic pathological alterations induced by schistosomiasis (El-Lakkany *et al.*, 2011). As the most severely affected organ during *Schistosoma mansoni* infection is the liver, treatment targeting schistosomiasis-associated hepatotoxicity remains a promising approach worth investigation (Abdel-Hafeez *et al.*, 2012).

A novel enaminone derivative of 4-hydroxyquinoline, BDHQ, was synthesized and previously screened for its therapeutic potential antischistosomal activity (El-Shennawy *et al.*, 2007). They proved that BDHQ exhibited major *In vivo* schistosomicidal effects against both mature and immature worms. However, the precise mechanism of action of BDHQ against schistosomiasis is still unknown.

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Although it is thought to be as a result of its role in immunologic mechanisms, which support the comparative advantage that BDHQ has over PZQ. This study revealed very encouraging results indicated that BDHQ produced statistically significant improvements of parasitological parameters and is a suitable candidate for treating schistosoma infection in a murine model. These previously reported results about the strong schistosomicidal effect of BDHQ prompted further research to investigate its potential role in prevention of hepatic damage induced by infection (El-Shennawy *et al.*, 2007).

It was suggested that generation of reactive oxygen (ROS) and nitrogen species (RNS) likely contribute to both onset and progression of *S. mansoni*-induced liver fibrosis. On the cellular level, generated free radicals can have deleterious effects on macromolecules causing peroxidation of cell lipids and DNA and protein oxidation (Muriel, 2009).

Elevated nitric oxide (NO) generation during inflammation has been found to mediate disease processes by inducing cell apoptosis in tissues, and causing damage to DNA by oxidation (Ohnishi *et al.*, 2013). Accumulation of DNA damage with time can lead to cellular gene modifications that may be mutagenic or carcinogenic.

In the context of our work, the emerging field of pharmacogenomics demands preclinical evaluations to determine which compounds are too toxic to bring into clinical testing and identify those medications that pose a risk to normal mitochondrial (mt) function (Pacheu-Grau *et al.*, 2010). Therefore, such drugs causing serious pathological mutations should be avoided. Although, the test systems used for evaluation of genotoxic potential of PZQ are diverse and the results contradictory (Montero and Ostrosky, 1997), the mutagenic effect of PZQ on mtDNA has not been studied yet. Mutagenesis of the mt genome plays a crucial role in altering mitochondria functions, leading to serious diseases (Cohen, 2010).

In addition, the noncoding displacement-loop (D loop) region in the mtDNA was found to be a mutational hotspot (Miyazono *et al.*, 2002). These findings warrant further investigations of the mutagenic potential of both drugs within mt genome, as a basis for possible clinical trials with humans (Cioli, 2000).

In several countries with major endemic infections, PZQ is not only widely available for treatment but is also being actively distributed to prevent or control disease ("morbidity control"). In high-prevalence areas, treatment is now given indiscriminately to the entire population (El Khoby *et al.*, 1998). Therefore, the purpose of this study was to evaluate the histopathological lesions, oxidative/nitrosative stress and genotoxic effects following treatment with PZQ and/or BDHQ in hepatic tissues of *S. mansoni* infected mice. In addition to study their effects on mtDNA mutations within D-loop fragment, using single strand conformation polymorphism (SSCP) technique followed by sequencing for mutant sample. For evaluating their safeness for applying in preventive chemotherapy, we aimed to

investigate the hepatic pathology in comparable groups of treated healthy mice using the same techniques.

MATERIAL AND METHODS

Chemicals and reagents

PZQ tablets (Distocide®) were supplied by Egyptian International Pharmaceutical Industries Company, EIPICO. Cremophore-El, agarose, absolute ethanol 99.5%, Tris-(hydroxymethyl)-amino methane (Tris-base), ethylenediamine-tetraacetic acid disodium salt (Na₂EDTA) and ethidium bromide (EtBr) were purchased from Sigma (St. Louis, MO, USA). All other reagents were of the highest grade commercially available.

Ethics Statement

The animal experiments were conducted in accordance with the ethical guidelines for animal handling and care as established and approved by the Ethical Research Committee of Theodor Bilharz Research Institute (TBRI), Giza, Egypt.

Animals and infection

Eighty male CD1 Swiss albino mice, average weight 20 g \pm 2, were from the Schistosome Biology Supply Center (SBSC) of the Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Mice were injected subcutaneously each with 100 \pm 10 cercariae (Egyptian strain), according to the technique described by Peters and Warren (1969).

Drug treatment

PZQ was administered to mice in its full curative dose of 500 mg/kg for 2 consecutive days (Gonnert and Andrews, 1977). BDHQ, a yellow water insoluble powder, was synthesized and characterized by our group previously (El-Shennawy *et al.*, 2007). It was given to mice in a dose of 600 mg/kg, which were also divided in half and given on two consecutive days. Both the drugs were administered 45 days post-infection (PI) in a fresh aqueous suspension of 2% Cremophor EL using stainless steel oral cannula. The dosage of each drug, in combined administration, was reduced to the half of each of their respective curative doses.

Animals were randomly allocated into 8 groups, each of 10 mice. The 1st group was infected and treated with drug vehicle (2 % Cremophor-EL), the 2nd, 3rd and 4th groups were treated with PZQ (500 mg/kg), BDHQ (600 mg/kg) and PZQ (250mg/kg) + BDHQ (300 mg/kg) respectively. Each dose for each drug was divided equally and administered on two consecutive days. Comparable groups of uninfected mice were subjected to the same treatment schedule to evaluate the safety of BDHQ and PZQ/BDHQ combination therapy. The last group was uninfected untreated control maintained in parallel with the previous seven groups. Five mice of each group were killed 47 days PI i.e., 1 day post-treatment, while the remaining 5 were sacrificed 9 weeks PI (2 weeks PT). All animals were killed by cervical dislocation. The excised livers were washed with cold phosphate buffer saline (PH 7.4), and pieces of the liver samples were fixed in 4% paraformaldehyde for histopathological examination. Other pieces

were prepared for obtaining tissue homogenate to be used for evaluating the activity of oxidative stress markers and antioxidant enzymes activity. The rest of the livers were stored at -80°C .

Worm burden

Comparable groups of infected treated mice were run in parallel with the main groups for evaluating worm burden after perfusion of the hepatic and portomesenteric vessels (Duvall and DeWitt, 1967).

Histopathology and granuloma measurements

The fixed liver specimens were dehydrated in a graded alcohol series and were processed for staining with hematoxyline-eosin (H&E) according to standard procedures. The diameter of hepatic granulomas was conducted according to a previous study as described by El-Lakkany *et al.*, (2012). Digital images were analyzed using "Image-Pro Plus 5.0" software. Granuloma structural configurations, including cellular components and associated hepatic histopathological alterations were also examined.

Estimation of lipid peroxide

The content of MDA (malonaldehyde), a compound produced during lipid peroxidation, was determined by thio barbituric acid reaction (TBARS) as described by Ohkawa *et al.*, (1979). The levels were expressed as nmoles of MDA/mg homogenate protein in liver. The protein amounts were evaluated as described by Lowry *et al.*, (1951).

Determination of tissue nitric oxide synthase (NOS) activity

Liver NOS activity was evaluated indirectly by determination of $\text{NO}_3^-/\text{NO}_2^-$ (NO_x ; nitrate/nitrite), stable end products of NO, concentrations. It was determined using Nitric Oxide Colorimetric Assay Kit (Biodiagnostic, Giza, Egypt) according to the protocol supplied by the manufacturer. The levels of total nitrite in hepatic tissues were expressed as $\mu\text{moles/mg}$ protein in liver.

Assay of enzymatic antioxidants

Superoxide dismutase (SOD) activity was assayed by the method of Nishikimi *et al.*, (1972) and Catalase (CAT) activity was measured according to method of (Aebi, 1984) (Biodiagnostic Co., Giza, Egypt).

DNA analysis

Quantization of DNA fragmentation was determined in liver according to the method described by Hickey *et al.*, (2001). The amount of DNA fragmentation was expressed as the percentage of total DNA appearing in the supernatant.

Mitochondrial DNA (mtDNA) extraction from liver tissue was based on procedures outlined previously by Ahmad and his coauthors (Ahmad *et al.*, 2007). Primer sequences (Bioneer, Seoul, Korea) for both D-loop 5' and 3' end fragments were as described by Dai *et al.*, (2005).

The final PCR products for D-loop 5' and 3' end fragments were about 342 bp and 437 bp, respectively. GoTaq® Green Master Mix (Promega, USA) was used for the polymerase chain reaction (PCR) in a 20 μl volume containing mtDNA (50–100 ng).

Denaturated PCR products were run along with the dye on a 9% polyacrylamide gel electrophoresis (acrylamide/bisacrylamide=49:1, v/v). The PCR products that showed mutation using SSCP were sequenced for detection of point mutation. Bands that abnormally shifted in the SSCP gel compared with their corresponding normal control were considered to harbor somatic mitochondrial mutations. The abnormal shifted bands were subjected to DNA sequencing and the spectrum of mutations was generated.

Analysis, for each group of animal, was repeated at least twice and in those in which a mutation was found the analysis was repeated from the original extracted mtDNA sample at least three more times.

Amplification products were purified using the QIAquick PCR purification kit (Qiagen, GmbH, Germany). Cycle sequencing of both strands was performed using the BigDye Terminator Kit version 3.1 (Applied Biosystems, Foster City, CA) on an ABI Prism 3730 Genetic Analyzer automated sequencer. Primers for sequencing are described by Dai *et al.*, (2005). Sequence data was analyzed using the Sequencher 4.1 software package (Gene Codes, MI). If the DNA sequence at a particular location in the mtDNA differed from the corresponding normal mtDNA, then it was defined as a somatic mutation.

Statistical analysis

All data are expressed as means \pm SEM. In general, the data were analyzed by two-way ANOVA followed by the Bonferroni test. Student's t test was used to test the significance of the difference between groups. *P* value of <0.05 was considered as statistically significant. All calculations were performed using GraphPad Prism software 5.01 (La Jolla, CA, USA).

RESULTS

Parasitological studies

Treatment of infected mice with BDHQ alone or combined with PZQ at 6 weeks PI resulted in a highly significant ($P<0.001$) reduction in the total worm burden; compared to the infected untreated and PZQ-treated groups, associated with a significant reduction ($P<0.001$) in the hepatic tissue egg load (Table 1).

Histopathological changes

Liver sections of untreated and PZQ + BDHQ treated uninfected mice showed normal hepatic lobular architecture with hepatocytes arranged in thin plates (Fig.1A, B). Only few degenerated hepatocytes with appearance of fat vacuoles and apoptotic bodies were observed two weeks PT in liver sections of uninfected-PZQ treated mice (Fig.1B).

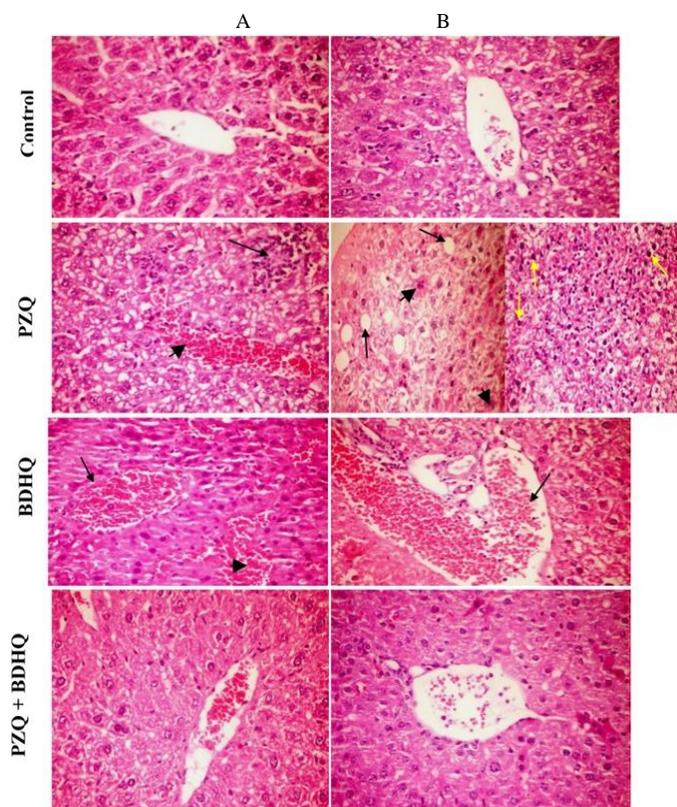


Fig. 1: Histopathological liver analysis. Sections from the liver of uninfected mice killed 1 day (A) and 2 weeks PI (B) examined by light microscopy ($\times 400$). Untreated mouse liver showing normal hepatic architecture and normal hepatocytes uninfected. Sections of mice treated with PZQ showing normal hepatocytes with congested central vein (A; arrow head), and focal area of necrosed hepatocytes infiltrated and replaced by mononuclear cell infiltration (A; arrow) and degenerated hepatocytes (B; arrows head) with appearance of fat vacuoles (B; arrows) and apoptotic bodies (b; yellow arrows). Sections of mice treated with BDHQ showing congested central vein (A; arrow), and blood sinusoids (A; arrow head) and congested hepatoportal vessels (B; arrow). Sections of mice treated with PZQ + BDHQ showing apparently healthy hepatic tissue.

Inflammatory granulomatous lesions with a typical large granuloma were mainly seen in the hepatic parenchyma and to a lesser extent in the portal tracts 7 and 9 weeks PI, in addition, to perivascular lymphocyte infiltrations focal areas of necrosis and aggregation (Fig.2A, B).

Fibrocellular granulomas constituted around 60% of the estimated granulomas with mean diameter of $218.46 \mu\text{m} \pm 26.16$ and $163.67 \mu\text{m} \pm 2.16$, at 7 and 9 weeks PI, respectively. With the progression of infection, granulomas were reduced in size with fewer inflammatory cells and more fibrous tissue content. Treatment with PZQ or BDHQ reduced both the granuloma size and inflammatory cells 7 and 9 weeks PI, compared to their corresponding infected untreated groups. Administration of PZQ and BDHQ combined resulted in a marked decrease in inflammatory cells in portal tracts and granulomas, and remarkable increase in degenerated ova within hepatic granuloma, which significantly ($P < 0.001$) decreased in diameter 9 weeks PI when compared to their corresponding PZQ treated groups (Fig.2, Table 2).

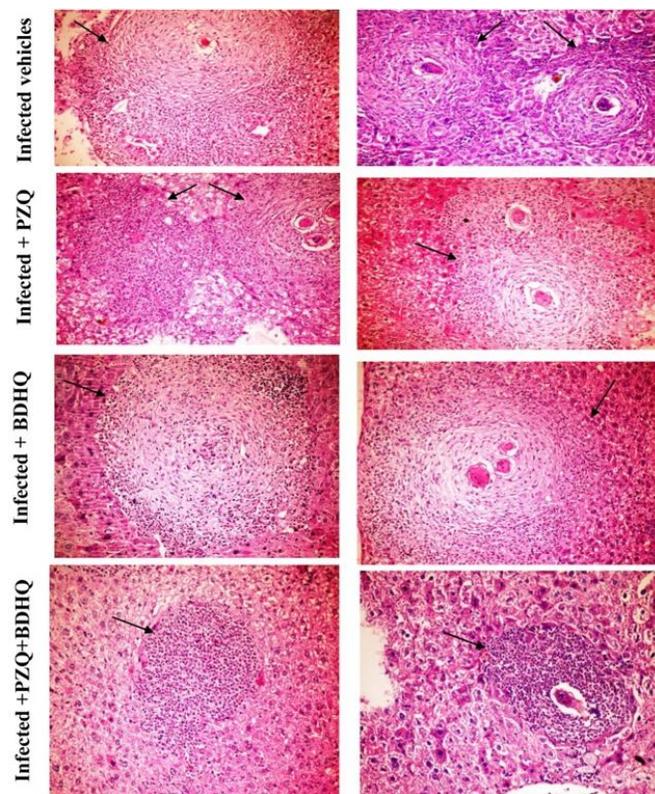


Fig. 2: Histopathological liver analysis. Sections from the liver of infected mice killed 7 (A) and 9 weeks PI (B) examined by light microscopy ($\times 400$) showing granulomas (arrows). Untreated mouse liver showing irregularly outlined large fibrocellular granuloma consisting of fibrous tissues surrounding one living intact ova with large peripheral zone of chronic inflammatory cells. Infected treated with either PZQ or BDHQ alone showing medium sized fibrocellular granuloma with starting ova degeneration. Infected treated with PZQ + BDHQ showing showing small sized fibrocellular granuloma with more ova degeneration and less inflammatory cells (A) and small well demarcated granuloma with more fibrous tissue and less inflammatory cells (B).

Evaluation of oxidative/nitrosative stress markers

Hepatic MDA and NOx concentrations, which are oxidative/nitritative stress markers, were evaluated. Treatment of uninfected mice with PZQ, BDHQ or PZQ + BDHQ doses did not alter the hepatic MDA and NOx levels (Fig. 3A and B).

Infection with *S. mansoni* significantly increased the tissue levels of MDA and NOx ($P < 0.001$) compared to the control (Fig. 3A & B). Although the MDA level was reduced in infected groups treated with either PZQ or BDHQ alone, it decreased more significantly ($P < 0.001$) with PZQ+BDHQ treatment to be close to that of the uninfected untreated control. However, the hepatic level of NOx was increased significantly ($P < 0.001$) in all infected treated groups compared to infected untreated animals. Moreover, a significant decrease in MDA level ($P < 0.01$) accompanied with a highly significant increase in NOx level was observed in PZQ+BDHQ-treated infected group compared to their respective PZQ-treated infected group.

Hepatic antioxidant enzyme activities

Treatment of uninfected animals with PZQ and/or BDHQ did not produce any significant change in the activities of SOD and CAT (catalase) compared to control animals (Fig.3C and D).

Table 1: Parasitological parameters in mice (n=5) treated with BDHQ with/without PZQ for 2 days at 7 and 9 weeks post-infection with *Schistosoma mansoni*.

Animal groups	Mean worm burden			Hepatic ova burden				
	7 weeks		9 weeks	7 weeks		9 weeks		
	Mean ± SEM	Reduction % ^a (P-value) ^b	Mean ± SEM	Reduction % ^a (P-value) ^b (P-value) ^c	Mean ± SEM	Reduction % ^a (P-value) ^b	Mean ± SEM	Reduction % ^a (P-value) ^b (P-value) ^c
Infected untreated	22.67 ± 1.20	----	25.00 ± 1.15	----	16 650 ± 77.75	----	19 226 ± 246.73	----
Infected + PZQ	3.09 ± 0.24	86.37% (<0.001)	1.32 ± 0.13	94.72% (<0.001) (NS)	7510 ± 117.82	54.89% (<0.001)	4037 ± 194.67	79.00% (<0.001) (<0.001)
Infected + BDHQ	4.71 ± 0.11	79.22% (<0.001)	1.92 ± 0.09	92.32% (<0.001) (<0.05)	5390 ± 163.79	67.63% (<0.001)	4403 ± 37.97	77.10% (<0.001) (<0.001)
Infected + PZQ + BDHQ	1.85 ± 0.27	91.84% (<0.001)	1.01 ± 0.08	95.54% (<0.001) (NS)	4120 ± 124.68	75.26% (<0.001)	2302 ± 40.25	88.04% (<0.001) (<0.001)

SEM, standard error of the mean for five mice.

^a Reduction% = [(Mean number in controls – mean number in test hosts)/mean number in controls] × 100.

^b P-value compared to infected untreated control.

^c P-value compared to their respective group at 7 weeks PI.

Table 2: Granuloma size in mice (n=5) treated with BDHQ with/without PZQ for 2 days at 7 and 9 weeks post-infection with *Schistosoma mansoni*.

Animal groups	7 weeks		9 weeks	
	Hepatic granuloma diameter (µm) ± SEM	Reduction % (P-value) ^b	Hepatic granuloma diameter (µm) ± SEM	Reduction % (P-value) ^b (P-value) ^c
Infected untreated	218.46 ± 26.16	----	163.67 ± 2.16	-----(<0.01)
Infected + PZQ	165.57 ± 0.57	24.21%(<0.01)	110.84 ± 0.31	9.6%(NS)(<0.01)
Infected + BDHQ	156.61 ± 7.07	28.31%(<0.001)	91.77 ± 3.48	31.34%(<0.001)(<0.001)
Infected + PZQ + BDHQ	140.44 ± 0.35	35.71%(<0.001)	59.83 ± 5.12	55.24%(<0.001)(<0.001)

SEM, standard error of the mean for five mice.

^a Reduction% = [(Mean number in controls – mean number in test hosts)/mean number in controls] × 100.

^b P-value compared to infected untreated control.

^c P-value compared to their respective group at 7 weeks PI.

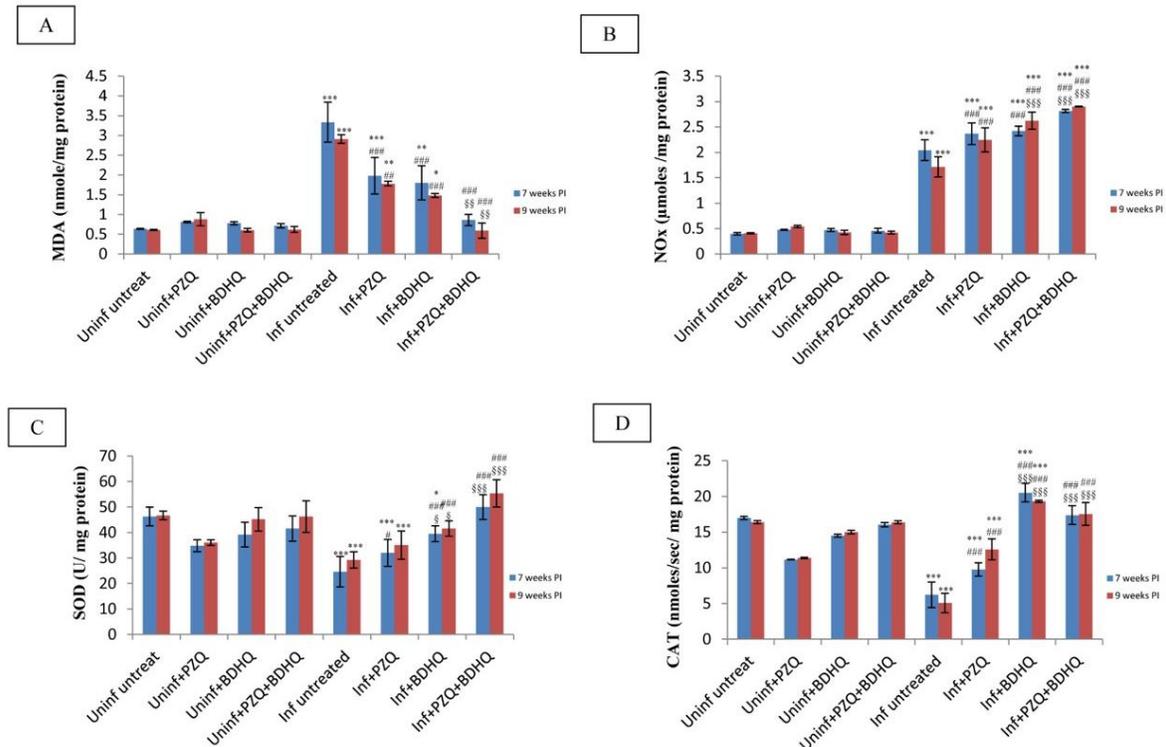


Fig. 3: Effect of BDHQ with/without PZQ on MDA (A), NOx (B), SOD (C) and CAT (D) in hepatic tissues of uninfected and *S. mansoni*-infected mice sacrificed seven and nine weeks PI (seven mice/group). Vertical bars indicate the standard error of the mean (SEM) of five mice.

* Significantly different from respective uninfected untreated group at P < 0.05, ** P < 0.01 and *** at P < 0.001. ## Significantly different from respective infected untreated (infect+veh) group at P < 0.01 and ### at P < 0.001. § Significantly different from respective infected PZQ-treated (infect+PZQ) group at P < 0.05, §§ at P < 0.01 and §§§ at P < 0.001.

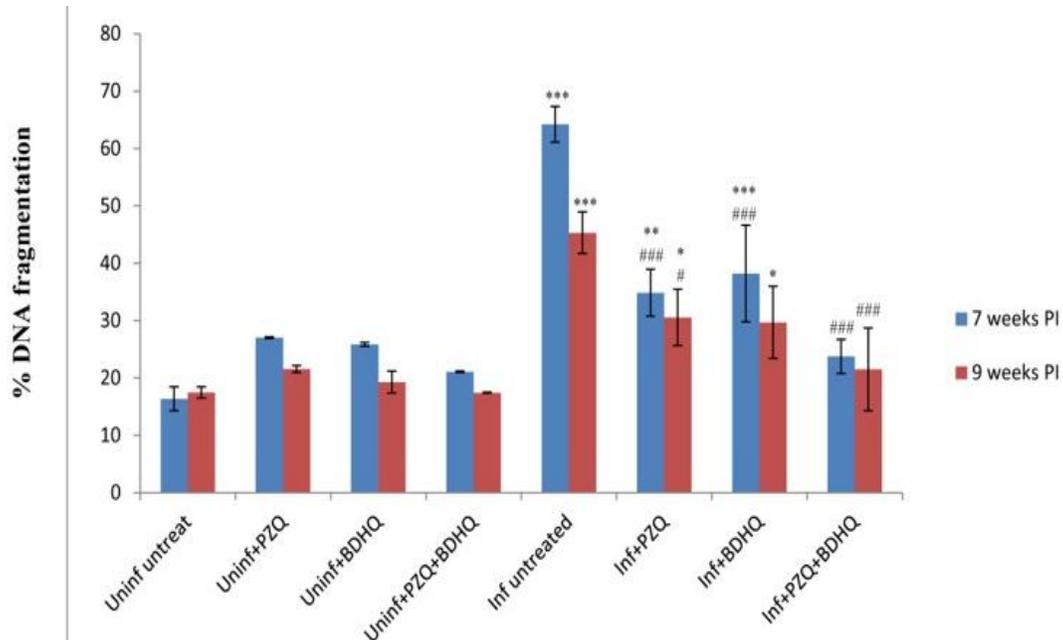


Fig. 4: Effect of BDHQ with/without PZQ on hepatic DNA fragmentation in uninfected and *S. mansoni*-infected mice sacrificed seven and nine weeks PI (seven mice/group). Vertical bars indicate the standard error of the mean (SEM). * Significantly different from respective uninfected untreated (control) group at $P < 0.05$, ** $P < 0.01$ and *** at $P < 0.001$. # Significantly different from respective infected untreated (infect+veh) group at $P < 0.05$ and ### at $P < 0.001$.

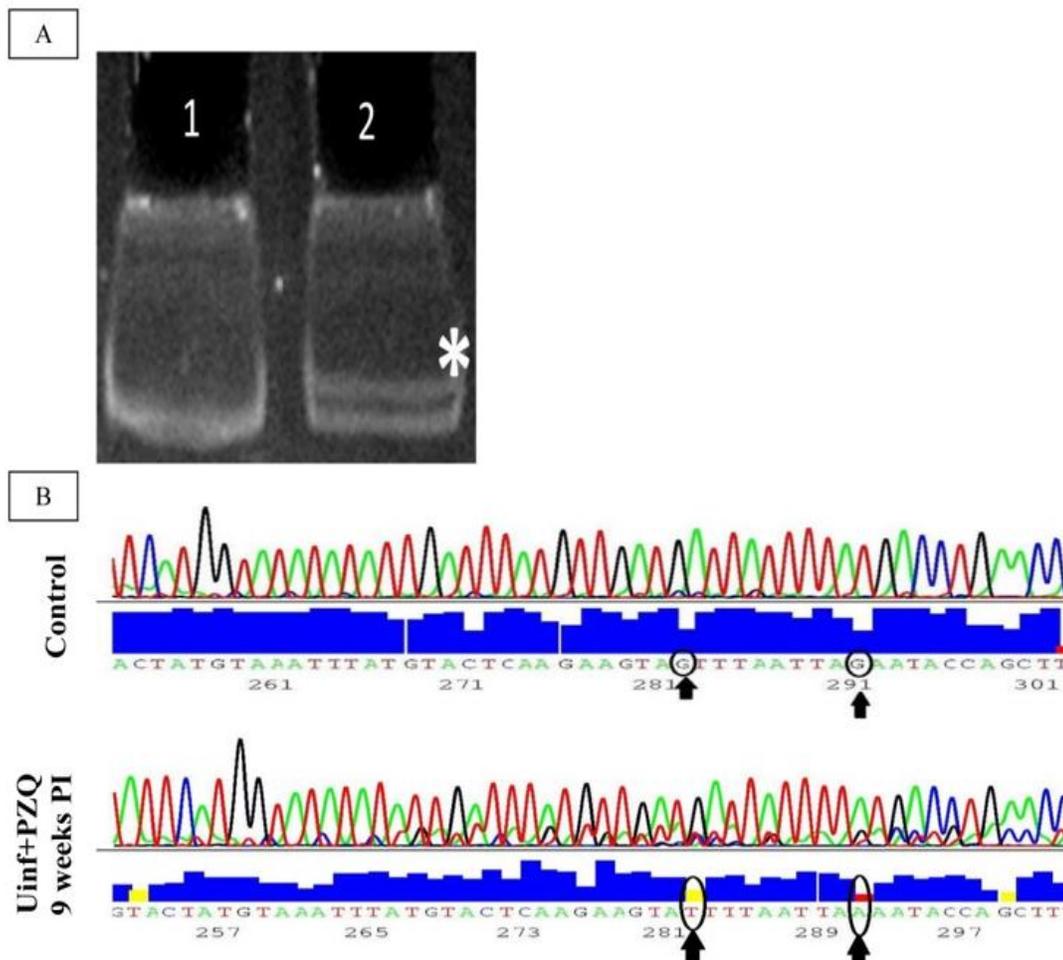


Fig. 5: (A): Representative 9% polyacrylamide gel showing PCR-SSCP pattern for D-loop 5' fragment (342 bp) with band shift (lane 2) in PZQ-treated uninfected animals 9 weeks PI, relative to that of hepatic tissues of the respective uninfected untreated control (lane 1). (B): DNA sequence chromatogram showing mutations in D-loop 5' fragment (342 bp) of hepatic mitochondrial mouse genome. The mutated bases in DNA from hepatic tissues harvested 9 weeks PI from uninfected animals treated with PZQ and its corresponding normal bases in DNA from passage-matched uninfected untreated animal are circled and indicated by arrows.

Infection of mice with *S. mansoni* produced significant reduction ($P < 0.001$) in the hepatic content of SOD and CAT enzymes when compared with their corresponding normal controls. Treatment of infected animals with PZQ led to significant elevation in the activity of SOD ($P < 0.05$) only 7 weeks PI and highly significant increase in CAT activity ($P < 0.001$) both at 7 and 9 weeks PI (Fig.3C and D). Treatment of infected mice with BDHQ alone or combined with PZQ produced a highly significant increase ($P < 0.001$) in the activities of both SOD and CAT. Combined treatment with PZQ and BDHQ resulted in a remarkable significant effect ($P < 0.001$) on both enzymes compared to that of the respective infected PZQ-treated group.

Hepatic DNA changes

Compared to untreated control mice, no significant changes in hepatic nuclear DNA fragmentation seen following different treatment of uninfected mice. (Fig.4). In contrast, *S. mansoni* infection caused highly significant ($P < 0.001$) degree of hepatic DNA fragmentation compared to the control mice. In addition, treatment with PZQ and BDHQ led to a significant decrease ($P < 0.001$) in hepatic DNA fragmentation 7 weeks PI, compared to infected untreated. Combined treatment with PZQ and BDHQ resulted in a remarkable significant ($P < 0.001$) decrease in % DNA fragmentation reaching a level close to that of control at both 7 and 9 weeks PI (Fig.4).

Hepatic mitochondrial DNA changes

The PCR products of mtDNA from hepatic tissues samples were analyzed by SSCP to identify DNA harboring mutations, which were identified as band shifts. Treatment of uninfected animals with PZQ exhibited an abnormal band migration 9 weeks PI in D-loop 5' fragment (342 bp) relative to normal tissue control (Fig.5A). No differences in the SSCP electrophoresis bands of the two D-loop fragments were observed in the other groups relative to control.

Point mutations were observed 9 weeks PI in uninfected mice treated with PZQ at nucleotide positions 15,403 (G→T) and 15,412 (G→A) of D-loop 5' fragment of hepatic mt mouse genome (Fig. 5B).

DISCUSSION

It is likely that oxidative stress is a key factor in the process of liver fibrosis. It was evidenced previously that oxygen-free radical damage leads to liver fibrosis in murine models of schistosomiasis (Muriel, 2009; Ohnishi *et al.*, 2013). Thus, new schistosomicidal drugs that ameliorate the activity of the oxidative stress system may effectively alleviate liver injury.

The marked elevation in the levels of MDA indicates enhanced lipid peroxidation leading to tissue injury and failure of the antioxidant defense mechanism to prevent the formation of excess free radicals (Muriel, 2009; Ohnishi *et al.*, 2013). The treatment with BDHQ brought back the elevated levels of MDA and documents the ability of BDHQ to prevent lipid peroxidation by acting as a good chain breaking antioxidant.

The free radical generation and accumulation of H_2O_2 during *S. mansoni* infection results in the inhibition of antiperoxidative enzymes such as SOD and CAT (Muriel, 2009; Ohnishi *et al.*, 2013). The reduced activities of these enzymes were normalized upon treatment with BDHQ with/without PZQ. The free radical scavenging effect of BDHQ supports its potent antioxidant property as evidenced previously for other 4-hydroxyquinoline derivatives (Liu *et al.*, 2002). This was associated with remarkable worm and egg reduction, reduced granuloma diameter, greater granuloma circumscription, more ova degeneration and fewer inflammatory cells. This antioxidant activity may be responsible for the less severe liver pathology observed in infected animals treated with BDHQ compared to untreated infected animals. It is well-known that the size of the granulomas decreases as the infection becomes more chronic. In the present study, we provided evidence that mice treated with BDHQ and/or PZQ showed a higher reduction in the average diameter of granulomas both 7 and 9 weeks PI.

Our data are in agreement with a previous study indicating that NO has been speculated to be a toxic substance only in the presence of potent toxic agents such as hydrogen peroxide and superoxide (Wink *et al.*, 1993). In this context, El shennawy *et al.*, (2007) reported a significant increase of interferon-gamma (IFN- γ) secreted by BDHQ-activated T lymphocytes. Furthermore, it was hypothesized that NO may be playing a host protective role in the liver at the time this organ cytokine milieu is still type 1 as shown by elevated levels of IFN- γ (Brunet *et al.*, 1999). The inflammatory response of the liver is directly affected by the parasite. Inflammation activates a variety of inflammatory cells and produce high concentrations of free radicals including RNS and ROS (Muriel, 2009; Ohnishi *et al.*, 2013). Overproduction of RNS and ROS can cause nitrate and oxidative stress which contributes to the damage of biomolecules such as DNA, RNA, lipid and proteins, leading to an increase in mutations, genomic instability, epigenetic changes, and protein dysfunction and play roles in the multistage carcinogenic process.

It was recently reported that elevated oxidative/nitrosative stress leads to fragmentation of nuclear DNA in liver, which contribute to hepatocellular apoptosis as well as necrosis (Mukhopadhyay *et al.*, 2011). In our study, we evaluated the level of DNA damage through quantification of fragmented DNA.

We reported high DNA fragmentation level quantified in hepatic tissues of infected mice potentially concurrent with the elevation in the level of oxidative/nitrosative stress parameters associated with inflammatory granulomatous reactions. Recent studies reported oxidative/nitrosative DNA damage in *S. haematobium*-associated bladder cancer supports our results of a strong correlation between *S. mansoni* infection and increased levels of nitrosative stress (Ma *et al.*, 2011). On the other hand BDHQ treatment with/without PZQ for infected animals decreased remarkably the level of fragmented DNA level compared to that treated with PZQ only coincided with the degree of lowering the oxidative/nitrosative stress.

In conclusion, BDHQ treatment alone or combined with PZQ appears to stimulate antioxidant activity within the liver resulting in reduced fibrosis following schistosome infection. Most measurements were markedly improved in the bitherapy intervention group, suggesting a synergistic effect of BDHQ with PZQ may slow progression of liver fibrosis in individuals affected by schistosomiasis. The genotoxic effects that were reported for PZQ, the reduced level of DNA damage in infected PZQ-treated animals compared to control ones, may be attributed to the elimination of the parasite and the reestablished immunological responsiveness of the host (Montero and Ostrosky, 1997).

Our results also showed that no mutations were detected in the two D-loop fragments of mtDNA of untreated as well as treated infected animals. Hence, it was postulated that mtDNA mutation seldom takes place with schistosome infection. Notably, our study revealed that the Egyptian strain of *S. mansoni* did not induce point mutations within the two selected D-loop fragments of mtDNA in the hepatic tissues of mice either with or without PZQ and/or BDHQ treatment.

In the present study, concomitant administration of BDHQ + PZQ at reduced doses exceeded the effect of the BDHQ alone, with combination therapy resulting in a very high level healing of hepatic granulomatous lesions, as evidenced by the ameliorative effects for all parameters studied. These effects are likely due to the dual actions of both the novel schistosomicidal BDHQ on the schistosomula and adult stages of the worm and the antischistosomal PZQ on the adult stages of schistosome worms. In order to evaluate the safety of BDHQ in preventive chemotherapy, we run comparable uninfected groups treated and evaluated the same as infected groups. Interestingly, no noticeable differences in the therapeutic efficacy for uninfected animals were observed between the untreated and BDHQ and/or PZQ-treated groups in all studied parameters. Yet for PZQ-treated group, that there was marked elevation in the markers of oxidative nitrosative stress along with some degree of genotoxicity and histopathological lesions. Interestingly, we proposed a novel mutations in D-loop 5' fragment in the mtDNA of hepatic tissues in uninfected PZQ-treated animals 9 weeks PI, which suggested that the D-Loop region is the main location where PZQ induced mtDNA mutations occur. Induction of mtDNA mutation in uninfected PZQ-treated groups, while not in infected treated ones, can be explained by the fact that in case of infection the action of PZQ principally acts on the parasite and, in addition, there is a possibility that schistosomiasis masked the mutagenic effect of PZQ found in mtDNA of hepatic tissues. This effect may be due to the alterations induced by the parasites and other complex variables such as biotransformation and metabolic activation of the drug within the animal tissue (Montero and Ostrosky, 1997).

In this investigation, the therapeutic efficacy of BDHQ alone or combined with PZQ was as safe as PZQ, except for the point mutation issue, with regard to all parameters studied in hepatic tissue of *S. mansoni* infected mice. These finding need more studies to elucidate the precise mechanism for this mutagenic effect performing on a wide range of doses on different time

scales. Therefore, there is an urgent need for studying the PZQ–cell interactions which might lead to a thresholded-type of dose response for mutations.

In conclusion, to best of our knowledge, this study is the first one which investigates the *In vivo* efficacy of concomitant administration of BDHQ with PZQ on improvement of hepatic pathology in schistosomiasis. The novel antischistosomal compound, BDHQ exhibited synergistic action with PZQ against hepatic schistosomiasis in murine model that is superior to the effect of each drug alone. These findings may be attributed to the different mechanism of action of both drugs that achieved the same or even higher levels of efficacy by using smaller doses of either agent, which might also result in fewer or milder side effects.

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