MAJOR ARTICLE

Inhibition of Heme Aggregation by Chloroquine Reduces *Schistosoma mansoni* Infection

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Adult *Schistosoma mansoni* digest large amounts of host hemoglobin and release potentially toxic heme inside their guts. We have previously demonstrated that free heme in *S. mansoni* is detoxified through aggregation, forming hemozoin (Hz). Possible mechanisms of heme aggregation and the effects of chloroquine (CLQ) on formation of Hz and on the viability of this parasite have now been investigated. Different fractions isolated from *S. mansoni,* such as crude whole-worm homogenates, total lipid extracts, and Hz itself promoted heme aggregation in vitro in a CLQ-sensitive manner. Treatment of *S. mansoni*–infected mice with CLQ led to remarkable decreases in total protein, Hz content, and viability of the worms, as well as in parasitemia and deposition of eggs in mouse livers. These results indicate that inhibition of formation of Hz in *S. mansoni,* by CLQ, led to an important decrease in the overall severity of experimental murine schistosomiasis. Taken together, the results presented here suggest that formation of Hz is a major mechanism of heme detoxification and a potential target for chemotherapy in *S. mansoni.*

Heme is a ubiquitous molecule found in all aerobic organisms and plays essential roles in various biological processes [1]. However, because of its ability to oxidize biomolecules, free heme is very toxic [2, 3]. Therefore, the maintenance of a low level of free heme in living cells is essential to avoid its deleterious effects [4]. A special situation is found in hematophagous organisms, which usually digest large amounts of blood, producing peptides, amino acids, and free heme. Thus, blood-feeding organisms must have very efficient protective mechanisms against heme toxicity. In *Plasmodium* parasites and in the bug *Rhodnius prolixus,* most of the free heme is detoxified by formation of a specific type of aggregate, a dark-brown crystalline material known as hemozoin (Hz) [5–8].

The blood fluke *Schistosoma mansoni* is the main causative agent of human schistosomiasis, which, among parasitoses, ranks second behind malaria in terms of public health importance, infecting ~200 million people worldwide. Adult *S. mansoni* ingest large amounts of blood to complete their developmental cycle, and hemoglobin is degraded in their gut through the action of several proteolytic enzymes [9, 10]. It has been proposed that heme is one of the end products of hemoglobin digestion in *S. mansoni* [11, 12]. However, recent studies showed that, similar to *Plasmodium* parasites and *R. prolixus,* one of the pathways of heme detoxification in *S. mansoni* is its aggregation into Hz [13, 14]. Aggregation of heme into an insoluble form in the gut of *S. mansoni,* followed by its elimination through regurgitation, appears to be the first line of defense against the toxicity of released heme.

The therapeutic effects of quinoline drugs are well described for *Plasmodium* parasites and have been extensively used in the treatment of malaria for >300

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years. However, their exact mechanism of action is not completely understood [15, 16]. It is known that quinolines affect heme-detoxification mechanisms; their association produces a complex that increases interaction of heme with membranes, enhancing its toxic effects, and several studies have also indicated that quinolines block heme aggregation in both *Plasmodium* parasites and *R. prolixus* [8, 15–18]. Because formation of Hz is a key target in malaria chemotherapy, it is conceivable that formation of Hz in *S. mansoni* could also represent a new therapeutic target in schistosomiasis. In the present study, we have investigated the possible mechanisms of Hz synthesis and its physiological role in *S. mansoni* and have examined the possible therapeutic use of chloroquine (CLQ) as an inhibitor of heme aggregation in schistosomiasis.

MATERIALS AND METHODS

Chemicals and reagents. Hemin chloride, benzamidine, soybean trypsin inhibitor, proteinase K, pepstatin, dithiothreitol, CLQ, artemisinin, quinine, quinidine, quinacrine, amodiaquine, praziquantel, Folin-phenol reagent, and MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl terazolium bromide) were purchased from Sigma Chemical. All other reagents were of analytical grade.

Parasites and animals. *S. mansoni* strain LE was maintained in *Biomphalaria glabrata* snails and in Syrian hamsters. Adult *S. mansoni* worms were obtained by mesenteric perfusion of Syrian hamsters 42 days after infection, as described elsewhere [19]. Cercariae were gathered from snails previously infected in the laboratory. Schistosomula were obtained by in vitro transformation of cercariae and were maintained for 8 days with human blood in culture, in accordance with methods described elsewhere [20]. Mice were treated in accordance with the Brazilian biosafety guidelines and were maintained in special facilities.

Hz extraction and quantification. Hz was extracted from *S. mansoni* and quantified in accordance with methods described elsewhere [13]. Protein contents of different *S. mansoni*–stage homogenates were measured by use of bovine serum albumin as a standard [21].

Lipid extraction. Total lipids were extracted in chloroform-methanol (2:1) from homogenates of adult *S. mansoni* females previously prepared in 0.15 mol/L phosphate buffer (pH 7.4). In brief, 1.0 mL of homogenate was mixed with a solution containing 4.0 mL of chloroform and 2.0 mL of methanol. The tubes were vigorously shaken for 5 min and subsequently were centrifuged at 1000 *g* for 15 min at room temperature. The organic phase was removed and dried in N_2, and the amount of lipids extracted was gravimetrically determined. The dried lipids were solubilized in 100% chloroform, and the tubes were sealed under N_2 atmosphere and kept at −20°C until the experiments were performed.

Heme aggregation assay. Unless otherwise stated, all the samples used in heme aggregation assays were obtained from recently perfused worms and were homogenized in 1.0 mL of 0.15 mol/L NaCl containing a cocktail of proteinase inhibitors, as described elsewhere [13]. After homogenization, samples were kept at −70°C until use. This material was centrifuged at 20,000 *g* for 20 min at 5°C, and the total protein content of the pellet was determined. These fractions were used to measure heme aggregation activity [8]. A sample corresponding to 20 μg of protein was incubated for 24 h at 37°C in 0.5 mol/L sodium acetate (pH 4.8), in the presence of 100 μmol/L hemin. After the incubation, the reaction mixture was centrifuged at 15,000 *g* for 15 min at 25°C. The pellet was washed 3 times with 1 mL of 0.1 mol/L $NaHCO_3$ plus 2.5% SDS (pH 9.1) and 2 times with deionized water. The final pellet was solubilized in 0.1 mol/L NaOH, and the amount of heme was determined spectrophotometrically at 400 nm. All experiments were repeated at least 3 times.

Viability assay. The viability of *S. mansoni* worms was evaluated by the reduction of MTT to formazan, as described elsewhere [22]. In brief, 6 males or females for each condition of treatment were washed in saline and incubated with 100 μL of 0.5 mg/mL MTT in 0.15 mol/L PBS (pH 7.4) for 1 h at 37°C, in a 96-well plate. After that, the supernatant was discarded, 200 μL of DMSO was added, and the plate was incubated for 30 min at room temperature. Viability was determined spectrophotometrically at 540 nm.

Treatment with CLQ. Adult Swiss mice were infected with *S. mansoni* by subcutaneous injection of living cercariae in the cervical region. Treatment with CLQ was performed according to 2 different experimental protocols. In the first protocol, mice were treated from day 42 to 49 after infection, with daily intraperitoneal (ip) injections of 50 mg/kg CLQ. In the second protocol, mice were treated from day 7 to 28 after infection, with ip injections of 60 mg/kg CLQ every other day. The worms obtained from the first protocol of treatment were collected by mesenteric perfusion at day 50 after infection, whereas the worms obtained from the second protocol were collected at day 42 after infection. The worms were separated by sex and were counted, and viability was determined. The live worms were then homogenized for determination of Hz and protein content.

Egg counting. After mesenteric perfusion, the liver of each mouse was weighed, sliced into small pieces, and subsequently digested overnight with a solution of 4.0% KOH at 37°C, in accordance with methods described elsewhere [23]. After that, 5-μL aliquots of the digested liver were placed onto a glass coverslip, and the number of eggs was determined by counting with a Zeiss stereomicroscope (Stemi SV11 MC80).

Aminotransferase assay. The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are markers of hepatocellular damage, were assayed in the

plasma of mice by use of a photometric method, as described elsewhere [24].

Statistical analysis. Comparisons between groups were done by the nonpaired Student's *t* test or 1-way analysis of variance (ANOVA) tests, by use of Microsoft Excel 2000. A posteriori Tukey's test was performed for pairwise comparisons, by use of Statistica software (Statsoft). For all tests, a difference of $P < .05$ was considered to be significant.

RESULTS

Heme aggregation induced by* S. mansoni *fractions. On the basis of previous reports that demonstrated that homogenates of *Plasmodium* parasites [18] and midgut content from *R. prolixus* [8] promote the formation of Hz in vitro, we initially investigated the capacities of soluble and particulate fractions from homogenates of *S. mansoni* males and females to promote heme aggregation. Figure 1*A* demonstrates that most of the activity responsible for formation of Hz is present in the particulate fractions from both males and females.

Considering that, during their developmental cycle, *S. mansoni* undergo profound metabolic and physiological changes, we attempted to determine the capacity of Hz synthesis at 3 different developmental stages. Figure 1*B* shows that heme aggregation activity is not uniformly present along the development of *S. mansoni,* being more intense in adult worms than in larval-stage worms (such as cercariae and schistosomula). This indicates that the capacity of heme aggregation in *S. mansoni* is regulated along the developmental cycle and may be correlated with the onset of hemoglobin digestion in the gut of the adult parasite. Moreover, the factor responsible for heme aggregation in adult females is heat labile; boiling of the particulate fraction practically abolishes the capacity to induce formation of Hz in vitro (figure 1*C*). Previous studies have shown that Hz itself can also promote heme aggregation, in an auto-catalytic reaction without the involvement of proteins from the parasite [25]. Figure 1*D* shows that heme aggregation activity induced by incubation of Hz previously isolated from adult *S. mansoni* females with free heme is heat resistant, similar to what has been described for *Plasmodium* parasites [25].

Previous studies reporting the induction of heme aggregation by total lipid extracts from *Plasmodium* parasites [26–28] led us to investigate whether total lipids isolated from *S. mansoni* would also be efficient catalysts of heme aggregation in vitro under acidic conditions. Total *S. mansoni* lipid fractions indeed induced heme aggregation in vitro (figure 1*E*). Similar to the auto-catalytic reaction mechanism described above and distinct from the heme aggregation activity promoted by total worm homogenates, such lipid extracts exhibited a heat-resistant activity; prior incubation of the extracts for 30 min at 90°C before the assay did not reduce the capacity to induce heme aggregation (figure 1*E*).

Effect of CLQ on heme aggregation in vitro. We next evaluated the effect of quinoline-derived drugs, such as CLQ and other antimalarials, on heme aggregation in vitro. Quinoline antimalarial drugs appear to exert their effects on *Plasmodium* parasites by forming a complex with heme, thus diverting it from detoxification pathways, such as heme oxygenase or formation of Hz [15]. We evaluated the effect of CLQ on heme aggregation induced by particulate fractions from adult female homogenates, by isolated Hz and by total lipid extracts. CLQ significantly inhibited heme aggregation induced by particulate fractions of *S. mansoni* female homogenates, in a dose-dependent manner, with an apparent IC_{50} of 30 μmol/L (figure 2*A*). Interestingly, CLQ also inhibited auto-catalytic Hz-induced heme aggregation (figure 2*B*), as well as heme aggregation promoted by total lipids extracted from adult females (figure 2*C*). Furthermore, the inhibition of heme aggregation was also observed with other quinoline-derived drugs, such as amodiaquine, quinacrine, quinidine, and quinine (figure 2*D*). The endoperoxide antimalarial artemisinin exhibited a less potent inhibitory activity, compared with that exhibited by quinoline-derived drugs (figure 2*D*). Interestingly, praziquantel (up to 1 mmol/L) had no effect on heme aggregation reactions in vitro induced by total female homogenates (data not shown).

Effect of CLQ on* S. mansoni *infection in mice. The next set of experiments were designed to evaluate the effect of ip injections of CLQ in vivo, by use of adult Swiss mice infected with *S. mansoni.* Two different treatment protocols were used: in the first protocol, mice were treated from day 42 to 49 after infection, with daily injections of 50 mg/kg CLQ; in the second protocol, mice were treated from day 7 to 28 after infection, with injections of 60 mg/kg CLQ every other day. These 2 protocols were designed to evaluate the effect of CLQ at different stages of development of *S. mansoni.* In the first protocol, the treatment began at day 42 after infection, when the worms were already in the adult stage (a period of intense ingestion of hemoglobin), whereas, in the second protocol, treatment began at day 7 after infection, when the worms were in the young lung stages (when the parasites begin to feed on blood). The concentration of CLQ used in these experiments was chosen on the basis of the maximum tolerated dose of CLQ (60 mg/kg/day) reported elsewhere for adult Swiss mice [29].

In the first protocol, there was a slight but not significant ($P = .19$) reduction in the Hz content of *S. mansoni* females (figure 3*A*). As reported elsewhere by us [13], the Hz content of males is very low, and CLQ did not interfere with heme aggregation (figure 3*A*). Interestingly, CLQ led to a drastic and significant ($P = .00107$, ANOVA) reduction of the viability of adult females, as assessed by the reduction of MTT (figure 3*B*). It is conceivable that an increase in the concentration of free heme due to the inhibition of Hz synthesis in vivo, by CLQ, may have led to an oxidative stress situation, which could ex-

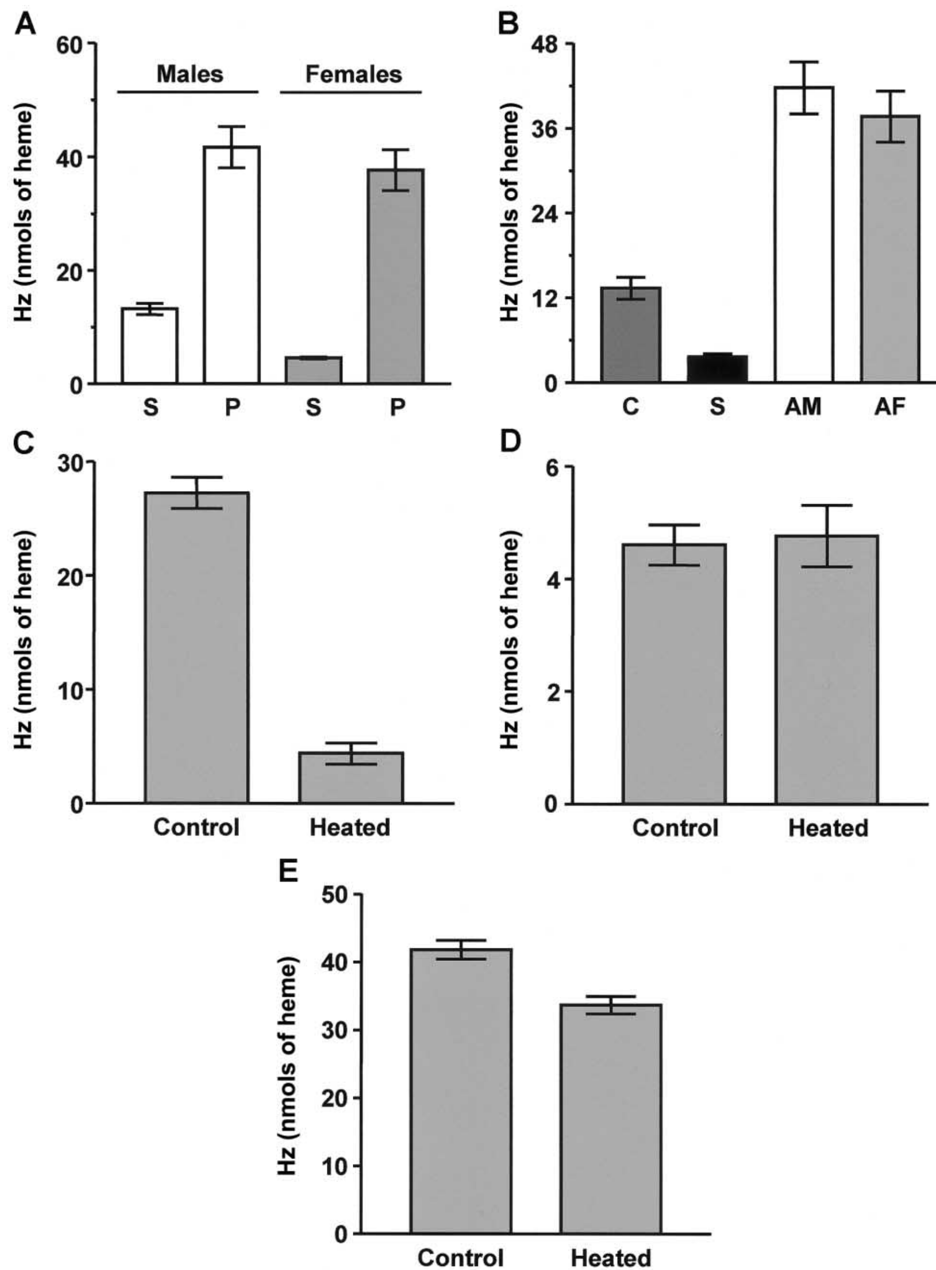

Figure 1. Characterization of heme aggregation activity in *Schistosoma mansoni. A,* Formation of hemozoin (Hz) induced by 20 μg of proteins from the supernatant (S) and particulate (P) fractions of adult male and female homogenates incubated with 100 μmol/L heme. *B,* Heme aggregation activity induced by 20 μg of proteins from P fractions obtained from cercariae (C), schistosomula (S), adult male (AM), and adult female (AF) homogenates incubated with 100 μmol/L heme. *C,* Effect of heating on Hz synthesis promoted by a total female homogenate. Hz synthesis was induced by incubation of 100 μmol/L heme with 20 μg of proteins from P fractions from females. Control, homogenate previously incubated for 30 min at 37°C; heated, homogenate previously incubated for 30 min at 90 °C. *D,* Effect of heating on the autocatalytic heme aggregation induced by Hz isolated from adult *S. mansoni* females. Hz synthesis was induced by incubation of 100 μmol/L heme with 20 nmol of isolated Hz, as described in the section on experimental procedures. Control, Hz previously incubated for 30 min at 37°C; heated, Hz previously incubated for 30 min at 90°C. *E,* Effect of heating on heme aggregation induced by total lipids isolated from adult females of *S. mansoni.* Hz synthesis was induced by incubation of 100 μmol/L heme with 80 μg of total lipids, as described in Materials and Methods. Control, total lipids previously incubated for 30 min at 37°C; heated, lipids previously incubated for 30 min at 90°C. In all the experiments, Hz was extracted and quantified as described in Materials and Methods. Results are means $\pm$ SEM ($n = 4$).

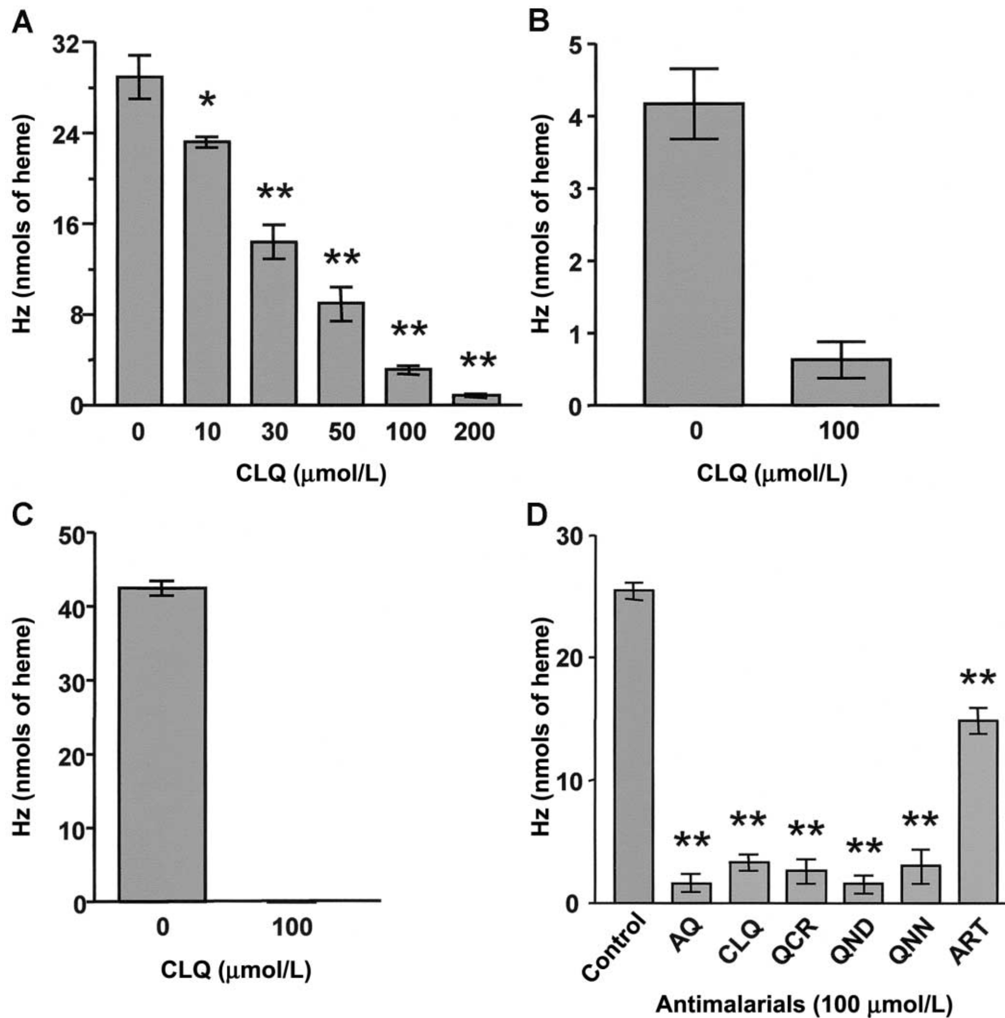

Figure 2. Inhibition of heme aggregation by chloroquine (CLQ) and other antimalarials. *A,* Heme aggregation induced by total female homogenate was inhibited by CLQ. Hemozoin (Hz) synthesis was induced by incubation of 100 μmol/L heme with the particulate fraction of female homogenate (20 μg of protein), in the presence of different concentrations of CLQ. *B,* Inhibition of autocatalytic Hz synthesis by CLQ. Hz synthesis was induced by incubation of 100 μmol/L heme with 20 nmol of isolated Hz, in the absence (control) or in the presence (CLQ) of 100 μmol/L CLQ. *C,* Hz synthesis induced by a total lipid extract from adult females was inhibited by CLQ. Hz synthesis was induced by incubation of 100 μmol/L heme with 80 μg of total lipids, in the presence of different concentrations of CLQ. *D,* Inhibition of heme aggregation by other antimalarial drugs. Hz synthesis was induced by incubation of 100 μmol/L heme with 20 μg of proteins from the particulate fraction of a female homogenate, in the absence (control) or in the presence of 100 μmol/L the following antimalarials: amodiaquine (AQ), CLQ, quinacrine (QCR), quinidine (QND), quinine (QNN), and artemisinin (ART). In all the experiments, Hz was extracted as described in Materials Methods. Results are means ± SEM ($n = 4$). *$P < .05$ and **$P < .0002$, for pairwise comparisons (1-way analysis of variance and posteriori Tukey's test).

plain the reduction in the number of viable worms. However, it is important to note that this treatment protocol had no significant effect on the total protein content of the worms, the parasitemia, the number of eggs deposited in the host liver, or the form and structure of the worms (data not shown). It is also important to note that this treatment protocol did not bring about any toxicity in the mice; the activities of the enzymes AST and ALT were not increased in the plasma of mice (data not shown).

When CLQ was administered to infected mice according to the second treatment protocol, remarkable changes in several biochemical and physiological parameters of the worms were noticed. Figure 4*A* shows that treatment with CLQ caused a significant inhibition ($P = .0060$, ANOVA) of Hz synthesis in *S. mansoni* females, whereas it did not affect Hz production in males. This treatment protocol also led to a reduction in the total protein content of worms, in which males were significantly more affected than females ($P_{\text{males}} = .00314$, ANOVA; $P_{\text{females}} = .08$, ANOVA) (figure 4*B*). When the viability of the worms was monitored by use of the MTT-reduction assay (figure 4*C*), we found that there was a small but not significant ($P = .5993$, ANOVA) reduction in viability of female worms

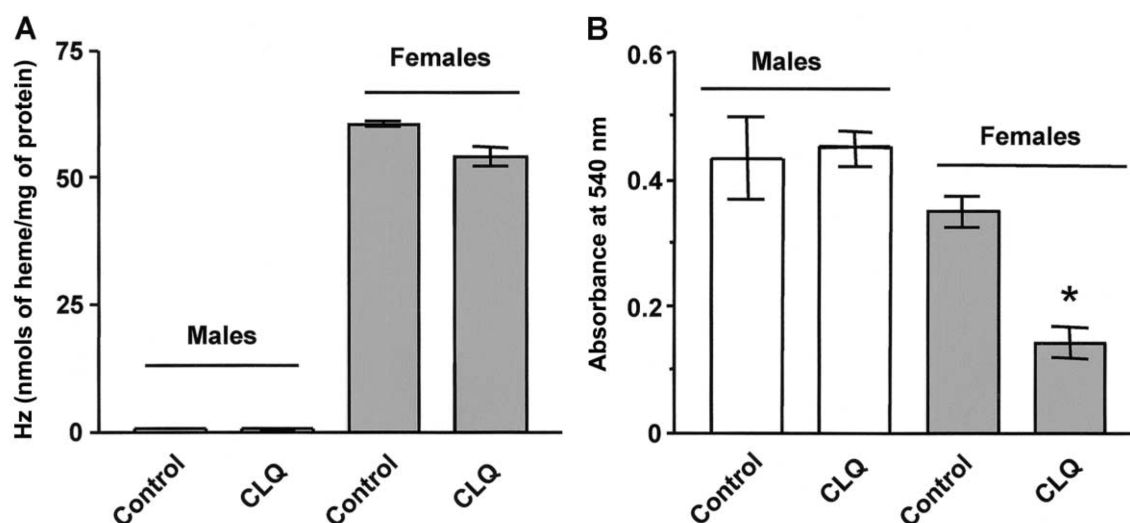

Figure 3. Effect of intraperitoneal injection of 50 mg/kg/day chloroquine (CLQ) from day 42 to 48 after infection in *Schistosoma mansoni*–infected mice. Control mice were treated with saline. *A,* Hemozoin (Hz) content in male and female *S. mansoni* worms. *B,* Viability of *S. mansoni* males and females assessed by the reduction of MTT. Results are means ± SEM ($n = 3$). $^*P < .002$, for pairwise comparisons (1-way analysis of variance and posteriori Tukey's test).

obtained from CLQ-treated mice. Of importance, figure 4*D* shows that the parasitemia was significantly affected by treatment with CLQ; the number of both male and female worms per mouse was drastically reduced ($P_{\text{males}} = .0313$, ANOVA; $P_{\text{females}} = .045$, ANOVA). The most striking result, however, was the inhibition of deposition of eggs in mouse livers. Figure 4*E* shows that CLQ significantly ($P = .005$, Student's *t* test) reduced the number of eggs deposited in the livers of infected mice, indicating that the inhibition of Hz synthesis in *S. mansoni,* by CLQ, markedly affects deposition of eggs. This treatment protocol, as well, did not result in any toxicity in the mice; the activities of 2 enzymatic markers of liver damage (AST and ALT) were not significantly ($P > .5$, ANOVA) altered after the treatment (figure 4*F*).

Figure 5*A* shows that treatment of infected mice with CLQ caused no morphological changes in adult *S. mansoni* males perfused from such mice. Remarkably, however, in female worms obtained from CLQ-treated mice, a dramatic reduction in the amount of Hz crystals inside their guts could be clearly detected (figure 5*B*).

DISCUSSION

Chemotherapy has been the main strategy for control of schistosomiasis, and praziquantel has been the drug used for this purpose for several decades [30]. However, recent reports indicate the development of resistance to praziquantel in *S. mansoni* isolated from human patients, and, for this reason, there is growing concern about the reliance on treatment with praziquantel alone [31–35]. Alternative drugs, such as arthemether, have been effectively used against *S. mansoni* [36, 37]. As an alternative to the current options in treatment, CLQ inhibits Hz synthesis in *S. mansoni,* leading to a reduction in worm burden and deposition of eggs in the liver.

Cohen et al. postulated a relationship between the antimalarial action of CLQ and its interaction with heme [38]. Other reports showed that heme is the main target of CLQ and that the CLQ-heme complex increases the affinity of heme by biological membranes, enhancing its toxic effects [17, 39, 40]. Slater and Cerami demonstrated that CLQ and other quinolines inhibit formation of Hz induced by *P. falciparum* extracts in vitro [18]. Quinolines also inhibit heme aggregation induced by different catalysts, such as histidine-rich proteins (HRPs), lipids, and Hz itself [25, 28, 41]. Previous studies showed that CLQ induces gut swelling in *S. mansoni* schistosomula, suggesting not only that this compartment has an acidic environment but also that CLQ accumulates therein [42]. Elslager et al. demonstrated that numerous hydrazine and hydroxylamine derivatives of CLQ and quinacrine were effective against *Plasmodium* parasites, but only 1 of them, a quinaldine analogue, exhibited antischistosomal effects. However, this activity was concluded to be remarkably structure specific, since several derivatives of this compound were tested, and none of them displayed significant activity against *S. mansoni* when administered at near toxic drug-diet doses to infected mice [43]. Epidemiological evidence from clinically controlled trials that treatment of malaria, in areas where both schistosomiasis and falciparum malaria are endemic, with quinoline-derived drugs affects schistosomes remains elusive. In the present study, we have demonstrated that formation of Hz is promoted by *S. mansoni* particulate fractions in a CLQ-sensitive manner. Sim-

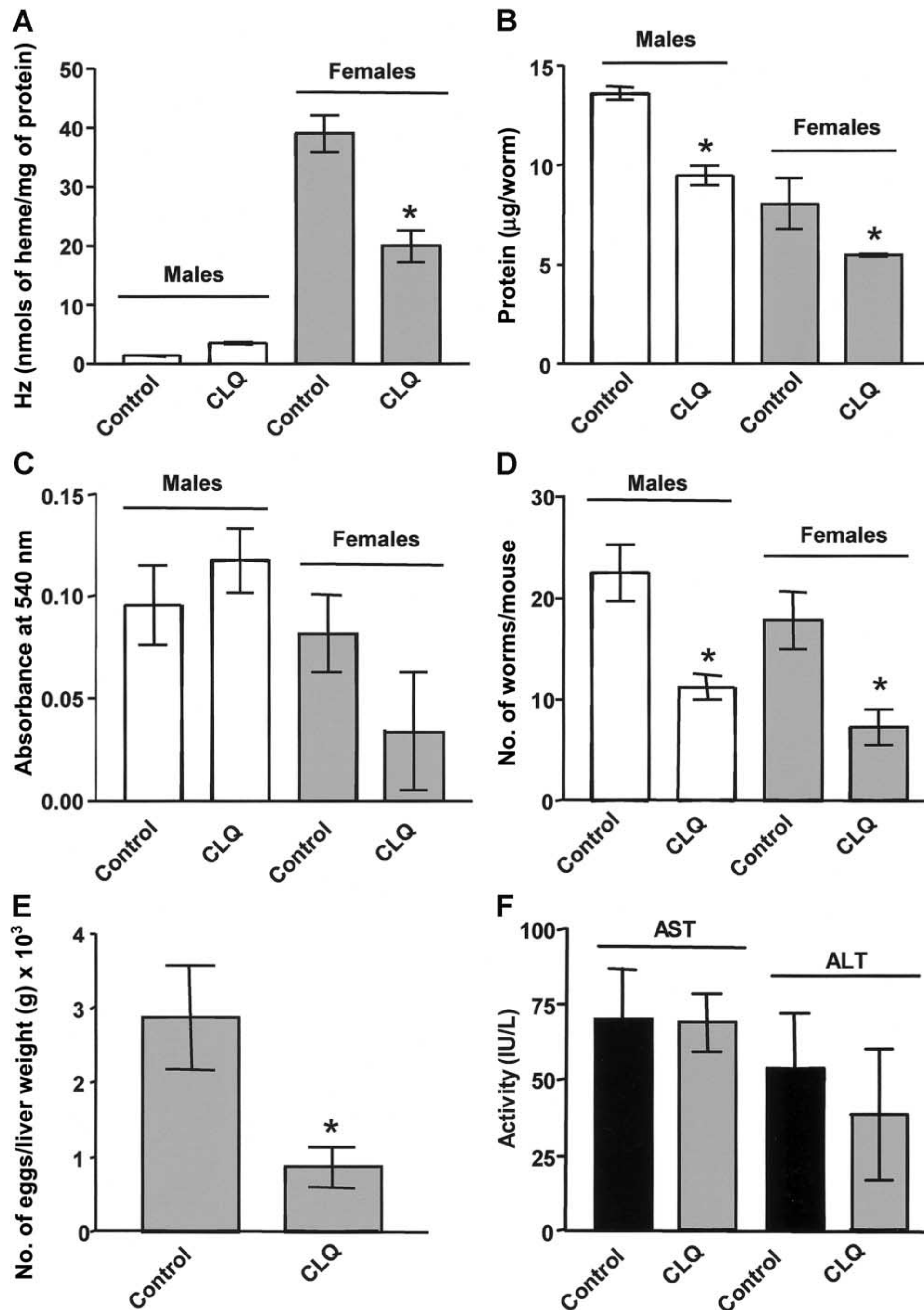

Figure 4. Effect of intraperitoneal injection of 60 mg/kg chloroquine (CLQ) every other day from day 7 to 28 after infection in *Schistosoma mansoni*–infected mice. Control mice were treated with saline. *A,* Hemozoin (Hz) content in male and female *S. mansoni* worms. *B,* Total protein content determination in *S. mansoni* males and females. *C,* Viability of *S. mansoni* males and females, assessed by the reduction of MTT. *D,* Total no. of *S. mansoni* males and females per mouse. *E,* Total no. of *S. mansoni* eggs deposited in liver parenchyma of mice. *F,* Enzymatic activities of plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of control and CLQ-treated mice. In all panels, results are means ± SEM ($n = 3$). $^*P < .05$, for pairwise comparisons between conditions (1-way analysis of variance and posteriori Tukey's test).

ilar to the activity present in *R. prolixus* midgut [8], the heme aggregation activity of *S. mansoni* is associated with particulate fractions (figure 1*A*). This indicates the involvement of membrane components in the catalysis of heme aggregation and is in contrast with the case of malaria parasites, in which soluble HRPs have been implicated in formation of Hz. Interestingly, searches for *P. falciparum* HRP-II and HRP-III sequences in the recently published *S. mansoni* transcriptome [44] did not

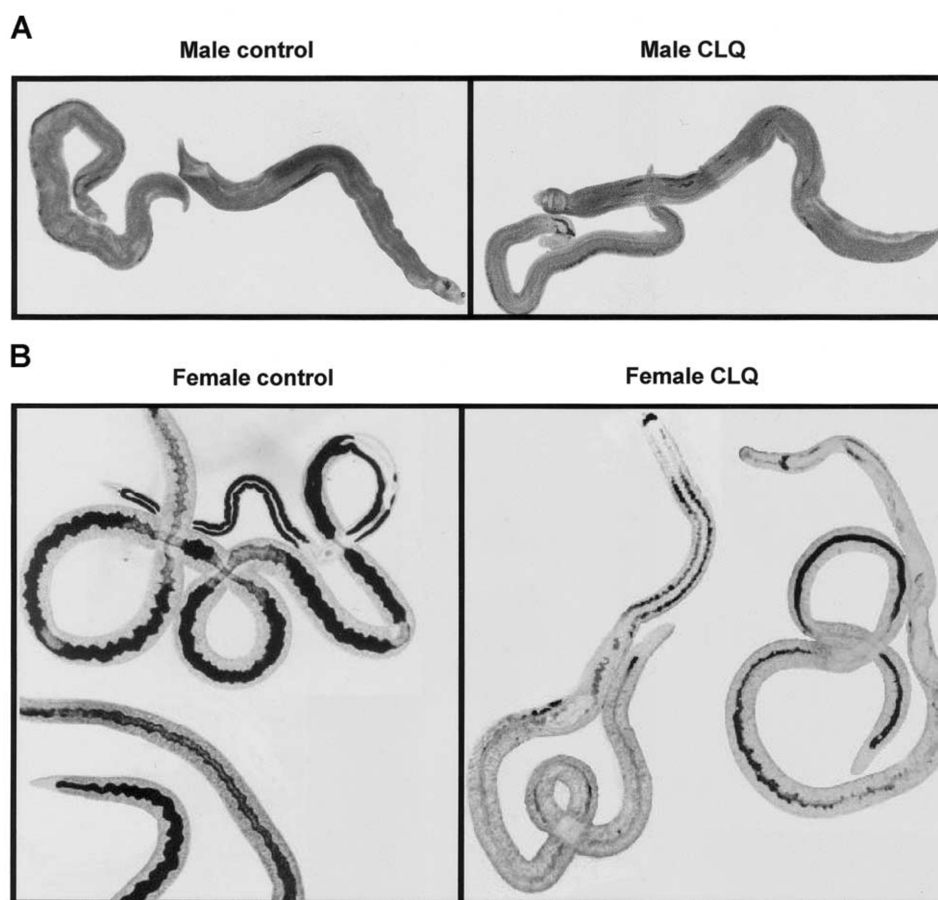

Figure 5. Effect of treatment of infected mice with chloroquine (CLQ) (60 mg/kg CLQ every other day from day 7 to 28 after infection) on form and structure of *Schistosoma mansoni.* Control mice were treated with saline. *A,* Adult males of *S. mansoni* obtained from infected mice. Four different individuals for the controls and 4 for CLQ-treated mice were observed. Original magnification, ×12. *B,* Adult females of *S. mansoni* obtained from infected mice. Five different individuals for the controls and 7 for CLQ-treated mice were observed. Original magnification, ×25. Worms obtained from control (6 pairs) and CLQ-treated (6 pairs) mice were examined and photographed with a Zeiss stereomicroscope (Stemi SV11 MC80) with Kodak Tmax film.

identify any homologous sequences (data not shown). Characterization of the components present in the membrane fraction of *S. mansoni* that could be involved in heme aggregation is currently underway in our laboratory.

Different from what has been described for *P. falciparum*, in which boiling trophozoite extracts did not affect the ability to induce formation of Hz [25], heme aggregation induced by *S. mansoni* homogenates is sensitive to heat denaturation, suggesting that at least part of this activity may be due to proteins present in *S. mansoni* gut, whereas heme aggregation driven by lipid extracts and by Hz itself was not affected by heating (figure 1*C*–1*E*). When heme aggregation induced by homogenates and total lipid extracts was compared with the autocatalytic activity of Hz, we found that the latter was significantly lower, suggesting that the autocatalytic reaction plays a secondary role as a promoter of heme aggregation. The mechanistic explanation for the induction of heme aggregation by total lipids may be based on the amphiphilic properties of free heme and its association with phospholipid membranes. It is conceivable that, in the lipid extract of *S. mansoni*, heme binds to and becomes locally concentrated in lipid micelles, providing a nucleation site from which autocatalytic synthesis continues extending Hz crystals. In line with these observations, it has been proposed that the parasitophorous vacuolar membranes are involved in formation of Hz in *Plasmodium* parasites [45].

We have shown that CLQ inhibits formation of Hz in *S. mansoni* (figure 2*A*–2*C*). The mechanism by which CLQ inhibits formation of Hz in *S. mansoni* has not been completely elucidated, but it is possible that CLQ binds free heme, which is diverted from the pathway of formation of Hz. Besides CLQ, other quinolines also inhibited heme aggregation induced by *S. mansoni* homogenates, whereas artemisinin was not as effective (figure 2*D*). Praziquantel did not inhibit formation of Hz in vitro, further suggesting that a combination of CLQ, or

other quinolines, with praziquantel may constitute an interesting therapeutic strategy, since these drugs seem to affect different targets in the parasite.

Treatment of experimentally infected mice with CLQ caused drastic alterations to the worms. A discrete reduction in Hz content and a drastic decrease in viability were observed only in female worms subjected to the first treatment protocol (figure 3*A* and 3*B*). It is possible that a slight reduction of heme aggregation in *S. mansoni* females, by CLQ, would be sufficient to produce relatively low levels of free heme, which would be enough to exert toxic effects on the parasites, leading to a reduction in viability of the females. Nevertheless, in this treatment protocol, total worm protein levels, parasitemia, and deposition of eggs in mouse livers were not affected by CLQ (data not shown). A second treatment protocol, performed from day 7 to 28 after infection, with ip injections of 60 mg/kg CLQ every other day, was also used. This approach resulted in a drastic reduction in Hz content, specifically in females, indicating that females are more susceptible to treatment with CLQ than are males. In addition, protein content in the CLQ-treated worms was reduced, suggesting that inhibition of heme detoxification mechanisms by CLQ affects essential metabolic pathways in *S. mansoni* (figure 4*B*). This treatment also resulted in an expressive reduction of viability of adult females, whereas males were not affected (figure 4*C*). Severe inhibition of Hz synthesis in vivo should be toxic to the parasites, because of increased levels of free heme and the consequent membrane damage and generation of free radicals [2–4]. Alternatively, accumulation of CLQ in the gut of *S. mansoni* might cause an increase in the pH of the digestive tract, leading to the inhibition of proteolytic enzymes and a reduction in hemoglobin digestion and heme release [46]. Whatever the precise mechanism involved, treatment with CLQ, according to this second protocol, resulted in a marked reduction of total parasitemia of infected mice (figure 4*D*). The most dramatic result of treatment with CLQ was related to deposition of eggs in the liver. Differences in the number of liver granulomas between control and CLQ-treated mice were observed after removal of the liver from the mice and were confirmed by alkaline digestion of the liver followed by egg counting [23]. This result has significant implications with regard to the treatment of the disease, since the eggs are the causative agents of the pathological deviations associated with schistosomiasis.

The results presented here indicate that, together with other mechanisms, formation of Hz in *S. mansoni* represents an important defense against heme toxicity. In conclusion, formation of Hz in *S. mansoni* can be inhibited by CLQ and possibly by other quinolines in vivo. Therefore, therapies based on the use of CLQ alone or in combination with other quinolines or other antischistosomal agents may offer new avenues for the control and treatment of human schistosomiasis. In this regard, it has been demonstrated that Hz is significantly less harmful as a pro-oxidant than is free heme [47]. In addition, the recently available transcriptomes of both *S. mansoni* and *S. japonicum* [44, 48] may reveal potential new targets enabling rational drug-design approaches directed not only against formation of Hz but also against other mechanisms involved in heme detoxification.

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