Modulation of the antioxidant defence in different developmental stages of *Schistosoma mansoni* by praziquantel and artemether

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ABSTRACT

Human schistosomiasis is a chronic and debilitating parasitic disease caused by parasitic trematode worms (schistosomes). Praziquantel (PZQ) is the drug of choice as it is active against all *Schistosoma* species, can be administered easily, has high cure and egg reduction rates, with no or only mild side effects. Rapid re-infection following treatment and the concerns about PZQ resistance has led to the search for new drugs to treat schistosomiasis. Significant progress has been made with artemisinin derivatives (e.g., artemether [ART]) that are used for chemoprophylaxis. This present study aims to look at the effects of ART and PZQ on the antioxidant defence of immature (three-week-old) and mature (six-week-old) stages of *S. mansoni*. The possible development of time- or concentration-dependent changes in oxidative stress is assessed by incubation with different sublethal drug concentrations (50, 75, 100 ng/mL for both ART and PZQ) and different time periods (one and three hours). The results indicated a time- and concentration-dependent depletion of glutathione (GSH), which was greater in the immature worms after incubation with ART. On addition of ART to the incubation medium of mature and immature worms, elevation in lipid peroxidation (TBARS) level was observed, which was time- and concentration-dependent, and more prominent in the immature schistosomes. Addition of PZQ to the incubation medium containing the immature schistosomes did not have a significant effect on TBARS level, except after three hours' incubation with the highest concentration used; however, a significant rise was seen in the mature worms. The PZQ had no effect on the activities of superoxide dismutase (SOD), glutathione peroxidase (tGPx, sGPx and nGPx) and glutathione transferase (GST) in mature or immature worms. While ART induced SOD activity in mature worms, it induced tGPx, nGPx and GST activities in a time- and concentration-dependent manner in both mature and immature worms. Activation was more prominent in the immature schistosomes. The results of the present study indicate that the immature schistosomes are more prone to oxidative killing, which probably participates in the mechanism of antischistosomal action of ART against the immature stage of *S. mansoni*. The results suggest that the mechanism of schistosomicidal action of PZQ is probably not substantially dependent on oxidative stress or oxidative killing.

KEY WORDS: Artemether. Oxidative stress. Praziquantel. Schistosoma mansoni.

Introduction

Among parasitic diseases, schistosomiasis ranks second only to malaria in terms of socioeconomic and public health importance. Globally, it is estimated that about 200 million subjects are afflicted with this disease, and more than 600 million are at risk of infection. A multifaceted approach comprising health education, sanitation and snail control has been used to control schistosomiasis.[1] Despite reported progress in vaccine development,[2] this area does not appear to offer the promise of a field-proven product in the near future. Accordingly, chemotherapy and chemoprophylaxis are main avenues available currently to combat the disease.

At present, praziquantel (PZQ) is the drug of choice for the treatment of schistosomiasis. Chemotherapy with PZQ is highly effective in reducing morbidity but fails to prevent re-infection.[3] It has high cure and egg-reduction rates with only mild side effects,[4] but suffers from two serious drawbacks. First, it is active only against the adult stages of the worm, which means that it exerts its effect after sexual maturation and oviposition. Despite parasitological cure, the patient may still suffer from the consequences of the progress of disease pathology. Second, a series of laboratory studies and clinical trials have raised concerns about the possible development of tolerance and/or resistance to praziquantel.[5]

These two serious problems have stimulated active search for an effective schistosomicidal agent that does not have the handicaps of praziquantel. Recently, derivatives of artemisinin have been shown to exhibit antischistosomal activity. Significant progress has been made with artemether (ART), the methyl ether derivative,[6] which is especially active against immature schistosomes.[7]

The schistosomes are most susceptible to immune elimination before sexual maturity and least susceptible as adults.[8] One mechanism that cells may employ to kill parasites is the release of toxic molecules and oxygen

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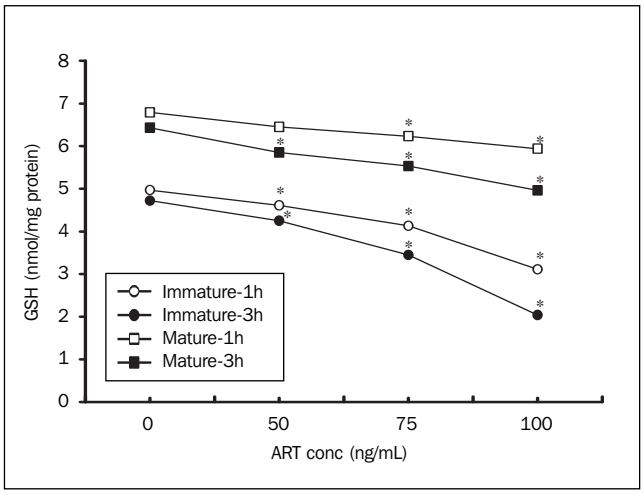

Fig. 1. Changes in GSH following incubation of immature and mature *S. mansoni* worms in culture media containing ART for one and three hours. *Significantly different from baseline values by ANOVA ($P<0.05$).

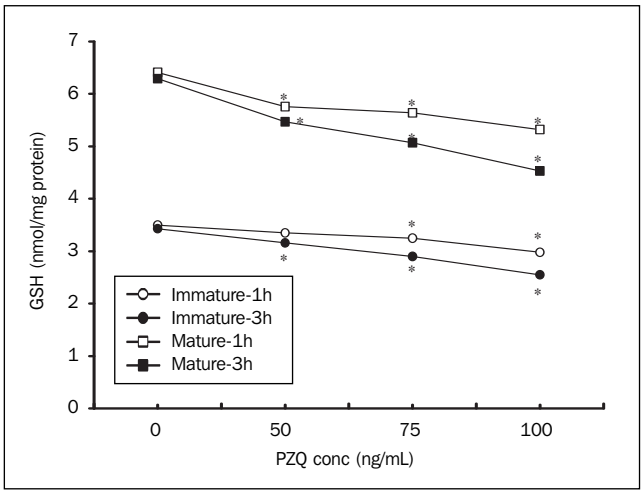

Fig. 2. Changes in GSH following incubation of immature and mature *S. mansoni* worms in culture media containing PZQ for one and three hours. *Significantly different from baseline values by ANOVA ($P<0.05$).

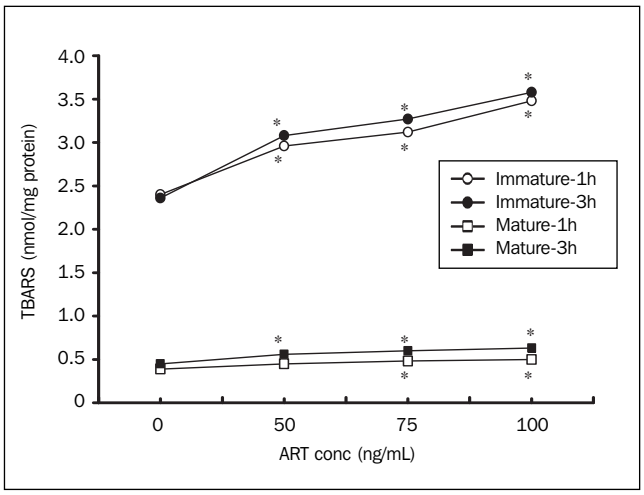

Fig. 3. Changes in TBARS following incubation of immature and mature *S. mansoni* worms in culture media containing ART for one and three hours. *Significantly different from baseline values by ANOVA ($P<0.05$).

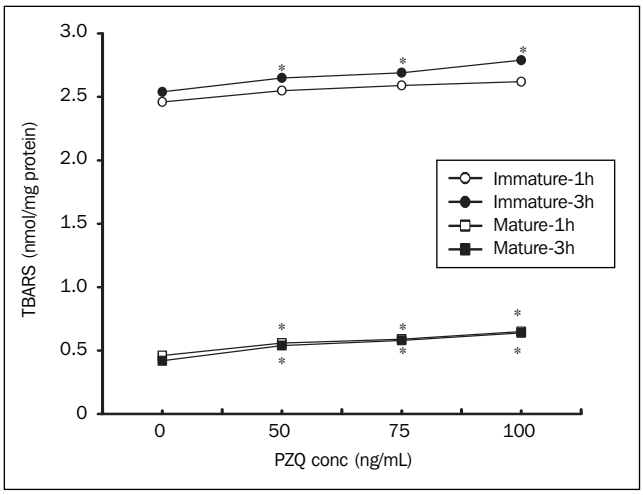

Fig. 4. Changes in TBARS following incubation of immature and mature *S. mansoni* worms in culture media containing PZQ for one and three hours. *Significantly different from baseline values by ANOVA ($P<0.05$).

radicals,[9–11] the latter being able to attack the parasite's lipid membrane and destroy its integrity. However, the schistosomes have evolved a number of immune evasion mechanisms.[12] One such mechanism is an antioxidant defence. Also reported is a stage-specific difference in the susceptibility of immature schistosomes and adult *S. mansoni* to killing by cell free generated oxidants.[13]

The aim of the present study is to assess the effects of praziquantel and artemether on the antioxidant defences in the immature and mature stages of *S. mansoni* worms.

Materials and methods

The study was performed on immature (three-week-old) and mature (six-week-old) *S. mansoni* worms provided by the Institute of Theodor Bilharz, Cairo. These worms were recovered from infected hamsters by liver perfusion. The worms were suspended in RPMI 1640 medium at 37°C. Each group of worms was divided into two subgroups, which were exposed to ART or PZQ separately.

Four sets of incubates were prepared similarly whereby 160 immature worms were transferred into each well of a culture plate. In two sets, ART was added to the incubation medium, while PZQ was added to the other two sets to give final concentrations of 50, 75 or 100 ng/mL in the individual wells. Each concentration was run in duplicate. One set each of ART and PZQ incubates were incubated for 1 h and the others for 3 h at 37°C in a humid atmosphere of 5% CO_2. Each experiment was repeated three times. Similar incubations were carried out with 50 pairs of mature worms.

At the end of the incubation period, the worms were washed (x2) and then homogenised in phosphate-buffered saline (PBS). The homogenates were used to determine reduced glutathione (GSH)[14] and malondialdehyde as

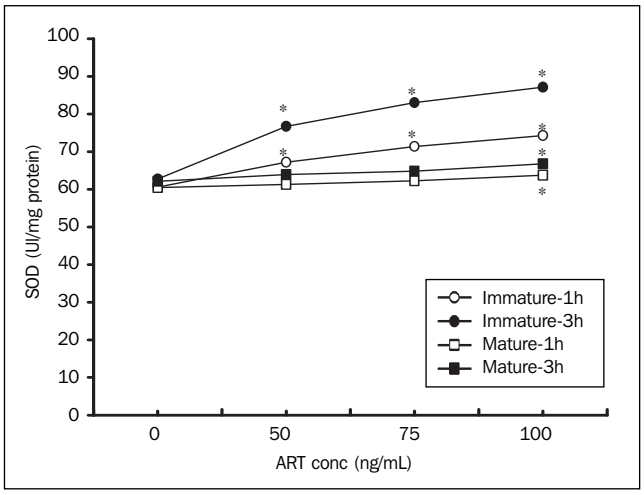

Fig. 5. Changes in SOD activity following incubation of immature and mature *S. mansoni* worms in culture media containing ART for one and three hours. *Significantly different from baseline values by ANOVA ($P<0.05$).

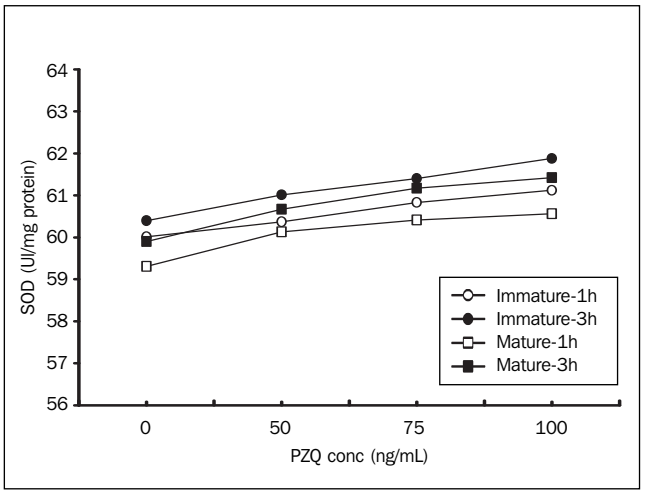

Fig. 6. Changes in SOD activity following incubation of immature and mature *S. mansoni* worms in culture media containing PZQ for one and three hours.

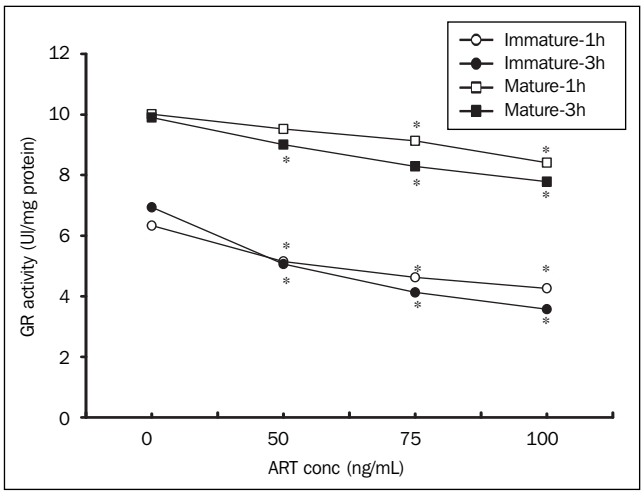

Fig. 7. Changes in GR activity following incubation of immature and mature *S. mansoni* worms in culture media containing ART for one and three hours. *Significantly different from baseline values by ANOVA ($P<0.05$).

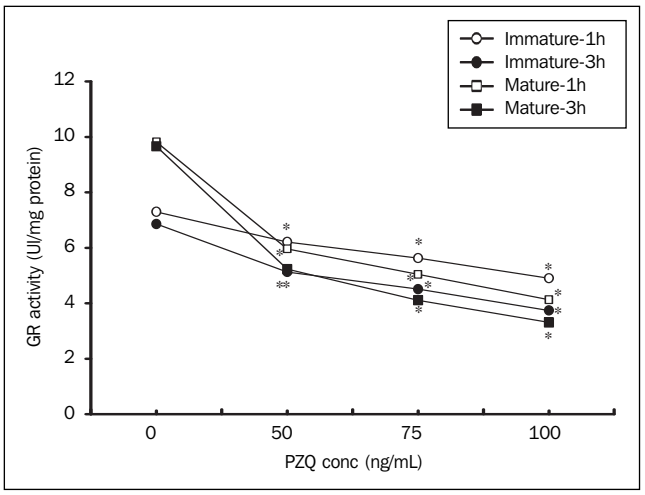

Fig. 8. Changes in GR activity following incubation of immature and mature *S. mansoni* worms in culture media containing ART for one and three hours. *Significantly different from baseline values by ANOVA ($P<0.05$).

thiobarbituric acid reactive substances (TBARS),[15] as well as the activities for SOD,[16] total and selenium-dependent glutathione peroxidase, glutathione-S-transferase (GST)[18] and glutathione reductase.[19] A modification of the method of Lowry *et al*.[20] was used to determine protein level in the samples.

In planning the series of experiments, it was decided to use *in vitro* incubation of worms, which would give the direct effect of the drugs without input from the host. Incubation was carried out in RPMI 1640 medium because decomposition of artemether to free radical reaction products proceeds more quickly in this medium than it does in other media suitable for the incubation of worms.[14]

All data are presented as mean ± standard deviation (SD). All analyses were carried out using SPSS software. One-way ANOVA was used to assess differences, and $P<0.05$ was considered to be statistically significant.

Results

The level of GSH in mature worms was 36–83% higher than that in immature worms. On addition of the drugs, GSH was depleted in a time- and concentration-dependent manner (Figs. 1 and 2). The effect was greater in the immature worms after incubation with ART (Fig. 1), while it was relatively higher in the mature worms after incubation with PZQ (Fig. 2).

Markers for oxidative stress include depletion of GSH and elevated TBARS levels. The average TBARS level in cultured immature schistosomes was about five times higher than that in adult schistosomes. After addition of ART to the incubation medium, elevation in TBARS was time- and concentration-dependent and was more prominent in the immature than in the mature schistosomes (Fig. 3).

Addition of PZQ to the incubation medium of immature schistosomes did not have a significant effect on TBARS level

Table 1. Effect of artemether on the activities of glutathione peroxidases and GST in immature *S. mansoni* worms.

	Concentration of ART			
	0	50 ng/mL	75 ng/mL	100 ng/mL
One-hour incubation				
tGPx mU/mg protein	3.60±0.09	3.84±0.08	4.18*±0.29	4.46*±0.13
sGPx mU/mg protein	1.50±0.08	1.52±0.05	1.54±0.04	1.55±0.10
nsGPx mU/mg protein	2.10±0.01	2.32*±0.03	2.64*±0.25	2.91*±0.04
GST U/mg protein	0.058±0.002	0.062±0.005	0.069*±0.002	0.073*±0.002
Three-hour incubation				
tGPx mU/mg protein	3.73±0.11	4.35*±0.20	4.57*±0.05	4.97*±0.16
sGPx mU/mg protein	1.63±0.04	1.66±0.05	1.69±0.05	1.71±0.03
nsGPx mU/mg protein	2.10±0.08	2.69*±0.16	2.88*±0.04	3.26*±0.13
GST U/mg protein	0.064±0.001	0.070*±0.002	0.080*±0.004	0.085*±0.003

Data presented as the mean±SD.

*Significantly different from baseline values ($P<0.05$).

Table 2. Effect of artemether on the activities of glutathione peroxidases and GST in mature *S. mansoni* worms.

	Concentration of ART			
	0	50 ng/mL	75 ng/mL	100 ng/mL
One-hour incubation				
tGPx mU/mg protein	26.00±0.33	26.65±0.09	28.17*±0.12	29.96*±0.33
sGPx mU/mg protein	11.40±0.12	11.43±0.13	11.51±0.10	11.56±0.11
nsGPx mU/mg protein	14.60±0.25	15.22*±0.04	16.66*±0.11	18.40*±0.22
GST U/mg protein	0.065±0.001	0.066±0.002	0.069*±0.003	0.071*±0.002
Three-hour incubation				
tGPx mU/mg protein	26.87±1.14	28.40*±1.09	31.17*±0.52	33.27*±0.43
sGPx mU/mg protein	11.57±0.13	11.65±0.11	11.72±0.14	11.81±0.11
nsGPx mU/mg protein	15.30±1.03	16.75*±1.00	19.45*± 0.44	21.46*±0.33
GST U/mg protein	0.072±0.001	0.075±0.002	0.078*±0.002	0.083*±0.003

Data presented as the mean±SD.

*Significantly different from baseline values ($P<0.05$).

except after three hours' incubation with the highest concentration used (100 ng/mL). In the mature worms this drug caused a significant rise in TBARS level (Fig. 4).

The similarity in SOD activity in the immature and mature worms, observed in the present study, indicates that SOD reaches its full expression and activity early in the life of *S. mansoni* (Figs. 5 and 6). Addition of PZQ to the incubation medium had no effect on SOD activity in either mature or immature schistosomes (Fig. 6). Although ART did not affect the SOD activity in the mature schistosomes, it did induce this enzyme in immature worms, with the increase in activity depending on drug concentration and duration of incubation (Fig. 5).

The present results show that tGPx activity in the immature schistosomes was only a fraction (14%) of that seen in the mature worms. However, tGPx activity showed the typical time- and drug concentration-dependent increase following incubation with ART (Tables 1 and 2). This increase was more apparent in the immature schistosomes. The activity of tGPx did not show any change following incubation with PZQ over the concentration range used (Tables 3 and 4).

The activity of sGPx and nsGPx in mature worms was seven times higher than that seen in the immature schistosomes. Incubation of either immature or mature stages of *S. mansoni* with ART or PZQ had no effect on sGPx activity (Tables 1–4); however, nsGPx activity was increased by addition of ART in a time- and concentration dependent manner in both immature and mature worms. Activation was more prominent in the immature schistosomes (Tables 1–4)

Activity of GST increases during maturation and incubation of immature worms with ART resulted in an increase in activity, which was almost the same as that seen in the mature schistosomes (Tables 1 and 2). Such changes were not seen following incubation with PZQ (Tables 3 and 4).

In contrast to other enzymes in the *S. mansoni* glutathione-related antioxidant system, GR was inhibited by incubation with either ART or PZQ in both immature and adult worms.

Table 3. Effect of praziquantel on the activities of glutathione peroxidases and GST in immature *S. mansoni* worms.

	Concentration of PZQ			
	0	50 ng/mL	75 ng/mL	100 ng/mL
One-hour incubation				
tGPx mU/mg protein	3.23±0.16	3.25±0.07	3.29±0.14	3.31±0.12
sGPx mU/mg protein	1.47±0.11	1.48±0.06	1.50±0.05	1.51±0.07
nsGPx mU/mg protein	1.76±0.06	1.77±0.03	1.79±0.09	1.80±0.06
GST U/mg protein	0.056 ±0.002	0.057±0.001	0.059±0.003	0.06±0.003
Three-hour incubation				
tGPx mU/mg protein	3.43±0.06	3.47±0.06	3.53±0.12	3.56±0.014
sGPx mU/mg protein	1.60±0.04	1.62±0.05	1.65±0.03	1.66±0.03
nsGPx mU/mg protein	1.83±0.03	1.85±0.02	1.88±0.08	1.90±0.12
GST U/mg protein	0.061±0.003	0.063±0.004	0.065±0.005	0.066±0.003

Data presented as the mean±SD.

Significantly different from baseline values by ANOVA ($P<0.05$).

Table 4. Effect of praziquantel on the activities of glutathione peroxidases and GST in mature *S. mansoni* worms.

	Concentration of PZQ			
	0	50 ng/mL	75 ng/mL	100 ng/mL
One-hour incubation				
tGPx mU/mg protein	26.33±0.67	26.60±1.22	26.87±0.95	27.03±0.44
sGPx mU/mg protein	11.43±0.12	11.51±0.10	11.58±0.19	11.62±0.23
nsGPx mU/mg protein	14.90±0.56	15.09±1.13	15.29±0.76	15.41±0.30
GST U/mg protein	0.068±0.001	0.069±0.003	0.071±0.001	0.072±0.004
Three-hour incubation				
tGPx mU/mg protein	26.80±0.99	27.26±2.18	27.67±1.59	27.87±1.03
sGPx mU/mg protein	11.53±0.11	11.63±0.11	11.71±0.16	11.76±0.14
nsGPx mU/mg protein	15.27±0.88	15.63±2.08	15.96±1.45	16.11±0.92
GST U/mg protein	0.074±0.003	0.076±0.001	0.079±0.004	0.080±0.004

Data presented as the mean±SD.

Significantly different from baseline values by ANOVA ($P<0.05$).

In the immature worms, the degree of inhibition was similar with both drugs. In contrast, greater inhibition of this enzyme in the adult schistosomes was seen with PZQ (Figs. 7 and 8)

Discussion

An overview of the oxidative stress status and antioxidant defence in sexually immature and mature *S. mansoni* incubated *in vitro* with sublethal concentrations of ART or PZQ showed that the antioxidant defence in the immature worms was inferior to that of the mature stage. Thus, immature schistosomes are more prone to oxidative killing, which probably participates, at least partly, in the mechanism of antischistosomal action of ART against the immature stage of *S. mansoni*. The results also suggest that the mechanism of schistosomicidal action of PZQ is not substantially dependent on oxidative stress or oxidative killing.

Adult worms contain significantly greater amounts of oxidant scavengers,[22] especially glutathione, which is a measure of protective thiol groups. The present study showed that the mature schistosomes had GSH concentrations higher than those in immature worms.

Incubation of the mature and immature schistosomes with sublethal concentrations of PZQ or ART caused an increase in oxidative stress, presenting as decreased levels of GSH and increased levels of TBARS. Studies show that ART, which has a peroxide group in its chemical structure, exhibits the highest activity against juvenile stages of parasites, while adult worms are significantly less susceptible.[6] On the other hand, PZQ acts against adult worms[7] and mature eggs in host tissues.[23] It has been proposed that GSH might play an important role in the defence of the worms against ART-generated toxic peroxides and free radicals.[24]

The observed elevation in TBARS, following addition of ART to the incubation medium, bears special significance because these toxic oxidation products are usually neutralised rapidly by intracellular free radical scavengers

such as GSH. The concentrations of carbonyl end products of lipid peroxidation increased with the increase in ART concentration or with the prolongation of the time of incubation. The results obtained following addition of PZQ to the incubation medium, and its effect on TBARS level, are similar to data reported in the literature.[25]

In the *S. mansoni* parasite, the defence mechanism against free radicals includes the enzymes SOD, GPx, GST, GR and cytochrome C oxidase.[26] Superoxide dismutase has a central role in the defence against oxidative stress. The equal activity of SOD in immature and mature worms, observed in the present study, indicates that SOD reaches its full expression and activity early in the life cycle of *S. mansoni*. This observation parallels published data to show that high levels of SOD are present in all schistosomal stages.[3] It also strengthens the proposal that SOD occupies a central position in the defence against oxidative stress in schistosomes.[27]

Various other enzymes are involved in the antioxidant protection of schistosomes, and GPx is an important antioxidant enzyme in the protection of *S. mansoni*. The activity of tGPx showed the typical time- and drug concentration-dependent increase following incubation with ART, which was more apparent in immature schistosomes. This induction of tGPx may constitute an important part of the defence mechanism, whereby the parasite endeavours to counteract the potential damage resulting from the increased lipid peroxidation. In this respect, it should be noted that the greatest increase in tGPx in immature worms caused by ART was still far below the level seen in mature schistosomes.

The selenium-dependent isoenzymes of GPx showed an activity three times higher in mature schistosomes compared with the immature stages in experiments conducted by Nare *et al*.[3] The higher activity of sGPx in mature schistosomes was confirmed in the present study. As with tGPx, the activity of sGPx in mature worms was seven times higher than in immature schistosomes. Consequently, adult worms should be expected to be more resistant to H_2O_2 attack.[28] Also, the activity of nsGPx in mature worms was seven times higher than in immature schistosomes. Incubation of either immature or mature stages of *S. mansoni* with ART or PZQ had no effect on sGPx activity. Accordingly, the increase in tGPx activity following incubation with ART must have resulted from induction of nsGPx, which is an isoform of GST.

The family of glutathione reductases is a component of the system responsible for maintenance of reduced glutathione pools. It plays a key role in the cellular defence against oxidative stress by preventing the accumulation of GSSG, thus maintaining the redox state.[29] Progressive increases in the levels of glutathione reductase have been observed with the development of *S. mansoni*.[3] In the present study, the level of GR activity in mature worms was about one and a half times that in immature worms.

Decreased GR activity should be a contributing factor to the depletion of GSH. With the increased GPx activity observed following incubation with ART, the inhibited GR enzyme will not be able to cope with the excess GSSG produced, which will not be reduced to GSH sufficiently quickly in order to maintain the GSH pool. Such inability to reduce oxidised glutathione is a factor contributing to the depletion of GSH in the worm. This should lead to a more oxidising intracellular environment and an inability to maintain an appropriate redox potential for proper survival of the parasite.[30]

The results of the present study indicate that incubation with ART causes an increase in oxidative stress, expressed as depletion of GSH and elevation of TBARS. Incubation with PZQ showed similar but milder effects in immature worms, but had a greater action in the mature stage. The addition of ART to the incubation medium induced antioxidant defence enzyme activity. This probably takes place as an adaptive response to protect the immature worm against oxidative killing, especially as the antioxidant defence at this parasite stage is not fully developed. □

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