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Plasmodium falciparum: selenium-induced cytotoxicity to *P. falciparum*

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Abstract

The in vitro antimalarial activity of sodium selenite (NaSe) was investigated and the mechanism of its action was studied. NaSe had antimalarial activity against both the chloroquine-susceptible strain FCR-3 and chloroquine-resistant strain K-1 of *Plasmodium falciparum*. The shrunken cytoplasm of the parasite was observed in a smear 12 h after treatment with NaSe. Co-treatment with copper sulfate ($CuSO_4$) in culture did not affect the antimalarial activity of NaSe, but NaSe cytotoxicity against the mammalian cell line Alexander was decreased significantly. The intracellular reduced glutathione level of parasitized red blood cells was decreased significantly by treatment with NaSe, and the decrease was consistent with their mortality. Treatment with NaSe had a strong inhibitory effect on plasmodial development, and NaSe cytotoxicity to human cells was decreased by co-treatment with $CuSO_4$. These results suggest that co-treatment with NaSe and $CuSO_4$ may be useful as a new antimalarial therapy.
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Index Descriptors and Abbreviations: Plasmodium falciparum; antiplasmodial activity; sodium selenite; pRBCs, parasitized red blood cells; RBCs, red blood cells; GSH, reduced glutathione; NaSe, sodium selenite; $CuSO_4$, copper sulfate

1. Introduction

Drug resistance in malaria is an important public health concern. *Plasmodium falciparum* has developed resistance to most of the commonly used antimalarial drugs including chloroquine, sulfadoxine–pyrimethamine, and mefloquine (Wongsrichanalai et al., 2002). Therefore, a search for new antimalarial drugs is very important.

Selenium is an essential trace element in mammals. Numerous epidemiological and experimental studies have found an anticarcinogenic activity of selenium (Clark et al., 1996; Combs and Gray, 1998; Comstock et al., 1992; Siwek et al., 1994; Spallholz, 1994; Spyrou et al., 1996; Thompson et al., 1994). Recently, it has been argued that the anticarcinogenic activity of selenium depends on its ability to generate superoxide, which is known for its DNA-damaging activity and induction of apoptosis (Lu et al., 1994).

There are some preliminary experiments suggesting that oxidative stress is the cause of selenium cytotoxicity and growth inhibition. Some investigators reported that the superoxide anion was generated by the reaction of sodium selenite (NaSe) with reduced glutathione (GSH) in a cell-free system (Davis and Spallholz, 1996). Other investigators reported that the cytotoxicity of selenium was decreased by co-treatment with copper sulfate ($CuSO_4$) (Jensen, 1975). But the mechanisms of these beneficial reactions are not known.

Since the reactive oxygen species is known to have the toxic effects for the malaria parasite and NaSe generate the superoxide and consume the intracellular GSH, we have evaluated the antimalarial effect of NaSe and investigated its interaction with intracellular GSH for development of new antimalarial drug to resolve drug resistance problem.

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2. Materials and methods

2.1. Parasites and cells

Chloroquine-susceptible *P. falciparum* strain FCR-3 and chloroquine-resistant *P. falciparum* strain K-1 were grown asynchronously, following the modified method of Trager and Jensen (1976), in RPMI 1640 medium supplemented with 10% human B serum, 25 mM Hepes, 25 μg/ml gentamicin (Sigma–Aldrich, St. Louis, MO), sodium bicarbonate, and human type O red blood cells (RBCs) in disposable sterile dishes under a controlled atmosphere of 5% CO_2 at 37 °C.

The human hepatocellular carcinoma cell line Alexander (Alex cells) was a kind gift of Dr. Takeaki Nagamine, Gunma University School of Health Sciences. Alex cells were grown continuously in complete Dulbecco's modified Eagle's medium (DMEM; BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (100 U/ml, 10 ml/liter; BRL, Grand Island, NY) under a 5% CO_2 atmosphere at 37 °C.

2.2. Investigation of in vitro plasmocidal effect of NaSe

Since several studies have shown effective anticarcinogenic activity of NaSe between 10 and 100 μM (Batist et al., 1986; Caffrey and Frenkel, 1992; Davis et al., 1998), RPMI 1640 medium was supplemented with either a 10 or 100 μM NaSe solution that was sterilized by filtration through a 0.22-μm filter. The NaSe-supplemented medium was changed every 24 h. Five hundred microliters of a parasitized-RBCs (pRBCs) suspension was placed in each well of a 24-well flat bottom culture plate (Corning Costar, New York, NY) at a hematocrit of 5% and an initial parasitemia of 0.1%. Thin-smeared specimens stained with Giemsa solution were made every 24 h and parasitemia was evaluated by counting the parasites in 3000 RBCs. Morphological changes of the parasites were monitored by observing Giemsa-stained thin-smeared specimens 2, 4, 12, and 24 h after treatment.

2.3. Determination of IC_{50} of NaSe against parasite

The pRBCs were synchronized with 5% D-sorbitol for 15 min at room temperature as described by Lambros and Vanderberg (1979) and then were washed three times with RPMI 1640 medium by centrifugation at 400*g* for 5 min. After washing, the pRBCs were resuspended in RPMI 1640 medium supplemented with 10% human B serum, 25 mM Hepes, 25 mM gentamicin, and sodium bicarbonate. Synchronous pRBCs having about 2% parasitemia were placed in 24-well cell culture plates as described above. Twenty microliters of NaSe solution was added to each well to give a series of concentrations from 10 to 100 μM. Six wells per plate served as NaSe-free controls to monitor parasite growth. After 24 h of incubation under a 5% CO_2 atmosphere at 37 °C, the control wells were checked for parasite growth. When the schizonts were fully grown in the control wells, the culture plate was removed from the incubator. Thin-smear specimens stained with Giemsa solution were made from the contents of each well. We first counted RBCs in the control smears until we encountered a total of 50 schizonts. The effect of NaSe on parasite growth was evaluated by comparing the number of schizonts in the same number of RBCs as was counted in the control cultures. The growth inhibition effect (%) was calculated as follows: (test well schizont count/control well schizont count) $\times$ 100. The NaSe concentration inhibiting parasite growth by 50% (IC_{50}) was calculated by the probit method (Inaba et al., 2001).

2.4. Detection of hemolysis caused by treatment with NaSe and $CuSO_4$

Hemolytic level was determined by measuring the hemoglobin that eluted into the medium with the SLS-hemoglobin method (Hemoglobin B test Wako, Wako Pharmaceutical, Osaka, Japan). Briefly, after exposure of pRBCs or RBCs to 40 μM NaSe that completely inhibited parasite growth and 2.5–40 μM $CuSO_4$, as described above, samples were centrifuged at 1000*g* for 5 min at 20 °C, and the supernatant was collected and analyzed. These Se:Cu ratios were shown to decrease NaSe toxicity against carcinoma cells (Shen et al., 2001).

2.5. Cytotoxicity of NaSe and $CuSO_4$ to P. falciparum and Alex cells

Synchronized FCR-3 parasitized erythrocytes having about 2% parasitemia were cultured in 24-well cell culture dishes. Each well, containing 500 μl of a 5%—hematocrit RBC suspension was supplemented with a 100% growth inhibition (IC_{100})—concentration of NaSe and various concentrations of $CuSO_4$ (2.5–40 μM). The remaining procedures were the same as described above. The growth inhibition effect was calculated as shown above. Morphological changes of the parasites in these conditions were monitored by observing Giemsa-stained thin-smeared specimens at 2, 4, 12, and 24 h after treatment.

To assess NaSe and $CuSO_4$ cytotoxicity to a mammalian cell line, the same experiment was carried out with Alex cells. Alex cells were seeded at 0.5×10^6 cells/ml in 24-well culture plates and incubated (37 °C, 5% CO_2) 24 h in 1 ml of DMEM supplemented with NaSe (40 μM) and $CuSO_4$. After the incubation period, detached cells were collected; attached cells were rinsed in the wells once with phosphate-buffered saline (PBS, pH 7.4) and then trypsinized to remove them from the solid

matrix. Cells were then collected by centrifugation (10 min, 830*g*, 25 °C). Cell viability was assessed by trypan blue exclusion.

2.6. GSH measurement in pRBCs, normal RBCs, and Alex cells

Intracellular GSH measurement was done according to Hissin and Hilf's method (Hissin and Hilf, 1976). Briefly, after treatment with NaSe and $CuSO_4$, cells were counted with a hemocytometer. After washing with PBS, homogenization buffer (4:1 mixture of 0.1 M sodium phosphate, 0.005 M EDTA buffer, pH 8, and 25% metaphosphoric acid) was added. All suspensions were homogenized on ice with a sonic wave homogenizer for 40 s. The homogenate was centrifuged (30 min, 21000*g*, 4 °C), and the supernatant was collected for analysis. After addition of fluorescent agent *o*-phthalaldehyde (0.1% in methanol), GSH level was determined with a multi-well plate reader (CytoFluor, Perspective Biosystem, Framingham, MA) at an excitation wavelength of 360 nm and an emission wavelength of 420 nm. Standards contained 0–2 μg GSH/ml.

2.7. Data analysis

Data are presented as means ± SEM from at least three sets of independent experiments. Student's *t* test was used for statistical analyses. A P value <0.05 was considered statistically significant.

3. Results

3.1. The plasmocidal activity of NaSe to chloroquine-susceptible and -resistant *P. falciparum*

To confirm the plasmocidal activity of NaSe, chloroquine-susceptible and -resistant *P. falciparum* were exposed to medium containing either 10 or 100 μM NaSe for 3 days and growth inhibition was monitored by determining the parasitemia (Fig. 1). In the presence of 10 μM NaSe, the growth of chloroquine-susceptible FCR-3 caused less parasitemia (1.14 ± 0.34%) than in the control culture (2.41 ± 0.30%, $P < 0.0005$, Fig. 1A). The growth of chloroquine-resistant K-1 also caused less parasitemia (0.20 ± 0.06%) than in the control culture (1.29 ± 0.01%, $P < 0.005$, Fig. 1B). In the presence of 100 μM NaSe, the growth of both strains was inhibited completely.

Results of the in vitro drug susceptibility tests using NaSe are shown in Fig. 1C. The IC_{50} values for NaSe were 21.26 ± 0.94 μM for FCR-3 and 15.50 ± 1.60 μM for K-1. There was no significant difference between IC_{50} values of chloroquine-susceptible FCR-3 and -resistant K-1 ($P > 0.05$). No intact parasites were observed after treatment with 40 μM NaSe. For this reason, 40 μM NaSe was applied in the following experiments.

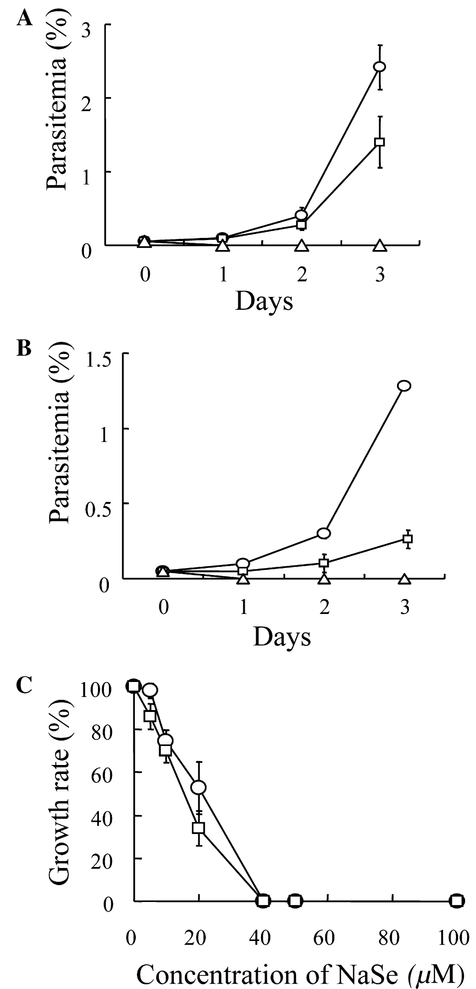

Fig. 1. Antimaralial effect of NaSe. NaSe was added to cultures of chloroquine-susceptible strain FCR-3 (A) and chloroquine-resistant strain K-1 (B) at concentrations of 100 μM (triangle), 10 μM (square), and 0 μM (circle). Parasitemia (%) was determined every 24 h. (C) Dose-dependent effect of NaSe on the inhibition of FCR-3 (circle) and K-1 (square) maturation in vitro. K-1 has less sensitivity than does FCR-3. Results are presented as means ± SEM. SE bars smaller than symbols are not shown.

3.2. Effect of $CuSO_4$ on NaSe toxicity to Alex cells or FCR-3

The effect of $CuSO_4$ in combination with NaSe on parasite growth was investigated. Although the mortality of the Alex cells was 30.01 ± 10.00% upon exposure to medium containing 40 μM NaSe, the mortality of Alex cells was decreased by co-treatment with $CuSO_4$ (Fig. 2). In the presence of either 2.5 or 40 μM $CuSO_4$, the susceptibility of the parasites to NaSe was not affected. Mortality of parasites was higher (50.0 ± 15.9%) than that of Alex cells (0.13 ± 1.50%) upon exposure to 20 μM $CuSO_4$ only.

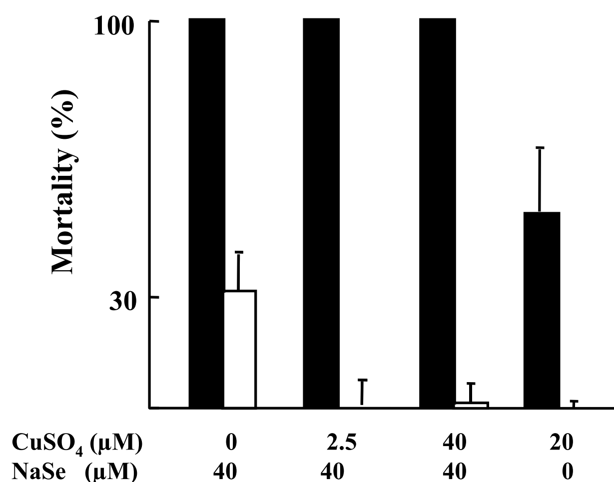

Fig. 2. Cell mortality after treatment with NaSe and $CuSO_4$ for 24 h. Trypan blue exclusion was used to assess mortality of Alexander cells (white bar). The effect of sodium selenite on *P. falciparum* growth was evaluated by comparing the number of schizonts in test erythrocytes with the number in the control cultures (black bar). The results are presented as means ± SEM.

3.3. Hemolysis caused by NaSe and $CuSO_4$

To detect hemolysis as an index of cytotoxicity, the concentration of hemoglobin in pRBCs and RBCs culture media was determined. No hemolysis was detected in the pRBCs culture medium in the presence of 40 μM NaSe only or of 20 μM $CuSO_4$ only. In the presence of 40 μM NaSe with 40 and 20 μM $CuSO_4$, the concentration of hemoglobin was significantly higher (6.53 ± 0.29 g/dl) than in the control culture (0.37 ± 0.05 g/dl, $P < 0.005$) (Table 1). At $CuSO_4$ concentrations less than 10 μM, the concentration of hemoglobin ranged from 0.53 ± 0.05 to 0.69 ± 0.22 g/dl, as compared to the control level of 0.37 ± 0.05 g/dl. These values were not significantly different ($P > 0.05$). Similar results were observed in RBCs culture.

Table 1
Lysis of pRBCs or RBCs induced by NaSe and $CuSO_4$

Concentration of NaSe/Cu (μM)	Hemoglobin (g/dl)[a]	
	pRBCs	RBCs
40/40	6.53 ± 0.29	12.08 ± 0.23
40/20	1.97 ± 0.15	4.16 ± 0.77
40/10	0.53 ± 0.05	0.63 ± 0.13
40/5	0.57 ± 0.08	0.56 ± 0.06
40/2.5	0.59 ± 0.13	0.73 ± 0.13
40/0	0.69 ± 0.22	0.59 ± 0.00
0/20	0.61 ± 0.19	0.50 ± 0.06
0/0	0.37 ± 0.05	0.49 ± 0.05

[a] Each value represents mean ± SEM.

3.4. Morphological changes of parasites exposed to NaSe and $CuSO_4$

Fig. 3 shows the morphological changes of the parasites when treated with 40 μM NaSe and 2.5 μM $CuSO_4$. In the control culture, only intact ring-form parasites were present after 4 h (Fig. 3A). Four hours after treatment with 40 μM NaSe and 2.5 μM $CuSO_4$, a small number of shrunken parasites were observed (Fig. 3B). In the presence of 40 μM NaSe and no $CuSO_4$, similar morphological changes of the parasites were observed (Fig. 3C). Twelve hours after treatment, most of the parasites were early and late trophozoites in culture with 0 μM NaSe and 0 μM $CuSO_4$ (Fig. 3D). Intact parasites were undetectable and almost all the parasites were shrunken after treatment with 40 μM NaSe and 2.5 μM $CuSO_4$ (Fig. 3E) and with 40 μM NaSe and 0 μM $CuSO_4$ (Fig. 3F).

3.5. Changes of intracellular GSH content after treatment with NaSe and $CuSO_4$

To evaluate the intracellular redox condition after treatment with NaSe and $CuSO_4$, intracellular GSH contents were determined. As shown in Fig. 4, intracellular GSH levels decreased significantly after a single treatment with 40 μM NaSe in pRBCs (33.2%), RBCs (27.5%), and Alex cells (36.2%), as compared with control GSH content ($P < 0.05$). $CuSO_4$ could prevent the depletion of intracellular GSH contents in Alex cells, even at 2.5 μM, but not in pRBCs and RBCs.

4. Discussion

Selenium is an essential trace element in humans. It exists in a number of forms with differing valence states, some of which have antineoplastic activity. The usual form of selenium used for supplementation is either selenite or selenomethionine; both have been given in doses up to 200 μg without toxicity. We studied the plasmocidal activity of NaSe against chloroquine-susceptible and -resistant *P. falciparum* strains. Our results showed that both *P. falciparum* strains were susceptible to the cytotoxic effects of NaSe and that the susceptibility to NaSe-induced cytotoxicity was unaffected by co-treatment with $CuSO_4$. In contrast, no cytotoxic effect of NaSe was found in Alex cells upon co-treatment with $CuSO_4$. These results suggest a selective inhibition of plasmodia by NaSe and $CuSO_4$. However, in considering the use of NaSe in antimalarial treatment, it will be a critical issue to select the appropriate dose to optimize both the plasmocidal effect and minimize adverse effects to host cells.

Growth inhibition by NaSe of all parasite developmental stages in vitro is obviously different from the

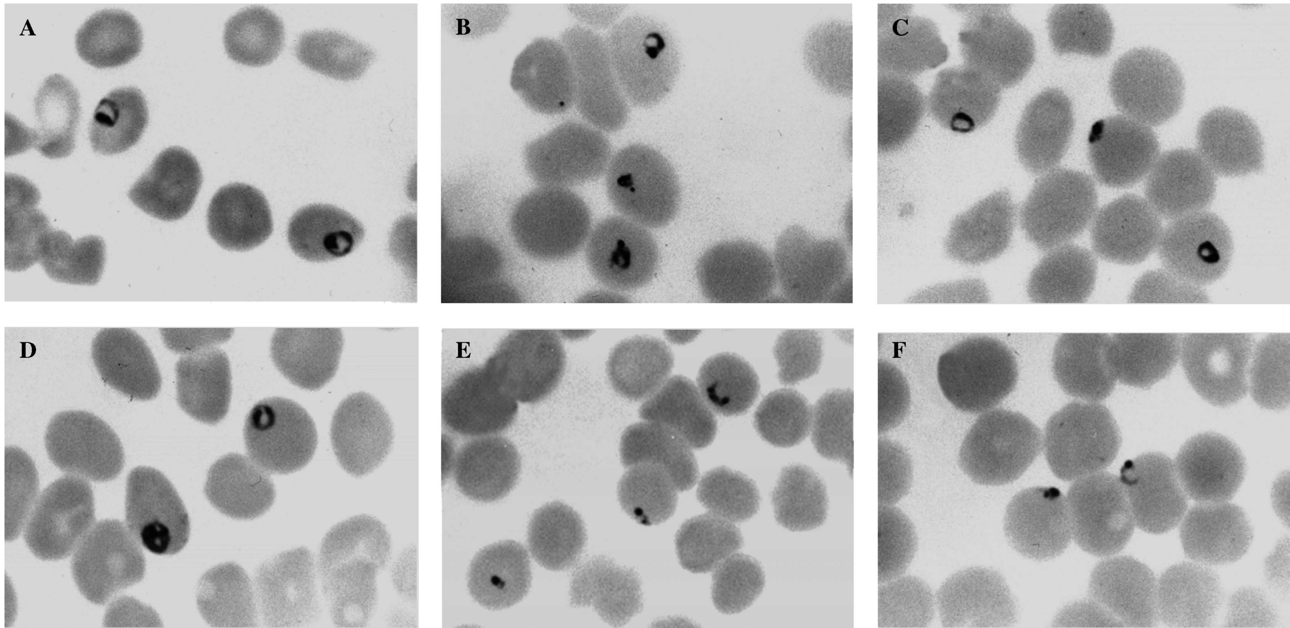

Fig. 3. Light micrographs of Giemsa-stained blood smears. (A) The parasite culture without NaSe or $CuSO_4$ for 4 h, (B) with 40 μM NaSe and 2.5 μM $CuSO_4$ for 4 h, (C) with 40 μM NaSe and 0 μM $CuSO_4$ for 4 h, (D) without NaSe or $CuSO_4$ for 12 h, (E) with 40 μM NaSe and 2.5 μM $CuSO_4$ for 12 h, and (F) with 40 μM NaSe and 0 μM $CuSO_4$ for 12 h (magnification, 1000×).

chloroquine effect on growth (data not shown). The shrunken and pyknotic appearance of parasites was observed by light microscopy (Fig. 3). Similar morphological changes of the parasites have been observed in owl monkeys infected with *P. falciparum* and treated with the Qhinghaosu derivative artemether (Kawai et al., 1993). Qhinghaosu derivatives, including artemether, are sesquiterpene lactone endoperoxide antimalarials and act through the generation of free radicals that alkylate parasite proteins (Meshnick et al., 1989). It is also well known that NaSe-induced cell death is induced by oxidative stress associated with selenite metabolism and that selenite metabolism is very sensitive to cell GSH (Davis and Spallholz, 1996; Seko and Imura, 1997; Shen et al., 2000). Morphological changes shown in this study and these facts indicate that the plasmocidal action induced by NaSe is mediated by mechanisms similar to those associated with Qhinghaosu derivatives.

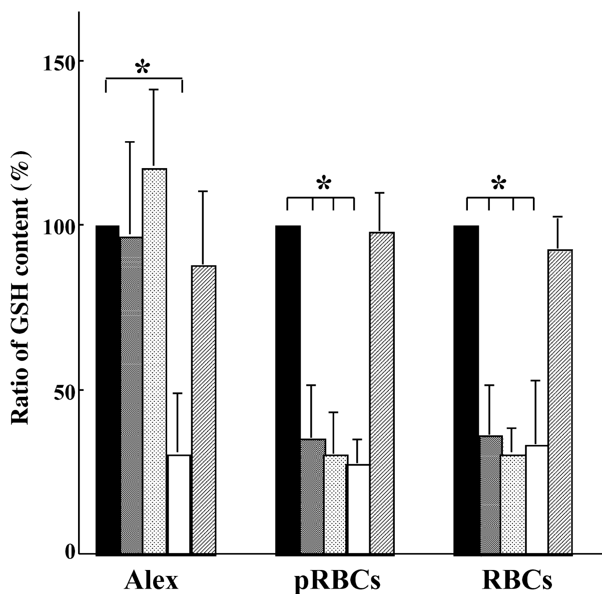

Fig. 4. The ratio of intracellular GSH content in Alexander (Alex) cells, pRBCs, and RBCs after treatment with NaSe and $CuSO_4$ to that in control cells. Results are presented as means ± SEM. The black filled column represents the control ratio for cells without NaSe and $CuSO_4$. The gray shaded column represents the ratio after treatment with 40 μM NaSe and 40 μM $CuSO_4$, the spotted column represents the ratio with 40 μM NaSe and 2.5 μM $CuSO_4$, the open column represents the ratio with 40 μM NaSe and 0 μM $CuSO_4$, and the hatched column represents the ratio with 0 μM NaSe and 20 μM $CuSO_4$. $^*P < 0.05$.

No different morphological changes of the parasites were observed between those treated with NaSe alone and with NaSe and $CuSO_4$. Treatment of human cells with NaSe and $CuSO_4$ is considered beneficial. The copper cation did not block selenite-induced oxidative stress in erythrocytes, as it does in other cells (Davis et al., 1998; Shen et al., 2001). Our data have also shown the prevention of GSH consumption by $CuSO_4$ in Alex cells. Several in vitro studies have reported that selenite treatments lead to a decline of intracellular GSH level, and co-treatment with copper prevents that decrease (Davis et al., 1998; Shen et al., 2000, 2001). However, in RBCs, GSH consumption by NaSe was not affected by $CuSO_4$. These results suggest that parasites might be killed in the same or a similar way as that seen after a single treatment with NaSe.

Detoxification of selenite toxicity in host cells by copper has been shown in in vitro (Davis et al., 1998; Shen et al., 2001) and in vivo (Jensen, 1975). It was suggested that the detoxification of selenite-induced cytotoxicity is caused by complexation with Cu^{2+}, forming GSSe–Cu^{2+}–SeSG or simply Cu^{2+}-selenide, that prevents the generation of oxygen-free radicals. It also was reported that Cu^{2+} acts as a superoxide dismutase mimic and can detoxify the selenite-induced cytotoxicity because inhibition of free radical generation was observed at a Cu:Se molar ratio of less than 1:1 (Davis and Spallholz, 1996). However, the treatment with NaSe and $CuSO_4$ caused marked cytotoxicity to *P. falciparum* but not to normal RBCs and Alex cells. This difference might be due to charge transfer from hemoglobin iron to copper in erythrocytes. Formation of methemoglobin prevented charge transfer to copper and took away its ability to complex with NaSe. Copper may act as a generator of free radical (Fernandes et al., 1988). Therefore, $CuSO_4$ may not work as a scavenger of superoxide or an inhibitor of the conversion of selenite to selenium by consumption of GSH in RBCs.

In conclusion, NaSe has a strong inhibitory effect on in vitro plasmodial development and is devoid of cytotoxicity towards human cells if there is co-treatment with $CuSO_4$. Co-treatment with NaSe and $CuSO_4$ may be a useful antimalarial regimen.

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