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Short Communication

تاثیر برخی از داروهای ضدمالاریا بر روی آنزیم گلوتاتیون -S-ترنسفراز *پلاسمودیوم برگیی*

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چکیده

گلوتاتیون-S-ترنسفرازها (GSTs) آنزیم‌هایی هستند که در تمامی ارگانیسم‌های زنده یافت می‌شوند و در سم‌زدایی ترکیبات زنوبیوتیک از طریق فاز II الحاق با گلوتاتیون عمل نموده و نقش مهمی را در مقاومت دارویی ایفا می‌کنند. فعالیت GST در سویه‌های مقاوم به کلروکین *پلاسمودیوم* جوندگان و انسانی به طور معنی داری در مقایسه با سویه های حساس بالاتر می‌باشد. در مطالعه حاضر فعالیت و سطح GST در موش‌های آلوده با *پلاسمودیوم برگیی* در هنگام تیمار با داروهای ضدمالاریا بررسی شد. آلوده سازی موش‌ها با *پلاسمودیوم برگیی* انجام گردید و مهار رشد انگل با استفاده از سه دوز داروهای ائوزین B و داروهای ضدمالاریای متداول مانند آرتمیسینین، سولفادوکسین-پیریمتامین با فاصله ۲٤ ساعت با استفاده از روش پیترز انجام شد. ۲ ساعت پس از آخرین دوز، خون‌گیری از موش‌ها انجام شده و اریتروسیت‌ها توسط ستون سلولز جدا گردید و *پلاسمودیوم برگیی* پس از شستشو با ساپونین آزاد شد. فعالیت آنزیم با استفاده از گلوتاتیون و CDNB به عنوان سوبسترا در ۳٤۰ نانومتر اندازه‌گیری شد. سطوح آنزیم GST با کیت Biotech اندازه‌گیری گردید. فعالیت ویژه و سطوح GST در *پلاسمودیوم برگیی* موش‌های آلوده در تیمار با ائوزین B ، سولفادوکسین-پیریمتامین و ترکیب آن‌ها کاهش پیدا کرد، اما در مورد آرتمیسینین و ترکیب آن با ائوزین B این کاهش مشاهده نشد. ترکیب ائوزین B به واسطه اثر کاهنده برGST که نقش مهمی در مقاومت دارویی انگل به داروهای ضدمالاریا دارد می‌تواند پتانسیل بالایی برای کاربرد در داروهای ترکیبی ضدمالاریا داشته باشد.

واژگان کلیدی: گلوتاتیون-S- ترنسفراز، *پلاسمودیوم برگیی*، ائوزینB، داروهای ضدمالاریا.

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Short Communication

The effect of some antimalarial drugs on *Plasmodium berghei* glutathione-S-transferase enzyme

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Abstract

Glutathione-S-transferase (GST) are a group of enzymes found in all living organisms that contribute to detoxification of xenobiotic compounds via Phase II conjugation with glutathione and play an important role in drug resistance. The GST activity in chloroquine-resistant strains of rodent and human *Plasmodium* is meaningfully higher than sensitive strains. In the present study, the GST activity and level were measured in those mice infected with *Plasmodium berghei* during the treatment with antimalarial drugs. Mice infections were carried out by *P. berghei* and inhibition growth of the parasite was performed through three doses of eosin B and prevalent antimalarial drugs similarly artemisinin and sulfadoxine-pyrimethamine in 24 h based on Peter's test. Two hours after the last dose, the mice were bled, RBCs were isolated by cellulose column, and *P. berghei* was released after washing with saponin. The enzyme function was measured using glutathione and CDNB as substrate at 340 nm. In addition, the GST levels were assessed by Biotech kit. The GST specific activities and levels of *P. berghei* in infected mice treated with eosin B and sulfadoxine-pyrimethamine and their combination were decreased while such change was not seen in those ones treated with atemisinin and in combination with eosin B. Owing to the reduction property of the combination of eosin B on the GST which has a fundamental role in the resistance of the parasite to antimalarial drugs, it could be considered as a promising procedure for application in antimalarial combination drugs.

Keywords: Glutathione-S-transferase, *Plasmodium berghei*, Eosin B, Combination drugs.
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Introduction

The human malaria parasites are exposed to metabolic stress during their development and efforts for malaria elimination have failed due to factors such as parasite resistance to existing drugs (1). A strategy for discovering new antimalarial drugs, is the targeting of antioxidant and redox systems which result in the reduction of parasites in the host and are considered as a valuable method in disease control (2). Oxidative stress plays a key role in the mortality of this disease, and at the same time it can play a promising role in chemotherapy against malaria. Detoxification of the "reactive oxygen species" (ROS) is itself a challenge to the *Plasmodium*-infected erythrocytes (3).

It is noteworthy that *Plasmodium* does not have the classical enzymes of catalase and GSH peroxidase (4). Glutathione-S-transferases (GST) include a family of eukaryotic and prokaryotic enzymes which help in detoxification of xenobiotic substrates mainly due to the reduced glutathione (GSH). In general, the most important functions of GSTs are: detoxification of endogenous and xenobiotic compounds, binding to toxicological compounds with a transitional role, enhancing drug resistance to anti-parasite compounds, insecticides etc. A class of GST enzymes have been identified as the cause of DDT resistance to *Anopheles gambia* (5) and proved to be the most important enzyme in the resistance to chloroquine in malaria parasite (6).

Laboratory stains such as methylene blue and eosin B (EO) are among the antimalarial drugs being tested (7). Methylene blue has been examined in combination with chloroquine on malaria patients (8). Eosin B is shown to inhibit the growth of *Plasmodium falciparum in vitro* due to its unique molecular structure. Eosin B also causes severe cytopathic effects in *Plasmodium falciparum* and prevents the growth of the parasite. In addition, this combination inhibits several enzymes including dihydrofolate reductase-thymidilate synthase (DHFR-TS), glutathione reductase and thiorodoxin reductase (9). Eosin B has an effect on *Plasmodium berghei in vivo* as shown earlier (10). In the present study, the activity of GST enzyme in murine malaria parasite *Plasmodium berghei* were tested with eosin B (EO) and its combinations with other malarial drugssuch as artemisinin (Art) and sulfadoxine-pyrimethamine (SP). As resistance of the parasite has been reported to the above drugs in different parts of the world, a study of its effect on the GST enzyme function and its levels in the parasite after drug treatment *in vivo* would help in further understanding their mechanism.

The effect of antimalarial drugs with eosin B was studied on GST in *Plasmodium berghei in vivo*.

Materials and methods

Parasite strain

Plasmodium berghei Haffkine strain was used in the study and were maintained cryopreserved in liquid nitrogen with Alservers solution. Ten Swiss NMRI mice 18-22 g (as per the guidelines of Genevacommittee for animal rights) were infected intraperitoneally (ip) with 10^9 *Plasmodium berghei* (11). After 5 days, the mice were checked for parasite growth by

Geimsa stained thin blood smears from the tail and if their parasite count had reached 30%, they were bled under euthanasia and 0.5 ml of the infected blood (10^7x2 erythrocytes/ml) were Injected ip into another 6 groups of mice (n=5).

Preparation of drugs

All chemicals were purchased from Sigma-Aldrich (USA). Eosin B, artemisinin, sulfadoxine-pyrimethamine were tested. A solution of 7% tween 80 and 3% ethanol is used as drug vehicle (DV). The dosage of drugs were 200mg/kg EO, 60mg/kg Art, 25:1.25 mg/kg SP (10). The combination drugs were tested at various dilutions and the following concentrations were seen to be effective: EO:Art (2.5:1.5mg/kg) and EO:SP (20:0.125:2.5mg/kg). The drugs were administered in the six groups of mice, with DV as negative control and each drug singly as positive controls (10).

Peter's suppression test of drugs on mice

The drugs were administered i.p. 2h after infection. The dosages were repeated 24, 48 and 72 h later. The mice were bled from the heart with heparin under euthanasia after 75 h and the infected RBCs collected (10).

Separation of Plasmodium from infected RBCs

2 ml of blood collected from each group of mice was centrifuged at 1200g for 10 min, its RBCs separated and mixed with sterile PBS. It was the passed through a 50 ml cellulose column and the RBCs without the leukocytes were collected. The resulting RBCs were suspended in 0.2% saponin w/v in PBS and placed on ice for 30 minutes. The mixture was then centrifuged at 600 g in 4 °C for 20 min, washed 3 times with PBS and stored at -80 °C (11).

Parasite extract preparation

Preparation of parasite extract was carried out by freeze thawing the pellet a number of times and finally suspending it in 200 µl of PBS.

Evaluation of GST activity by spectrophotometry

The GST enzyme assay was carried out using reduced glutathione and CDNB as substrates for the GST enzyme, the reaction absorbs at 340nm.

GSH+CDNB→GS-DNB Conjugate + HCl

Enzyme mix was prepared with 980µL of Dulbecco's Phosphate Buffered Saline, 10µL of resuspended 100mM glutathione and 10µl of 100mM CDNB in an Eppendorf tube. 100µL of the enzymatic reaction was added to each well of ELISA plate and mixed with 100µL parasite extract from each group. The plate was incubated at 37ºC and the change in absorption was recorded for 20 min and compared with DV as negative control and GST standard as positive control using the following equations (12):

$\Delta A340/min = (A340_f - A340_i)/t$

Where $A340_f$ and $A340_i$ are the final and initial reading respectively with t as the time in min.

Specific Activity= $(\Delta A_{340}/min \times V \times dil)/(\varepsilon_{mM} \times V_{enz})$ (12).

Where dil=dilution factor of the original sample, $\varepsilon_{mM}(mM^{-1}cm^{-1})$ is the extinction coefficient for CDNB conjugate at 340nm, V_{enz} is the volume of the enzyme sample tested.

GST Levels

Levels of GST in *Plasmodium berghei* were measured for mice treated with combination drugs, the single drugs and DV as positive and negative controls respectively. 100µL of parasite extract from the groups were tested by standard Sunlong Biotech kit with biotin avidin markers in accordance with the manufacturer's instructions (13).

Statistical analysis

Statistical analysis was carried out using ANOVA test with Graph Pad prism version 6. P values <0.001 were considered as significant.

Results

The results of the specific activity of the enzyme GST are shown in Fig. 1. Specific activity of GST in *Plasmodium berghei* has shown decrease with eosin B and SP as compared to DV and increase with artemisinin. GST levels of *P. berghei* was decreased with eosin B and SP in *Plasmodium berghei*, but increased with artemisinin and combination drugs of eosin B with artemisinin or SP (Fig 2).

Discussion

There are two super-families of GSTs, one of which is soluble and the other attached to the cell membrane. Soluble GSTs are found in all aerobic organisms both in the microsomal form and in the cytosol. The superfamily of the mammalian GST include cytosolic dimeric isozymes with a molecular weight of 45 to 55 kilodaltons (kD) named as α, µ, π and θ. Even though, the similarity of primary structures of GSTs are less than 10%, the tertiary and quaternary structures of these proteins are remarkably similar. *Plasmodium falciparum* GST has been estimated to represent >1% of the total protein in the cytosol and was thought of comprising of a single isotype (14, 15).

The GST levels of *Plasmodium berghei* with the antimalarial drugs singly clearly showed that two of the drugs, eosin B and SP significantly reduced GST levels in parasites from infected and treated mice resulting in its inhibition. This is similar to methylene blue another laboratory dye which is seen to affect glutathione reductase an enzyme participating in the glutathione detoxification cycle (16).

The results of this study showed that the

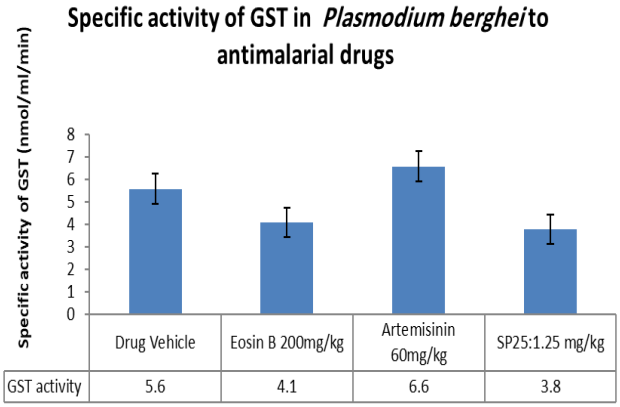

Figure 1. Specific activity of GST in *Plasmodium berghei* to antimalarial drugs (p<0.01).

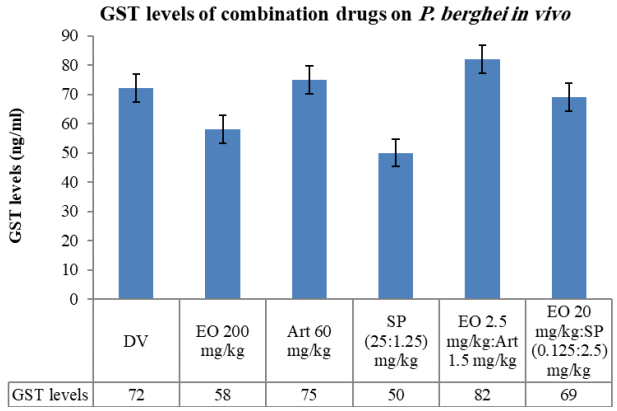

Figure 2. GST level of combination drugs on *Plasmodium berghei in vivo* (p<0.01).

specific activity GST has reduced significantly in infected mice treated with these two drugs which could be the result of accumulation of toxic ferriprotoporphyrin IX.

However, in the combination treated EO:SP group the GST levels had not reduced as much as the single drug and this can be attributed to the fact that the doses used for eosin B and SP were very low although it resulted in inhibition of parasitemia (data not shown).

Over the past decade GST has been considered as a potential target of common antimalarial drugs. In fact, there have been reports of stage dependent inhibition of GST in *Plasmodium knowelsi* extracts by the antimalarial drugs (chloroquine, arteminine and primaquine). However, their work was not reproduced *in vivo* and it was concluded that these drugs did not act on *Pf* GST (17).

Later work on membrane associated GSTs in *Plasmodium* spp. reveal their seeming relevance to the recently observed loss of artemisinin efficacy in field and clinical studies and this could be due to the fact that they make up a significant part of a parasite defense system to withstand exposure to artemisinins. (2)

In this study, the GST activity and level of of artemisinin on *Plasmodium berghei in vivo* has shown an increase both singly and in combination with eosin B. This result is similar to that of Srivastava who showed very poor inhibition of GST activity by artemisinin as compared to that of chloroquine. Also, the mechanism of action of artemisinin and its derivatives is based on their action on calcium pumps similar to SERCA and do not appear to have a significant effect on the GST enzyme.

The effect of eosin B and SP singly resulted in comparable lowering of GST levels in *Plasmodium berghei*, unlike that of artemisinin. Hence, it can be deduced that GST enzyme is not the direct target of these drugs. Eosin B alone inhibited GST but the combination drugs did not show a great lowering of GST activity which could be due to lower dosages used therefore this enzyme will not be activated greatly under drug pressure and will not result in resistance through this mechanism and so it can be used as target of other antimalarials.

Conclusion

Considering the above, seen that eosin B and SP inhibited GST activity and levels but artemisinin did not. The combination drugs however increased GST levels and activity significantly.

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