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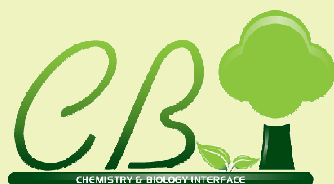

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Research Article

Artemisinin and its derivatives as inhibitors of antioxidant system of malarial parasite: *Plasmodium yoelii*.

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Keywords: Reactive Oxygen Species (ROS), Artemisinin, Arteether, Artemether, Artesunic acid Dihydroartemisinin (DHA), Chloroquine (CQ), *Plasmodium yoelii,* Antioxidant system.

Abstract: The intra-erythrocytic stages of malarial parasite encounter reactive oxygen species produced either by erythrocytes or host immune cells. In order to prevent oxidative damage, the parasite utilizes host enzymes and induces its own antioxidant enzymes. The erythrocytes itself have potent antioxidant defences to counteract production of ROS by oxidation of haemoglobin to methaemoglobin. The effect of Artemisinin and its derivatives was studied on antioxidant system of rodent malarial parasite *P. yoelii.* The Artemisinin and its derivatives inhibited the antioxidant enzymes of infected erythrocytes. The antioxidant system of *P. yoelii* was affected to different extent by antimalarials. Incubation of infected blood for one hour with these antimalarials exerted significant inhibitory effect on the antioxidant system of the erythrocyte as well as parasite. The increased levels of N-acetylglucosaminidase, Nitrite and H_2O_2 in macrophages obtained from infected animals were reduced significantly by treatment with Artemisinin and its derivatives.

Introduction

Malaria is amongst the most prevalent parasitic diseases in the tropics [1]. 95% of the malarial cases are due to *P. falciparum* and *P. vivax.* The antimalarials viz., 4-aminoquinolines, 8-aminoquinolines, amino alcohols and antifolates have been used for treatment of the disease, however due to increasing incidences of drug resistance in recent years development of new antimalarials is needed [2].

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Quinine is the drug of choice for the treatment of severe malaria except in South East Asia, where it is being replaced by derivatives of a compound called Artemisinin [3-4]. Quinine resistance in *P. falciparum* has been reported from Brazil and South East Asia [5]. Chloroquine resistant strains are often sensitive to quinine suggesting that the mechanism of resistance to chloroquine and quinine are different [6]. Artemisinin (Qinghaosu), a natural Chinese medicine is an effective chemotherapeutic drug for the treatment of multi-drug resistant strains of malaria [7]. Artemisinin has been

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isolated from the Artemisia annua leaves, a medicinal plant and its activity against the malaria parasite *Plasmodium falciparum* was subsequently demonstrated. A number of semi-synthetic derivatives have been prepared for use in malaria combat programmes and therapeutic usefulness has been repeatedly shown [8]. Best known among the different derivatives are Artemether, Arteether, Artesunate and β-dihydroArtemisinin (DHA) [9]. The mechanism of action of Artemisinin involves the generation of carbon-centered free radicals, due to presence of endoperoxide linkage [10, 11]. Among other mechanisms, it was proposed that reductive scission of the peroxide by ferroprotoporphyrin IX gives rise to an active intermediate which can alkylate both heme and essential parasite proteins leading to parasite death [12-14].

Oxidative stress is an important mechanism for the destruction of malaria and other intracellular parasites [15, 16]. It is commonly accepted that since intra erythrocytic stages of malaria parasites must encounter reactive oxygen species (ROS) produced either by erythrocyte or host immune cells, they must build antioxidative defense against these ROS [17]. In order to prevent oxidative damage, the parasite would both commandeer host enzymes and induces its own antioxidant enzymes. The erythrocyte itself has potent antioxidant defenses to counteract the constant production of ROS by oxidation of haemoglobin (Fe^{2+}) to methaemoglobin (Fe^{3+}) [18].

The present paper reports the effect of Arteether, Artemether, Artesunic acid and Dihydroartemisinin (DHA) on the antioxidant enzymes of malarial parasites, infected erythrocytes and also on the reducing power, lipid peroxidation and macrophage function.

Materials and Methods

Animals and chemicals:

Male albino mice (Swiss strain) weighing 22–25 g, were obtained from Division of Laboratory Animals, Central Drug Research Institute, Lucknow, India. Mice were housed in the animal house at the institute and care was provided as per the guidelines laid down by the ethics committee. Animals were divided into control and experimental groups, each group contained five mice. All the chemicals used were of analytical grade. Artemisinin, Arteether, Artemether, Artesunic acid and Dihydroartmisinin (DHA) were obtained from division of Medicinal and Process Chemistry, CDRI.

Maintaince of *P. yoelii* infection in swiss mice and isolation of infected RBCs and free parasites:

The experimental groups animals were inoculated intraperitoneally with 10^5 *P. yoelii nigeriensis* – infected erythrocytes as reported earlier [19]. The blood was collected at 5%, 20% and 60% parasitemia in citrate saline. The leucocytes and platelets were removed by passing through CF-11 column and free parasites were isolated by saponin lysis [20]. Briefly infected erythrocytes were suspended in equal volume of 0.15 % saponin at 4^0C for 20 min and free parasites were isolated by centrifugation at 3000 rpm for 15 min at 4^0C. The parasites were resuspended in five volume of PBS and sonicated at 15% amplitude. The supernatant obtained by centrifugation at 12000 rpm for 30 min was used for the determination of enzyme activities. Protein was estimated by the method of lowery *et. al* [21].

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Effect of Artemisinin & their derivatives on antioxidant system of infected erythrocytes and *P. yoelii* parasites:

The antioxidant enzymes viz; Catalase (CAT), Glutathione reductase (GR), Superoxide dismutase (SOD), Glutathione Peroxidase (GPx) were assayed asccording to their respective methods [22-27]. The effect of antimalarials was measured by incubating the infected erythrocytes and malarial parasites at 100 μM concentration for 10 min. The infected blood (~60%) parasitemia was incubated with Artemisinin and their derivatives for one hour and RBCs and plasma were used for estimating antioxidant status. Reducing power and lipid peroxidation were estimated by the method of Oyaizu *et. al*. [28] and Ohkawa *et. al.*. [29] respectively.

Isolation of peritoneal macrophages (PEC) and effect of Artemisinin derivatives on macrophage functions:

Macrophages were obtained from mice peritoneal cavity as reported earlier [30] and cell number was adjusted to 3 x 10^6 cells /ml. The suspension was sonicated at 10% amplitude and supernatant was used for measuring N-acetylglucosaminidase activity by the method of Baggilini *et. al.* [31]. H_2O_2 was estimated by the method of Meloan *et. al.*[32] and nitrite concentration was estimated by the method of Hageman *et. al.*[33]. Macrophages were incubated with Artemisinin and its analogs at 100-μM concentration for 1 hour & various parameters in treated macrophages were estimated as mentioned above.

Results

The status of antioxidant enzymes in *P. yoelii* infected mice at 5%, 20% and 60% parasitemia is shown in (Table 1). The catalase activity was decreased by 1.29, 1.41 and 1.46 folds while SOD activity was decreased by 2.15, 2.57 and 3.03 folds as compared with normal RBCs at different levels of parasitemia respectively. The glutathione reductase activity was increased by 1.33, 2 and 2.66 folds while glutathione peroxidase activity was increased by 1.33, 1.91 and 2.33 folds as compared with normal RBCs at different levels of parasitemia respectively. The levels of H_2O_2 and lipid peroxidation were increased by 2 and 4.6 folds in infected RBCs as compared to normal RBCs (Table 2). The nitrite and reducing power levels in infected RBCs were decreased by 1.17 and 1.56 folds as compared to normal RBCs. The levels of nitrite, H_2O_2 and reducing power in *P. yoelii* parasites were decreased by 10.46, 2.36 and 26 folds, while lipid peroxidation was 14.8 folds higher in *P. yoelii* lysate (Table 2).

In another set of experiment, the Artemisinin and its derivatives were incubated for one hour with infected blood (~60% parasitemia) at 100μM and after centrifugation antioxidant properties were studied in RBCs and plasma. **Catalase activity:** In drug treated RBCs, Arteether, Artemether, DHA and Artesunic acid at 100 μM conc. showed 16%, 15%, 36% and 40% inhibition of catalase activity respectively. In Plasma, Artesunic acid & DHA at 100μM conc. showed 30% and 32% inhibition of catalase activity, whereas Arteether & Artemether showed 12% and 11% inhibition at same conc.(Table 3). **Superoxide dismutase activity:** Arteether, Artemether and Artesunic acid at 100 μM conc. showed 22%, 20% and 10% inhibition of SOD activity respectively, whereas, DHA had no effect on drug treated RBCs (Table 3). In Plasma, Artesunic acid & DHA at 100μM conc. showed 20% and 34% inhibition of SOD activity, whereas, Arteether & Artemether showed 11% and 8% inhibition

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at the same conc. (Table3). **Glutathione reductase activity:** Arteether, Artemeether, DHA & Artesunic acid at 100 µM conc showed 5%, 10%, 18 % and 15% inhibition of glutathione reductase activity respectively in infected RBCs and had no effect on plasma glutathione reductase activity (Table 3).

Glutathione Peroxidase activity: Arteether, Artemeether, DHA & Artesunic acid at 100 µM conc. showed 22%, 50%, 34% and 45% inhibition of glutathione peroxidase activity in treated RBCs respectively (Table 3). In Plasma Arteether, Artesunic acid & DHA at 100µM conc. showed 53%, 68% and 55% inhibition of glutathione peroxidase, whereas Artemether had no effect on the activity (Table 3).

Similar experiments were also conducted in *P. yoelii* lysate. Arteether, DHA and Artesunic acid at 100µM conc. showed no inhibition of catalase activity (Table 3) while Artemether caused a minor inhibition. Arteether, Artemether, DHA and Artesunic acid at 100µM conc. caused 23%, 24%, 14% and 25% inhibition of SOD activity. Arteether, Artemether, DHA and Artesunic acid at 100µM conc. caused 22%, 23%, 20% and 18% inhibition of glutathione reductase activity respectivily. Artesunic acid at 100µM conc. showed 39% inhibition on the glutathione peroxidase activity, whereas Arteether, Artemether and DHA at 100µM conc. showed 33%, 23%, 22% inhibition respectively (Table 3).

The levels N-acetylglucosaminidase, nitrite and H_2O_2 were increased by 1.5, 3.33 and 2.29 folds respectively in macrophages obtained from infected animals as compared to normal animals (Table 4). *In vitro* effect of Arteether, Artemether, Artesunic acid & DHA was also studied on the N-acetylglucosaminidase activity, Nitrite and H_2O_2 levels in infected macrophages at 100µM concentrations. Arteether, Artemether, DHA and Artesunic acid showed 19%, 31%, 27% and 31.5% inhibition of N-acetylglucosaminidase activity respectively, as compared to macrophages isolated from infected animals (Table 4). The nitrite level was decreased by 3%, 10% and 14.5% by Artemether, DHA and Artesunic acid respectivily, while Arteether had no effect (Table 4). The H_2O_2 level was decreased by 5%, 11%, 33% and 21% by Arteether, Artemether, DHA and Artesunic acid respectively (Table 4).

Discussion

Artemisinin is the only drug used for the treatment of resistant malaria while the mechanisms of its antimalarial effects are still not so clear. As a very specific target for the activity of Artemisinin, the sarco/endoplasmatic reticulum $Ca2^{+}$-ATPase (SERCA) has recently been suggested [34]. Artemisinin derivatives cause rapid clearance of the blood stage malaria along with another asset of their apparently excellent human safety and tolerability [35-37].

Our investigation suggests that *P. yoelii* infection resulted in increased generation of reactive oxygen species (ROS) which is evident by increased activity of hepatic xanthine oxidase of the host [38]. The ROS generated in host-parasite interactions can cause lysis of host erythrocytes and alteration in major antioxidants of erythrocytes. Higher production of free radicals leads to an increased rate of lipid peroxidation which causes impairment of membrane functioning and inactivation of membrane bound receptors and enzymes [39]. It has been also shown that *Plasmodium* infected RBCs produce higher concentration of H_2O_2 and free radicals [40],

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which further results in increased breakdown of heme and finally leading to oxidative stress.

P. yoelii infection caused decrease in catalase and SOD activity which may be due to low level of superoxide ions during hemoglobin digestion. The decline in catalase and SOD activity may also be due to development of higher oxygen tension conditions due to parasitic oxidative stress [41]. The increased activity of glutathione reductase and glutathione peroxidase in *P. yoelii* infected RBCs may be an attempt of the host to neutralize the environment of oxidative stress generated due to the presence of the parasite. Increased level of GSH has been reported in many studies [42-43], which play a significant role in protecting cells against oxidative stress mediated by parasites.

Treatment with Artemisinin derivatives resulted in a significant decrease in the levels of antioxidant enzymes and has been shown to affect from other oxidant drugs. Glutathione peroxidase activity was highly suppressed in infected RBCs, plasma and *P. yoelii* parasites upon treatment with Artemisinin derivatives under *in-vitro* conditions indicating its major role in antioxidant defense. The level of nitrite, H_2O_2 and N-acetylglucosaminidase activity were also decreased after treatment with Artemisinin derivatives. Lysosomes are intracellular membrane bound collection of hydrolytic enzymes and are mainly activated in macrophage during endotoxemia and help in phagocytosis [44]. Alterations in macrophage function and impairment of antigen processing have been reported during *P. yoelii* and *P. berghei* infection in mice [30]. The macrophage hydrolytic enzymes may have some advantage for the host and serve as adaptive response to infection [45]. The activity of N-acetyl D-glucosaminidase has been reported to decrease considerably in parasitized erythrocytes membranes [46].

The results of present study suggest that Artemisinin derivatives cause the significant effect on oxidative stress and antioxidant defense indices. Artemisinin combination therapy is currently recommended for the treatment of malaria which may prevent the induction of drug resistance. However, due to multiple mode of action of drugs, the antagonistic effects should be studied.

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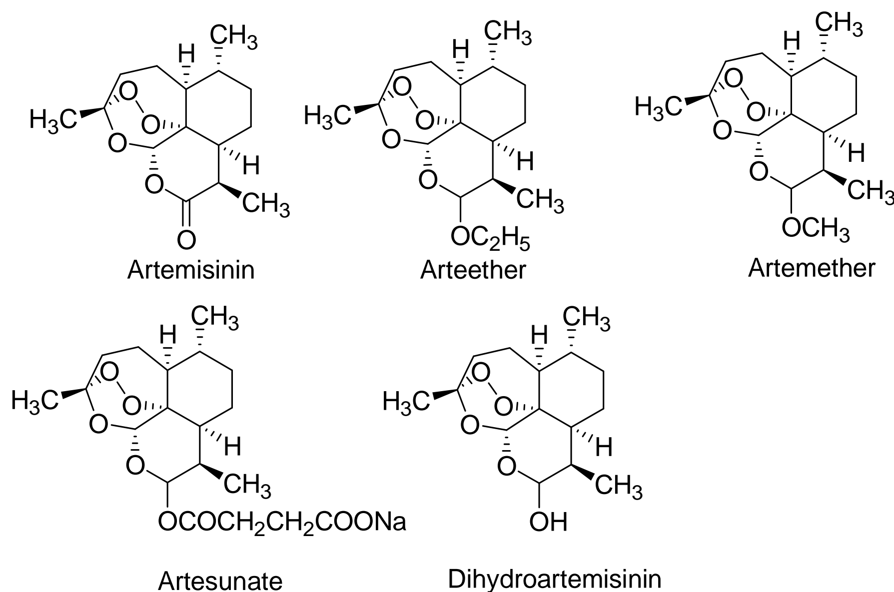

Table 1: Status of antioxidant enzymes during progressive level of parasitemia in *P. yoelii* infected RBCs.

Enzymes	Normal RBC	5%	20 %	60%
CAT	527.95 ± 7.03	407.35 ± 8.0	373.56 ± 4.7	360.58 ± 3.52
SOD	7.76 ± 0.08	3.6 ± 0.04	3.01 ± 0.05	2.56 ± 0.052
GR	0.003 ± 0.00022	0.004 ±0.00014	0.006 ±0.00007	0.008 ± 0.0007
GPx	0.024 ± 0.0075	0.032±0.00750	0.046 ± 0.00605	0.056 ± 0.00315

Values are mean ± SD of triplicate readings.

Catalase units are expressed as μ mole H_2O_2 reduced / min/ ml

SOD units are expressed as amount of enzyme that inhibits the formation of formazone by 50%.

Glutathione reductase units are expressed as μmole NADPH oxidized / min/ ml

Glutathione peroxidase units are expressed as μmole NADPH oxidized / min/ ml

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Table 2: Status of oxidants in normal, infected erythrocytes and *Plasmodium Yoelii*

Samples	Lipid Peroxidation	Nitrite	H_2O_2	Reducing Power
Normal	0.5×10^{-7}	276.3	19.9	3.9
Infected	2.3×10^{-7}	235.5	38.4	2.5
Parasite lysate	7.4×10^{-7}	26.4	8.4	0.15

Nitrite: units are expressed as µM equivalents of Nitrite /ml

H_2O_2: units are expressed as µg /ml

Reducing Power: units are expressed as ∆ OD /ml

Lipid peroxidation: units are expressed as n moles of malonyldehyde formed/hr/mg protein/ml

Table 3: Effects of Artemisinin derivatives on antioxidant system of *P. yoelii* infected RBCs, plasma and parasites lysate.

Samples	Arteether	Artemether	DHA	Artesunic acid
		(Percent inhibition)		
Infected RBCs				
Catalase	16	15	36	40
Superoxide dismutase	22	20	NI	10
Glutathione reductase	5	10	18	15
Glutathione peroxidase	22	50	34	45
Infected plasma				
Catalase	12	11	32	30
Superoxide dismutase	11	8	34	20
Glutathione reductase	NI	NI	NI	NI
Glutathione peroxidase	53	NI	55	68
Parasite lysate				
Catalase	NI	10	NI	NI

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Superoxide dismutase	23	24	14	25
Glutathione reductase	22	23	20	18
Glutathione peroxidase	33	23	22	39

The RBCs, plasma and parasites were collected at 60% parasitaemia and treated with antimalarials at 100 µM for 60 min. The units of antioxidant enzymes are mentioned in table 1.

Table 4: Status of N-acetylglucosaminidase activity, Nitrite and H_2O_2 in normal, infected and drug treated macrophages at (~60% parasitemia).

Macrophages	N-Acetylglucosaminidase	Nitrite	H_2O_2
Normal	26.25	10.59	2.67
Infected	39.37	35.27	6.13
Drug treated			
Arteether (100µM)	31.88 (19%)	35.25 (NI)	5.82 (5%)
Artemether (100µM)	27.16 (31%)	34.21 (3%)	5.45 (11%)
DHA (100µM)	28.62 (27%)	31.74 (10%)	4.10 (33%)
Artesunic acid (100µM)	26.96 (31.5%)	30.15 (14.5%)	4.84 (21%)

N-acetylglucosaminidase units are expressed as µM equivalents of p-nitrophenol /mg protein

The values of Nitrite and H_2O_2 are expressed as mentioned in Table 2. The values in parenthesis represent the percent decrease as compared to macrophages obtained from infected animals.

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