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Oxidative Stress and Micronutrient Therapy in Malaria: An *In vivo* Study in *Plasmodium berghei* Infected Mice

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Abstract: Free radical production from oxidative stress induced by malaria infection plays a major role in the pathogenesis of malaria. However, the use of agents with antioxidant activity may interfere with malaria progression. The study involves an *in vivo* evaluation of the role of some antioxidant micronutrients in the modulation of malaria infection. Rodent malaria model using *Plasmodium berghei* NK-65 strain (chloroquine sensitive) was used for the study. Forty five mice of either sex weighing 20.05±0.02 g were procured for the study. Forty mice were inoculated intraperitoneally with 1×10⁷ million *Plasmodium berghei* infected erythrocyte and were administered with 0.2 mL of distilled water, 0.2 mL of vehicle; Tween 80 (control and vehicle group), chloroquine 25 mg kg⁻¹ and artesunate 4 mg kg⁻¹ (standard drug group), vitamin A 60 mg kg⁻¹, vitamin E 100 mg kg⁻¹, selenium 1 mg kg⁻¹, zinc 100 mg kg⁻¹ (test group F, G, H and I, respectively) 72 hours post inoculation. Antioxidant micronutrients demonstrated significant (p<0.05) schizonticidal activity when compared with negative control during the 4 day curative test. Erythrocyte membrane distability was most markedly elevated in the tween 80 group (426.15%), followed closely by the chloroquine (373.85%) treated group and artesunate group (329.23%) and least in the zinc treated group (32.31%). There was no significant (p>0.05) difference in MCFI values (0.115±0.002; 0.114±0.002 g dL⁻¹) between vitamin A treated group and selenium treated group respectively. However, this was significant (p<0.05) between the micronutrient treated groups and the control (negative, positive and vehicle). Conclusively, antioxidant micronutrients have antimalarial activity which may be due potentiation of erythrocyte membrane stabilization.

Key words: Oxidative stress, micronutrient therapy, erythrocyte membrane stability, potentiation, malaria, free radicals

INTRODUCTION

Micronutrients are known to be an integral part of endogenous antioxidants and are known to influence malaria progression in man. A randomized trial in Papua New Guinea has shown that periodic supplementation with vitamin A reduced the incidence of febrile episodes and parasitaemia due to *Plasmodium falciparum* (Hussey and Clements, 1996; Shankar *et al.*, 2000). Also, vitamin A is essential for normal immune function and has been shown to influence both antibody response and cell-mediated immunity (Semba, 1998). Other studies have documented that antioxidants such as carotenoids, vitamins C and E could provide protection against oxidative stress induced by malaria infection (Adelekan *et al.*, 1997). Zinc deficiency has been observed to decrease the ability of the body to respond

to infection, affecting both cell mediated immune responses as well as humoral immune responses (Okochi and Okpuzor, 2005). In another study, the morbidity and outcome of avian malaria infection with *Plasmodium spartani* was more severe in ducklings fed with vitamin E and selenium deficient diets than in ducklings fed with vitamin E and selenium adequate diets (Yarrington *et al.*, 1973). Free radicals produced from oxidative stress are aggravated in malarial infection which leads to decrease in the antioxidant defense system. Consequently, oxidative stress in malaria infection can result in the development of malarial anemia (Kremsner *et al.*, 2000; Clark and Hunt, 1983). Hence, there is need to establish the mechanism that underlies the antimalarial activity of antioxidant micronutrients as well as determine its role in malarial induced oxidative stress.

MATERIALS AND METHODS

Materials: Chemicals and equipments: Heparinised capillary tubes, Light Microscope (Olympus, Japan), EDTA bottles, Feeding trochars, Syringes (1 mL, 5 mL), Cotton wool, Microscopic slides (Olympus, China), Hand gloves, Giemsa stain (Sigma), 98% Methanol (Sigma) and Tween 80 (sigma).

Drugs: Vitamin A (Clarion Medical Pharmaceuticals, Nigeria), Vitamin E (Clarion Medical Pharmaceuticals, Nigeria), Zinc gluconate (Mason Vitamins Incorporated USA), Selenium-organic (Mason Vitamins Incorporated USA), Chloroquine (Emzor Pharmaceuticals, Nigeria) and Artesunate (Emzor Pharmaceuticals, Nigeria).

Preparation of animals: Forty five in bred and pure Swiss albino mice of either sex weighing between 18- 25 g was used for the study. They were obtained from the animal house of the Nigerian Institute of Medical Research, Yaba Lagos State and housed in stainless steel cages with wire screen top. The animals were about 7-8 weeks old and were maintained on commercial feeds (Vital feeds, Jos) and tap water *ad libitum* for the entire duration of the study. The mice were allowed to acclimatize for 1 week in the laboratory environment under a controlled temperature of 20°C and at optimum humidity before being subjected to the experiment (Obernier and Baldwin, 2007). Good hygiene was maintained by constant cleaning and removal of faeces and spilled feeds from the cages daily.

Preparation of Inoculum of Chloroquine Sensitive Strain of *Plasmodium berghei*: *Plasmodium berghei* NK 65 strain maintained in the laboratory of Nigerian Institute of Medical Research, Yaba by serial blood passage from mouse to mouse was used for the study. Donor mouse with a rising parasitaemia of 20-30% confirmed by thin and thick blood film microscopy was used. Blood (0.2 mL) was collected in a heparinized tube from the auxiliary plexus of veins in the donor mouse using heparinized capillary tubes. The blood was diluted with 5 mL of Phosphate Buffer Solution (PBS) pH 7.2 so that each 0.2 mL contained approximately 1×10^7 infected red cells (Peter *et al.*, 1975; Fidock *et al.*, 2004). Each animal received inocula of about 10 million parasites per kilogram body weight, which is expected to produce a steadily rising infection in mice.

Preparation of drugs

Chloroquine: Fifty milligram of powdered chloroquine sulphate were dissolved in 20 mL of distilled water. So that 1 mL will contain 2.5 mg of chloroquine sulphate. Dosage administered to the animals in the standard drug group (A) was 25 mg kg⁻¹. Hence the 0.2 mL of solution administered contained 0.5 mg of chloroquine sulphate.

Artesunate: Four milligram of artesunate powder was dissolved in 10 mL of distilled water. Hence, 1 mL of distilled water contained 0.4 mg of artesunate. Dosage administered was 4 mg kg⁻¹ equivalent to 0.2 mL of solution.

Vitamin A: Two hundred thousand International Units of vitamin A caplet, which is equivalent to 60 mg of vitamin A, was used to prepare the dose administered (60 mg kg⁻¹). The drug was dissolved in 0.2 mL of Tween 80 used as a vehicle and distilled water in a ratio of 0.2:0.2:9.6 to make up a total volume of 10 mL. The final volume of drug administered was 0.2 mL, which is equivalent to 0.495 mg of vitamin A.

Vitamin E: One hundred milligram of vitamin E caplet was dissolved in 0.2 mL of Tween 80 and distilled water in a ratio of 0.2:0.2:9.6 making up a total volume of 10 mL. The dose administered to the animal was 100 mg kg⁻¹. Hence, the final volume of drug administered to the animal was 0.2 mL, which is equivalent to 1.6 mg of vitamin E.

Selenium: One milligram of selenium was dissolved in 10 mL of distilled water in its powdered form. A dose of 1 mg kg⁻¹ b.wt. was administered to the animals. The final volume of drug administered was 0.2 mL equivalent to 0.0145 mg of selenium.

Zinc: The dose of zinc administered was 100 mg kg⁻¹. 100 mg of zinc was dissolved in 10 mL of distilled water in its powdered form. 0.2 mL of the solution was administered which is equivalent to 1.91 mg of zinc.

Drugs/micronutrient administration: A 4-day curative test was performed using the methods of Peters, (1965). Peter *et al.* (1975) and Fidock *et al.* (2004). Mice were grouped into nine groups of 5 and drug/micronutrient administration was done daily for 4 days as shown in Table 1. Antioxidant micronutrients were administered orally using doses based on LD₅₀ values as reported by Schrauzer (2000), Oncu *et al.* (2002) and Oreagba and Ashorobi (2006), while the standard dose of chloroquine and artesunate were used.

At the end of the 4 day curative treatment (day 5 post treatment) blood samples were collected via the auxiliary vein into EDTA specimen bottles for laboratory analysis.

Laboratory analysis

Osmotic fragility test: Erythrocyte osmotic fragility was determined as described by Azeez and Oyewale (2010); Alanazi (2010). 0.02 mL of blood was added to tubes containing increasing concentration of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4 (0, 0.1, 0.3, 0.5, 0.7, 0.8 and 0.9%). The tubes were mixed and incubated at room temperature (29°C) for 30 min. The content of each tube was then centrifuged at 3500 rev min⁻¹ for 10 min. The relative amount of hemoglobin released into the supernatant was determined spectrophotometrically at a maximum wave length of 540 nm. The quotient of absorbance of the content of individual test tubes that caused 50% lyses of red blood cells was the Mean Corpuscular Fragility Index (MCFI) (Chikezie, 2007). This was extrapolated from the Osmotic Fragility Curve (OFT) obtained by plotting the percentage lysis against saline concentrations. The relative capacity of the antimalarials and antioxidant micronutrients to stabilize or destabilize red blood cell membrane was evaluated as percentage of the quotient of the difference between the MCFI values of the test and control samples (Chikezie, 2007). Thus:

$$\% \text{ Stability or distability} = \frac{\text{MCFI test} - \text{MCFI control}}{\text{MCFI control}} \times 100$$

Data analysis: Statistical analyses of the data were performed using statistical soft ware package SPSS version 17.0. Student's t-test and one way ANOVA were used to compare the mean of laboratory data between groups. The statistical significance level was set at 95% confidence interval and p-value<0.05 was considered significant.

RESULTS

Table 2 revealed the mean % hemolysis in the treated groups at varying saline concentration (0.0-0.9 g dL⁻¹). This was used to plot the osmotic fragility curves as shown in Fig. 1a-i. The osmotic fragility curve showed a similar sigmoidal pattern from which the MCFI was extrapolated after a 4 day curative treatment of established *Plasmodium berghei* infection in mice. The general hemolytic trend observed was a decrease in % hemolysis with increasing saline concentration in all the micronutrient treated groups when compared to the negative and vehicle control groups.

As shown in Table 3, there was a significant difference in the Mean Corpuscular Fragility Index (MCFI) between groups (F = 2275.65; p<0.05). This determines the % erythrocyte membrane stability or disability of the treatment groups. However, there was no statistically significant difference (p>0.05) in MCFI values of vitamin A and selenium treated groups. Erythrocyte membrane disability (Fig. 2) was most marked in the tween 80 group (426.15%), followed closely by the chloroquine (373.85%) treated group and artesunate group (329.23%) and least in the zinc treated group (32.31%).

DISCUSSION

The findings of the present study connote erythrocyte membrane protection from oxidative stress

Table 1: Drug administration (per os) in animals

Groups	Drugs/Micronurients	Dosage
A: Uninfected mice (control group). Not inoculated, no drugs administered	-	-
B: Parasitized mice (negative control group)	Distilled water	0.2 mL
C: Parasitized mice (vehicle control group)	Tween 80	0.2 mL
D: Parasitized mice (positive control treated)	Chloroquine sulphate	25 mg kg ⁻¹
E: Parasitized mice (positive control)	Artesunate	4 mg kg ⁻¹
F: Parasitized mice (test group 1)	Vitamin A	60 mg kg ⁻¹
G: Parasitized mice (test group 2)	Vitamin E	100 mg kg ⁻¹
H: Parasitized mice (test group 3)	Selenium	1 mg kg ⁻¹
I: Parasitized mice (test group 4)	Zinc	100 mg kg ⁻¹

Table 2: Percent hemolysis in different groups after 4 days curative test in *P. berghei* parasitized mice

Conc. Nacl g dL ⁻¹	Hemolysis (%)								
	Control(not inoculated)	Dist. H ₂ O	Tween-80	CQ	Arte	Vit A	Vit E	Selenium	Zinc
0	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00
0.1	17.94±0.34	41.2±2.69	40.62±1.07	42.7±0.84	41.74±0.20	28.02±0.42	25.38±1.42	22.70±0.93	23.48±0.74
0.2	14.94±0.38	30.90±0.38	39.48±0.49	39.76±0.47	33.16±1.72	23.42±0.42	22.38±0.31	27.50±0.58	24.92±0.90
0.3	27.44±0.58	38.24±0.30	39.20±0.89	39.98±1.26	49.86±1.02	33.10±2.18	30.54±0.98	28.60±0.56	24.64±0.91
0.4	31.40±0.99	34.82±1.05	56.28±2.57	45.88±1.22	38.16±0.48	26.30±0.95	42.12±2.08	28.90±0.49	27.04±0.43
0.5	33.54±1.69	33.98±1.23	55.30±2.29	46.70±0.43	47.10±0.60	22.52±0.36	25.04±4.52	26.06±0.22	24.36±0.92
0.6	25.82±1.49	34.62±0.55	41.52±0.18	38.86±0.33	30.44±0.23	24.02±1.88	18.70±0.81	26.72±0.94	25.66±1.09
0.7	24.08±0.96	31.46±0.40	35.22±0.28	30.90±0.25	20.56±0.36	27.16±0.52	7.60±0.51	19.92±1.54	23.34±0.37
0.8	6.78±0.80	17.10±1.08	13.82±0.57	20.50±0.33	13.94±0.36	21.96±0.28	14.98±9.76	12.68±1.45	17.86±0.82
0.9	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

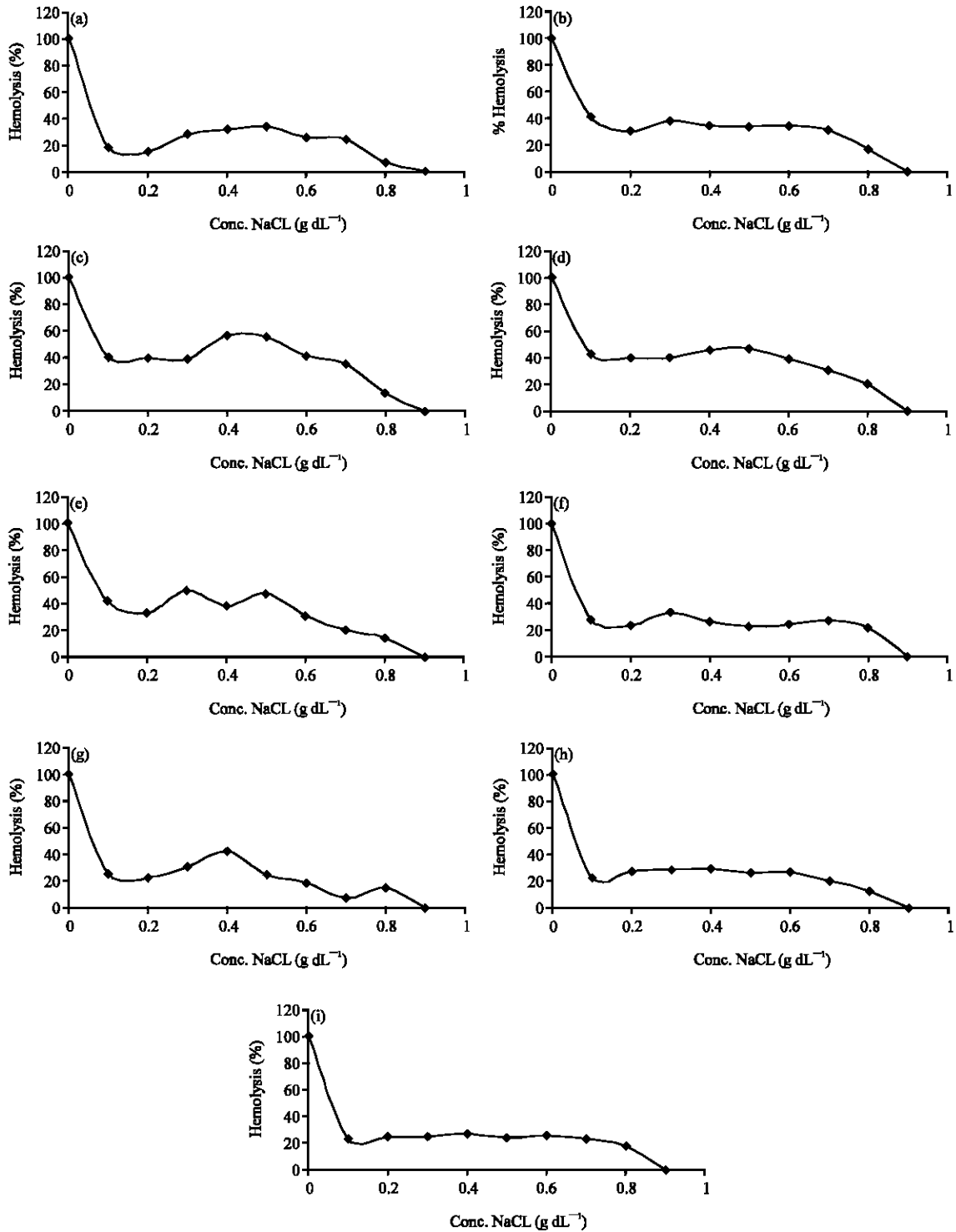


Fig. 1(a-i): Osmotic fragility curve in, (a) Control group MCFI = 0.065, (b) Dist. H2O Group MCFI = 0.239, (c) Tween 80 Group MCFI = 0.343, (d) Chloroquine Group MCFI = 0.308, (e) Artesunate Group MCFI = 0.279, (f) Vitamin A Group MCFI = 0.115, (g) Vitamin E Group MCFI = 0.129, (h) Selenium Group MCFI = 0.114, (i) Zinc Group MCFI = 0.08

Table 3: Mean Corpuscular Fragility Index and % Membrane Disability after 4 Day Curative Test in *P. berghei* Parasitized Mice (n=5mice each)

Group	Dose mg kg ⁻¹	Parasitemia (%) (g dL ⁻¹)	MCFI	Membrane Distability(%)
Control (not inoculated/no treatment)	-	-	0.065±.003	0.00
Negative control (Distilled H ₂ O)	0.2 mL	24.14±2.59	0.239±0.03	267.69
Tween-80	25	0.00±0.00	0.308±0.001	373.85
Positive control (Chloroquine)	4	0.00±0.00	0.279±0.002	329.23
Artesunate	60	8.90±2.78	0.115±0.002 ^{a*}	76.92
Vitamin A	100	11.61±2.78	0.129±0.002 ^{a*}	98.46
Vitamin E	1	5.08±1.85	0.086±0.003 ^{a*}	75.38
Selenium zinc	100	9.89±1.11	0.114±0.002 ^{a*}	32.31
One way anova			F = 2275.65 p<0.05	

Values are expressed as Mean±SEM. df = 4, Mean difference is significant at *p<0.05 when compared with control (negative, positive and vehicle). ^aNo significant difference in MCFI value between vitamin A and selenium treated groups

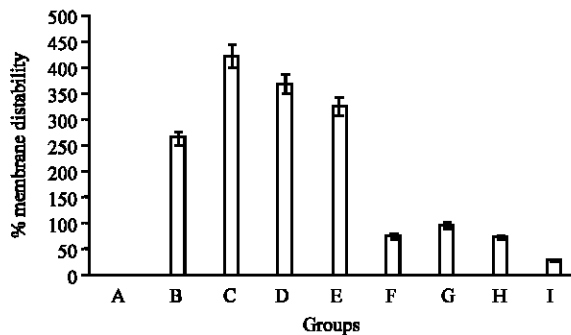


Fig. 2: Percent Membrane distability in different groups after 4 day curative test in parasitized mice, Key: A: Control (no inoculation, no treatment), B: Dist. H₂O, C: Tween-80, D: Chloroquine, E: Artesunate, F: Vit A, G: Vit E, H: Selenium, I: Zinc

during malaria infection. This proposed mechanism is further corroborated by the work of Stocker *et al.* (1985) which revealed that erythrocyte membranes are better protected by antioxidants than parasite membrane. According to Kraus *et al.* (1997), vitamins C and E supplementation reduced erythrocyte osmotic fragility and oxidative damage in rats. Similarly, exercise stress, heat stress and other forms of oxidative stress have been associated with increased erythrocyte osmotic fragility, concurrently with elevated levels of Thiobarbituric Acid Reacting Substances (TBARS) and Malondialdehyde (MDA) which are products of lipid peroxidation in the erythrocyte membrane (Kelle *et al.*, 1999; Ozturk and Gumuslu, 2004). Damage to the erythrocyte membrane proteins and lipids due to oxidative stress leads to

hemolysis as a result of destruction of the spectrin bands which is the base of the erythrocyte cytoskeleton (Reid and Mohandas, 2004). Also damaged by oxidative stress according to Reid and Mohandas (2004) are band 3, glycophorin C and RhAG; the membrane proteins that link the lipid bilayer to the spectrin cytoskeleton. These linkages play a significant role in regulating cohesion between the lipid bilayer and the cytoskeleton; the loss of which results in lipid loss, decreased membrane surface area and loss of deformability of erythrocytes. Erythrocyte membrane appears to be protected by the antioxidant micronutrients (vitamin A, E, Zinc and Selenium) as evidenced by a significantly lower MCFI in the antioxidant treated groups when compared to the infected control groups. This proposed mechanism is further corroborated by the work of Stocker *et al.* (1985) which revealed that erythrocyte membranes are better protected by antioxidants than parasite membrane. According to Kraus *et al.* (1997), vitamins C and E supplementation reduced erythrocyte osmotic fragility and oxidative damage in rats. Similarly, exercise stress, heat stress and other forms of oxidative stress have been associated with increased erythrocyte osmotic fragility, concurrently with elevated levels of Thiobarbituric Acid Reacting Substances (TBARS) and Malondialdehyde (MDA) which are products of lipid peroxidation in the erythrocyte membrane (Kelle *et al.*, 1999; Ozturk and Gumuslu, 2004). Additionally, the % membrane distability was also significantly lower in the antioxidant groups when compared to infected control. Also among the micronutrient treated groups, MCFI appears to be lower in the zinc treated group when compared to other micronutrient groups. This finding is supported by other studies which revealed that dietary zinc deficiency in rats is associated with increased hemolysis of erythrocytes in hypotonic saline (O'Dell *et al.*, 1987; Paterson and Bettger, 1985; Roth and Kirchgessner, 1994) and in the presence of various detergents, alcohols and toxins (Paterson and Bettger, 1985). Additionally, alterations in the composition of the erythrocyte membrane have been detected in zinc-deficient rats (Avery and Bettger, 1988, 1992; Driscoll and Bettger, 1991; Johanning and O'Dell, 1989; Paterson *et al.*, 1987). These findings corroborate the finding of the present study which revealed that zinc had the lowest MCFI value among the antioxidants used for treatment of malaria. *In vitro* addition of zinc to red blood cells is also protective against hemolysins (Avigad and Bernheimer, 1976; Takeda *et al.*, 1977). Consequent upon these findings, it is pertinent to state that with membrane stabilization, hemolysis is impaired, merozoite release is reduced and progressive parasitization of uninfected RBC is also significantly

reduced. It has been revealed that the trace element zinc plays an important role in the structure and function of biological membranes (Bettger and O'Dell 1993) which also corroborates the present study. Oxidative modifications of the membrane increases fragility of red blood cells (Stern 1986; Wagner *et al.*, 1988). Because there is some evidence for a physiological role of zinc as an antioxidant (Bray and Bettger, 1990), greater oxidative damage in zinc deficiency could be responsible for impaired stability of erythrocytes. In a previous study by Kraus *et al.* (1997), enrichment of the diet with antioxidants in combination (vitamin C, vitamin E and β -carotene) prevented the elevated osmotic fragility of erythrocytes in zinc-deficient rats. Indeed, this suggested an important role of oxidative damage in the impaired stability of erythrocytes in zinc deficiency. Vitamin E on the other hand is a natural constituent of biological membranes; it acts as an antioxidant by donating hydrogen atom at 6-hydroxyl group on the chromatin ring and by scavenging singlet oxygen and other reactive species (Lee *et al.*, 2004; Powers and Jackson, 2008). An association between Reactive Oxygen Species (ROS) generation and erythrocyte loss has been observed in malarial infection, when such markers were measured in the erythrocyte (Das and Nanda, 1999). The malarial parasite is known to perturb the lipid composition of the host RBC (Sherman, 1979), with RBCs in *in vitro* culture showing an increase in total lipid content and a decrease in percentage PUFA in the erythrocyte membrane (Hsiao *et al.*, 1991). The effect of ROS on erythrocytes is probably a balance between the parasite and host response. The significant reduction in membrane percentage PUFA and α -tocopherol concentration in malarial subjects supports oxidative stress. Wo and Yang (1986) observed that during the ageing of erythrocyte membrane, the spectrin content, Na/K-ATPase activity as well as the lipid fluidity were obviously decreased. However, supplementation of a trace amount of a selenium compound (Na_2SeO_3) in the medium prevented the dissociation of spectrin from membrane and delayed the changes of Na/K-ATPase activity and lipid fluidity. The effectiveness is proportional to selenium concentration within the range of 0.1-1.0 ppm (Wo and Yang, 1986). A similar effect of supplementation of selenium on the intact erythrocytes during ageing has also been observed (Wo and Yang 1986). This supports the potent membrane protective role of selenium as evidenced in the present study which showed a significantly lower MCFI and % membrane distability when compared to other treated groups. The protective action of selenium on biomembranes is generally interpreted in terms of the

activity of selenium-containing Glutathione Peroxidase ($\text{GP}\times$). Findings from this present study has further reiterated the fact that antioxidant play a significant role in membrane protection. This is further corroborated by the findings of Wambi *et al.* (2008) which observed that dietary antioxidant supplements increased survival when administered as a preventive measure prior to radiation exposure as well as when given as treatment after radiation exposure. The administration of dietary antioxidants prior to the radiation exposure was associated with significant protective effects against radiation-induced leukocyte depletion in peripheral blood and bone marrow, suggesting that antioxidants may improve the survival of irradiated animals by attenuating the deleterious effects of radiation on the host immune system. Earlier studies have previously demonstrated the preventive effect of antioxidants against radiation-induced oxidative stress *in vitro* and *in vivo*, which was measured by radiation-induced reductions of serum or plasma total antioxidant status in animals (Guan *et al.*, 2004, 2006; Kennedy *et al.*, 2004; Wan *et al.*, 2005, 2006).

CONCLUSION

Consequent upon these findings, it is pertinent to state that the antimalarial activity exhibited by these micronutrients may be due to membrane stabilization, impaired hemolysis, impaired merozoite release and the inhibition of progressive parasitisation of uninfected RBC.

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