Original Article

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Biochemistry Section

Role of Oxidative Stress on Malarial Anaemia: Significance of Oxidative Stress Index in Patients with Malarial Infection

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ABSTRACT

Introduction: An alteration of oxidant and antioxidant levels is thought to be involved in the pathogenesis of malaria. Limited data is found on association between oxidant and antioxidant status and significance of oxidative stress index in patients with malarial infection and anaemia in south Indian population.

Aim: To measure the levels of oxidants and antioxidants and oxidative stress index in patients with malaria and their association with anaemia.

Materials and Methods: The present case-control study was conducted in Department of Biochemistry, Osmania General Hospital, Hyderabad, India. A total of 50 subjects of malaria aged 20-45 years were included as cases based on inclusion criteria and exclusion criteria. Control group comprised of 50 healthy age and gender matched subjects. Haemoglobin (Hb), serum Malondialdehyde (MDA), Superoxide Dismutase (SOD), Total Antioxidant Capacity (TAC) was estimated in both the groups by standard methods using Ultraviolet Spectrophotometer. Independent sample t-test was used to test for the difference in all biochemical parameters among study and control groups. The study of orrelation among the parameters was done by Pearson's correlation.

Results: Both the groups were age and gender matched (p=0.39). MDA was significantly elevated in cases as compared to controls (p<0.0001). SOD, Ferric Reducing Ability of Plasma (FRAP), Hb were significantly lower in cases as compared to controls with p-value <0.0001. In the present study, it was found that there was a significant negative correlation between Hb and oxidative stress index (MDA/FRAP=-0.480) with p-value=0.0001 in patients with malaria.

Conclusion: The present study concluded that oxidative stress might be a cause of malarial anaemia. Alterations in oxidant and antioxidant levels might have a role in pathogenesis of malaria. As compared to individual markers, oxidative stress index was found to be significantly associated with malarial anaemia denoting its role as a better marker in these patients. Antimalarial therapy targeting the above factors might decrease the pathogenesis, morbidity and mortality in patients with malaria.

Keywords: Antioxidants, Malaria, Malondialdehyde, Total antioxidant capacity

INTRODUCTION

Malaria is a mosquito-borne infectious disease of the blood caused by the parasite *Plasmodium*. Five species of *Plasmodium* that infect human are known. They are *Plasmodium falciparum*, *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae,* and *Plasmodium knowlesi* [1]. Malaria is estimated to affect approximately 200 million people each year and remains a leading cause of death worldwide, it is estimated to account for more than 500 thousand deaths per year with the major burden of disease occurring in resource poor areas of the world [2].

Reactive Oxygen Species (ROS) is a collective noun for oxidising compounds such as superoxide oxygen radical (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^·$), lipid peroxides, and other related species [3]. Production of these occurs as a physiological response to a specific noxa. Alteration in oxidant and antioxidant levels is seen in patients with malaria. Increased oxidative stress is one of the major reasons for development of malarial anaemia [4]. The sources of generation of ROS (oxidative stress) during malaria infection might be in appropriate host immune response, release from macrophages during phagocytosis and by the malarial parasite itself during degradation of haemoglobin. These ROS being non specific in nature, cause lipid peroxidation and death of even non parasitised red blood cells. More so a decrease in antioxidant defense system was observed in these subjects probably due to usage of erythrocytic antioxidants by parasite to counter the defense mechanism of host [2,5].

Recently, the study was done by Tyagi AG et al., they opined that erythrocyte antioxidant enzymes, reduced Glutathione (GSH) and Malondialdehyde (MDA) may be considered to be reliable biochemical markers for diagnostic and therapeutic potential in malaria [1]. Association between oxidant and antioxidant status and significance of oxidative stress index in patients with malarial infection with anaemia patients in South Indian population is not known. Hence, the present study was aimed at determining the alterations of oxidants (MDA) and antioxidants (SOD and TAC) in patients with malaria, and the relationship between oxidants and antioxidant status with anaemia in patients with malaria.

MATERIALS AND METHODS

The present study was a case-control study which was conducted in Department of Biochemistry, Osmania General Hospital and Osmania Medical College in collaboration with Malaria department of Sir Ronald Ross Institute of Tropical and Communicable Diseases, Hyderabad during the period of April 2012 to 2017. Institutional Ethical Committee clearance was obtained prior to start of the study (IEC approval no:26/116/2011).

Sample size was calculated (n=2/d2*Cp Power) based on previous literature [5,6]. A total no. of 100 subjects were included in this study. Case group consisted of 50 malaria patients diagnosed on the basis of clinical findings and positive peripheral blood smear for malarial parasite. Control group consisted of 50, age and gender matched healthy subjects. All the subjects were between the age group of 20-45 years. The reason for selecting this age group was to avoid the effect of changes in oxidative stress and antioxidative capacity with regards to age.

Inclusion and Exclusion criteria: Newly diagnosed smear positive cases of malaria before the start of antimalarial therapy were

included in the study. Selection of patients was based on positive clinical findings for malaria and smear positivity for malarial parasite. A total of 416 patients were suspected for malaria based on clinical symptoms of which 12% (50 out of 416) showed a thin blood smear examination positivity among which 6.6% were positive for *Plasmodium falciparum*, 4.6% were positive for *Plasmodium vivax* and 0.8% was mixed. The average duration of illness being 16 days. Patients who were already undergoing treatment for malaria, patients who were on any form of antioxidant supplementation, pregnant women, patients who had recently undergone surgery. Critically ill patients, patients with tuberculosis, granulomatous diseases, cancer, immuno compromised patients were excluded from the study. Informed consent was obtained prior to sampling.

Procedure

Under aseptic conditions, before giving antimalarial therapy for patients, 4 mL of random venous blood in plain tube and 3 mL in ethylenediaminetetraacetic acid (EDTA) tube was collected from antecubital vein from all the study participants. Additive free sample was allowed to clot for 20 minutes and centrifuged at 3000 rpm (revolutions per minute) for 10 minutes and serum was separated within two hours of collection of blood. Appropriate sample handling was done to prevent sample haemolysis. Sample rejection criteria included the non acceptance of icteric and lipemic samples. The serum sample was used for analysis of MDA by thiobarbituric acid reactive substances method by U/V spectrophotometer [7,8], serum SOD was estimated by the inhibition of auto oxidation of adrenaline method [9], Serum TAC-FRAP was analysed by spectrophotometric method by U/V Spectrophotometer [10]. Whole Blood (EDTA) was analysed for Hb by an automated hematology analyser [11]. All the above parameters were analysed on the same day of sample collection.

STATISTICAL ANALYSIS

Study of data distribution was done by using Kolmogorov-smirnov test. Data obtained was expressed as Mean±Standard Deviation (SD) for data showing a normal distributed, median inter quartile range for data which showed a non normal distribution. Parametric independent sample t-test was used to test for the difference in all biochemical parameters among study and control groups. The study of correlation among the parameters was done by Pearson's correlation or Spearman rank correlation analysis as appropriately. Statistical analysis was performed using Microsoft Excel Spread Sheet (Microsoft Redmond, USA), and Statistical Package for the Social Sciences (SPSS) for windows version 16.0 (SPSS Inc, Chicago, IL, USA). A p-value of <0.05 was considered as statistically significant.

RESULTS

Both the groups were age and gender matched (p=0.39) [Table/Fig-1]. Hb was significantly lowered in patients with malaria compared with controls (p<0.0001). MDA levels were significantly higher in patients with malaria compared with controls (p<0.0001). Hb, SOD and FRAP levels were significantly lower in cases when compared to control group with p-value <0.0001 [Table/Fig-2]. [Table/Fig-3] showed that no significant correlations were observed between Hb and markers of oxidant and antioxidant status in patients with malaria. Hb was negatively correlated with MDA/FRAP, which was statistically significant (r=-0.480, p=0.0001). [Table/Fig-4] shows sensitivity and specificity. MDA and SOD showed 100% sensitivity, specificity of 98% and diagnostic efficiency of 99%.

Parameters	Cases	Controls	p-value
Age (years) Mean±SD	32.88±8.63	31.66±5.36	0.39
Gender (M:F)	34:16	30:20	NA
Ratio (M:F)	2.1:1	1.5:1	NA

[Table/Fig-1]: Demographic data of patients with malaria and controls. n=50.
SD: Standard deviation; n: Number of samples; p: Probability value; M: male; F: Female; M/F: Male/Female ratio

Parameters	Controls	Cases	p-value
Hb (gm/dL)	13.1±1.2	10.8±1.2	<0.0001*
MDA (n.mol/dL)	205.2±39.7	607.1±215.2	<0.0001*
SOD (units/g protein)	99.6±17.7	52.4±12.6	<0.0001*
TAC (FRAP) (µmol/L)	899.8±110.8	694.6±139.0	<0.0001*
UA (mg/dL)	4.90±1.01	7.25±0.99	<0.0001*
Total proteins (g/dL)	7.206±0.616	7.208±0.758	0.988

[Table/Fig-2]: Oxidant and antioxidant status in patients with malaria compared with controls. n=50.
Student t-test was used; *: statistically significant; n: Number of samples; gm/dL: Grams per deciliter; n.mol/dL: Nano mole per deciliter; units/g. protein: Units/gram protein; µmol/L: Micro mole per liter; Hb: Haemoglobin; MDA: Malondialdehyde; SOD: Superoxide dismutase; FRAP: Ferric reducing ability of plasma; UA: Uric acid; *: statistically significant; NS: Not significant

Parameter	Markers	r-value	p-value
Haemoglobin	MDA	0.258	0.070
	SOD	-0.234	0.102
	FRAP	-0.118	0.416
	MDA/FRAP	-0.480	0.0001**

[Table/Fig-3]: Pearson correlation analysis of markers with haemoglobin in patients with malaria.
**Correlation is significant at the 0.01 level (2-tailed); r: Pearson correlation coefficient; NS: not significant; Hb: Haemoglobin, MDA: Malondialdehyde; SOD: Superoxide dismutase, FRAP: Ferric reducing ability of plasma

Markers	Area under curve	Best cut-off	Sensitivity %	Specificity %	Diagnostic efficiency %
MDA (n.mol/L)	0.998	282.6650	100	98	99
Total proteins (gm/dL)	0.520	7.2500	48	62	55
UA (mg/dL)	0.949	6.0500	88	90	87
Hb (gm/dL)	0.900	11.6000	90	82	86
SOD (units/gm. protein)	0.998	78.0750	100	98	99
TAC (µmol/L)	0.867	749.5000	88	68	73

[Table/Fig-4]: Area under the curve, best cut-off values, sensitivity, specificity, diagnostic. efficiency at best cut-off values in discriminating controls and malaria cases.
Hb: Haemoglobin; MDA: Malondialdehyde; SOD: Superoxide dismutase; FRAP: Ferric reducing ability of plasma; UA: Uric acid, Hb: Haemoglobin; TAC: Total antioxidant capacity; µmol/L: Micro mole per liter; gm/dL: Gram per deciliter; m.mol/L: Nano mole per liter

DISCUSSION

In developing countries approximately 2-3 million deaths per year are due to malaria signifying it as one of the major health problems. Alteration in oxidant and antioxidant status has been observed in patients with malaria [5]. Anaemia is seen in patients with malaria. In the present study, a significant decrease in Hb was seen in cases as compared to controls. In the life cycle of malaria man is considered as a secondary host. Sporozoites inoculated by bite of female anopheles mosquito reach the blood stream and later invade hepatocytes. Here they multiply and produce merozoites. These merozoites invade red blood cells and continue further cycle of asexual reproduction known as erythrocyte schizogony [12,13]. The parasite degrades haemoglobin and uses it as a food material for fulfillment of its need for amino acids [6]. Loria P et al,. observed that Hb concentration of a parasitised erythrocyte is only 25% as compared to non parasitised erythrocyte [14]. Moreover, oxidative stress causes haemolysis and is a major reason for development of malarial anaemia [4,15]. Hence, both haemolysis and Hb degradation contribute to malarial anaemia. The present study findings were in accordance with other studies [16,17].

A spurious increase in oxidative stress is seen in malaria patients. In the present study, MDA was studied as a marker of lipid peroxidation. Infection by *P. falciparum* even in absence of oxidative stress can cause lipid peroxidation of membrane by activated monocytes present in the blood. An increase in the MDA levels were observed in patients with malaria as compared to healthy controls and this was in accordance with other studies [5,16]. The pathogenic mechanisms

for increase in ROS might be: due to the production by host immune system as a defense mechanism but may be inappropriate which might damage the normal tissues, their release during phagocytosis of malarial pigment containing erythrocytes by the host macrophages which might even effect the non parasitised red blood cells causing their damage, their release by parasitised erythrocyte, their production during the degradation of Hb by parasite [4,17]. This generated ROS cause lipid peroxidation of normal cells causing the death of host cells. Moreover, patients with malaria are suspected to have pro-oxidants. Haemoglobin can act as a pro-oxidant by catalysing the decomposition of lipid hydroperoxides by enhancing chain reaction. Heme break down can be caused by hydrogen peroxide resulting in free iron ions release which form hydroxyl radicals via fenton reaction [18]. Hence, Hb and hydrogen peroxide enhance lipid peroxidation.

An alteration in antioxidant level is seen in patients with malaria. In the present study, there was a significant decrease in antioxidants serum SOD and serum TAC level in concert with the increase in oxidative stress in malaria patients. This decrease was in accordance with other studies [16,17]. This might be due to the utilisation of the host's antioxidant defence such as erythrocytic SOD by the malarial parasites to counteract the oxidative damage [19]. Moreover, the antioxidant enzymes are degraded by malarial parasite to derive aminoacids, and cannot be replenished by red blood cells due to lack of protein synthesis which might be one of the reasons behind overall decrease in SOD activity in malaria patients as compared to controls [15].

The present study found that higher levels of uric acid was found in patients compared to controls. Due to absence of uricase activity in human serum, the final product of hypoxanthine degradation in humans is uric acid. Mutation of the enzyme activity through out the evolution might be the cause for its absence. Hence, higher serum uric acid levels (40-60 µg/mL) are seen in human serum with a lower capacity to control its increase. This may result in an increased prominence of this pathway in the inflammatory response to malaria in humans. The present study supports the view that increased concentrations of soluble uric acid might induce the release of inflammatory mediators from different cell types, suggesting that the soluble uric acid formed via hypoxanthine degradation could be the contributing factor to malaria induced inflammatory response [20-22].

In the present study, it was observed that MDA and SOD shared highest sensitivity and specificity as markers. The uric acid, Hb and TAC showed good discriminatory capacity [Table/Fig-4]. Hence, these markers can be used as multiple markers to identify pathophysiology of malaria. It was found that there were no significant correlations observed between Hb and markers of oxidant and antioxidant status in patients with malaria. In the present study, Hb was negatively correlated with MDA/FRAP, which was statistically significant (r=-0.480, p=0.0001) and which was in agreement with previous studies [4,15]. Oxidative stress triggers haemolysis and is a main cause for development of malarial anaemia. Hence, Hb degradation contribute to malarial anaemia [4,15]. It was found that oxidative stress index (MDA/FRAP) was better and reliable indicator of the disease rather than individual markers of oxidant and antioxidant status. The previous studies on oxidative stress indices have proven to be reliable, practical, and with clinical utility [23].

Limitation(s)

Large scale study can be carried out with administration of nutritional antioxidants such as vitamin E and C which might have a benefit in the treatment and prognosis of the disease. After an adequate treatment for parasitic clearance, administration of Vitamin E might be helpful to avoid malarial anaemia particularly in cases of *P. falciparum* malaria.

CONCLUSION(S)

The present study concluded that oxidative stress might be a cause of malarial anaemia. Alterations in antioxidant levels were observed in patients with malaria. Oxidative stress index was found to be reliable marker compared to individual markers of oxidative stress. Antimalarial therapy targeting the above factors might decrease the pathogenesis, morbidity and mortality in patients with malaria.

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