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Differential Oxidative Stress and Antioxidant responses in Mild and Severe Malaria

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ABSTRACT:

The aim of this study was to assess the extent of oxidative and antioxidant stress responses during malaria in the endemic settings of Mangaluru in India. We also assesed the possible role of oxidative stressduring mild and severe malaria, malarial anemia and malarial thrombocytopenia. The blood samples from 54healthy controls,202mild malaria and 72 severe malaria patients with *P. falciparum*, *P. vivax,* and mixed infectionswere analyzed for the levels of malondialdehyde, uric acid, superoxide dismutase, TNF-α, IL-6 and IL-10. The data was analysed by Kruskal-Wallis, Mann-Whitney U test and were correlated by Pearson correlation and spearman rank correlations.Compared to healthy controls, the mean malondialdehyde and uric acid levels were significantly higher in malaria patients, especially during severe*P. falciparum* cases. The malondialdehyde levels were directly proportional to increase in parasitic burden, C-reactive protein, uric acid and TNF-α levels. In contrast, an inverse relation was observed between malondialdehyde and hemoglobin levels as well asbetween malondialdehyde and platelet levels. The superoxide dismutase levels were also significantly lower in severe malaria cases and were inversely proportional to increased parasitic burden and C-reactive protein levels.The increased levels of oxidative stress products such as malondialdehyde, uric acid and decreased levels of antioxidants such as superoxide dismutase might have a potential role in progression of mild form of malaria into severemalaria. Thus, we specuulate thatanti-malarial drug treatment coupled with antioxidant supplements is likely to improve the adverse outcomes of malaria infection.

KEY WORDS: Oxidative stress, Antioxidants,*P. falciparum*, *P. vivax*, Severe malaria.

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INTRODUCTION

Oxidative stress (OS) is a result of increased oxidants and decreased antioxidant levels in the host[1]. During the erythrocytic stages of malaria infection, the innate immune cells, especially neutrophils and macrophages, produce reactive oxygen species (ROS), such as anion superoxide radical ($O2^{-\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical (•OH), and reactive nitrogen species (RNS) such as nitric oxide radical (NO•), nitrogen dioxide radical ($NO_2^{\bullet}$) and anion peroxynitrite ($ONOO-$)[1]. ROS plays both protective and pathological roles during malaria infections in a context-dependent manner[1]. Although the ROS production is considered to be an important host defense mechanism in killing parasites, its increased levels can result in anemia[2], thrombocytopenia[3], upregulation of endothelial cell adhesion molecules leading to cerebral malaria and other severe malaria complications[4].

Malondialdehyde (MDA) is a known reactive aldehyde, formed by the degradation of polyunsaturated lipids by ROS. MDA is known to form anadduct between deoxyribonucleic acid (DNA) and proteins. Thus, MDA is known to cause cellular damage and is also considered to be mutagenic. MDA and other lipid peroxidation products, 4-hydroxynonenal (4-HNE), and 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) form oxidation-specific epitopes (OSEs), are recognized by immune receptors such as damage-associated molecular patterns (DAMPS), on immune cells, triggering inflammatory responses[5,6]. These inflammatory cytokines are known to play an important role in immunopathology of malaria. Measurement of MDA levels is most widely used as a biomarker of OS in several disease conditions[7, 8].

Uric acid (UA) is a physiological by-product of nucleic acid metabolism and is an important parasite derived factor that contributes to malaria pathogenesis[9]. UA biosynthesis is catalyzed by xanthine oxidase, which produces reactive oxygen species (ROS) as a by-product[10]. During the growth of blood stage parasite, hypoxathine and xanthine, the precursors of UA are accumulated inside red blood cells (RBCs) and are released upon the rupture of schizont. These precursors are then degraded to UA upon cell senescence. Various *in vitro* studies have identified UA as an important parasite derived factor that induces a strong inflammatory response, leading to the release of cytokines such as TNF-α, IL-1β, IL-6 and IL-10 from innate immune cells[11, 12].

Antioxidants function as an important defense system against pathogenesis by scavenging free radicals[3]. The decreased production of antioxidants in malaria-infected individuals results in increased levels of oxidative products, exerting oxidative stress. Important antioxidants include vitamins A, C and

E, β-carotene, the reduced form of glutathione molecule (GSH), catalase and superoxide dismutase (SOD)[13]. Recent studies suggest SOD as a surrogate marker of severe *vivax* malaria[14].

In the present study, blood samples from mild and severe malaria patients who were treated at the Government Wenlock Hospital in Mangaluru city, India, were analyzed to assess the extent of OS, by measuring the circulating levels of MDA, UA and SOD in patients suffering from mild and severe *P. falciparum* (Pf), *P. vivax* (Pv) and mixed infections. Additionally, the relationship between the OS markers with parasitic burden, inflammatory cytokines, malaria anemia and thrombocytopenia were analyzed and the results are presented here.

MATERIALS AND METHODS

Ethics: The study protocol was approved by the ethical committee of Kuvempu University, Shivamogga, Karnataka; the central ethics committee of NITTE University, Mangaluru, Karnataka, and the Institutional Review Board of Pennsylvania State University College of Medicine, Hershey, PA, USA.

Study site, design and population

A hospital based cross-sectional study was conducted at the Government Wenlock Hospital in Mangaluru city during November 2014 to October 2015. A simple random sampling technique was used to recruit study participants irrespective of their socioeconomic status, sex, and ethnicity. The patients seeking medical attention at the malaria outpatient clinic, as well as inpatients and healthy individuals attending blood bank for blood donations were invited to participate in the study upon obtaining signed informed consent.The sample size was estimated as reported in earlier[15]. Considering a prevalence rate of 23%, a total of 274 infected patients were recruited in the study. A total 54 adults, who were blood donors at the blood bank of the Wenlock Hospital and tested negative for malaria infections, were enrolled as healthy controls (HC); the male to female ratio and age range in the HC group were similar to those of malaria patient group.

A semi-structured questionnaire was used to collect information on age, socio-demographic profile, economic status, education level, occupation, and clinical presentation of patients. Inclusion criteria were adults (>16 years of age), all types of *Plasmodium* (i.e., Pf, Pvand mixed) infections with mild and severe malaria. Exclusion criteria were children, pregnant women and individuals testing positive for dengue, typhoid, hepatitis B and C, HIV and those who used antipyretics prior to diagnosis.

The malarial diagnosis was performed by microscopic examination of Giemsa-stained peripheral blood smears. Briefly, two thick and thin blood slides were prepared from each study participant, stained

with 4% Giemsa stain and observed under the microscope for the presence of *Plasmodium*, and the species type. Parasite densities were quantified as parasites/µl of blood (number of parasites counted/ number of white blood cells (WBCs) counted × total number of WBCs per 1µl of blood) or (number of parasites counted/number of RBCs counted × total number of RBCs per 1µl of blood).

Sample collection and hematological analysis

Upon confirmation of malarial infection, about 3 ml of venous blood samples were drawn from patients before they were given any anti-malarial medications. The blood samples were aseptically drawn into heparin-coated vacutainers for plasma and to clot activator tubes for serum preparation and kept at 4°C until further use. Within 60 minutes of collection, the blood samples were used for hematological analysis and the serum or plasma samples were prepared by centrifugation at room temperature, labelled and stored at -80°C until further use. Hematological analysis of contents such as hemoglobin (Hb), red blood cells (RBCs), and platelets were performed by hematology analyzer (Mind Ray-Biomedical, Shenzhen, China). The blood sugar and C-reactive protein (CRP) levels were determined using commercially available kits (Agappe, India). The samples were also screened for hepatitis B, C, and HIV infections using commercially available ELISA kits (J. Mitra& Co, New Delhi).

Classification of study participants

The healthy individuals visiting blood bank for blood donations and testing negative for malaria by peripheral blood smear were grouped into HC. Malaria patients having low-grade fever, headache or chills, and without symptoms of severe malaria were considered as mild malaria (MM) cases. Patients suffering from severe malarial complications, as defined in WHO guidelines[8], were admitted into the hospital for supportive treatment and were considered as severe malaria (SM) cases.

Measurement of oxidative stress and antioxidant biomarkers

Estimation of MDA levels

The levels of MDA were measured by thiobarbituric acid reactive substances (TBARS) method[9,16].Briefly, MDA in the serum (100µl) was measured by the addition of 0.6% thiobarbituric acid (150µl), 15% trichloroacetic acid (150µl) and 0.2N HCl (100µl). The samples were incubated at 90°C for 10 minutes, allowed to cool at room temperature and centrifuged for 15 minutes at 1200xg. The absorbance of supernatant was measured at 535nm using a spectrophotometer (70UV/VIS Spectrometer, Shimadzu Analytical Pvt. Ltd, New Delhi). The concentration of MDA (nmoles/100ml) is calculated by the formula: OD of sample X total reaction volume/ nanomolar extinction coefficient X sample volume.

Estimation of UA

The uric acid levels were quantified by the commercially available kit (Agape India). Briefly, the serum samples (25µl) and reagent mixture (1ml) were mixed and incubated for 5 minutes at 37°C and the absorbance was measured at 546nm using a spectrophotometer.

Estimation of SOD

The SOD activity in plasma was measured by commercially available kit (Cayman chemical, Ann Arbor, MI, USA). Briefly, radical detector and standards/ samples were added to wells of a 96 well plate (Thermo Fisher Scientific Pvt. Ltd, USA). The reaction was initiated by the addition of xanthine oxidase, incubated at room temperature for 30 minutes and the absorbance was read at 450nm.

Analysis of in flammatory cytokines

The plasma levels of cytokines such as TNF-α, IL-6 and IL-10 were measured by sandwich ELISA, using commercially available kits from R&D Biotech, USA.

Statistical analysis

Statistical analysis was performed by entering the data in Microsoft Excel spreadsheet and analyzed by using GraphpadPrism version-6 (Graphpad Prism software, Inc., San Diego California, USA). Summary statistics were calculated for baseline demographics and quantitative variables. For comparison between various groups, non-parametric Kruskal-Wallis test was used. Pair-wise comparison between two groups was conducted by Mann-Whitney U test, and P values were corrected for multiple comparison error by Bonferroni correction. Correlations between two continuous variables were calculated by Pearson correlation and spearman rank correlations. The P values of <0.05 were considered to be significant.

RESULTS

The demographics of study participants

A total of three hundred twenty-eight (n=328) individuals were enrolled in this study at Government Wenlock Hospital in Mangaluru city. Among the study participants, 54 (16.5% of the total recruited) individuals who attended the blood bank of Wenlock hospital for blood donations and tested negative for malarial infections were considered as HC. Among the 274 malaria infected patients, 202 (73.7%) had MM and were treated at outpatient clinic and sent home, whereas 72 (26.3%) patients suffered from severe malarial complications and were hospitalized for supportive care. The mean age of study participants was 33.9 (ranged 16-65) years. The gender distribution of the patients was 162 (59.1%) males and 112 (40.9%) females. A majority of the infected patients were immigrants (196,

71.5%) who lived in densely populated malaria hotspot areas in Mangaluru city, and had migrated from the northern states of India, including Odisha, Bihar and Assam.

Most of these infected immigrants (138, 50.4%) worked at construction sites of building and roads,26 (9.5%) worked at hotels, 75 (27.4%) were housewives, 24 (8.8%) were students and 11 (4.0%) were into other forms of employment such as business, salesmen etc. A majority of the infected individuals (151, 55.1%) did not have basic education, 74 (27.0%) had primary level of education, 35 (12.8%) had secondary level of education and 14 (5.1%) had college level of education. Most of the infected patients (179, 65.3%) were found to beunaware of the prevention measures to be taken against malarial infections; only 57 (20.8%) used mosquito nets, 77 (28.1%) claimed to remove stagnant water to destroy mosquito breeding sources, 47 (17.2%) had windows covered with mosquito nets, and 79 (28.8%) used mosquito repellents while sleeping (Table 1).

Table 1: Socio-demographic profile of the study participants.

	HC	*P. vivax*	*P. falciparum*	Mixed	Overall (infected)
Total	54	103 (37.6)	107 (39.1)	64 (23.4)	274 (100)
Mild malaria - MM		70 (34.7)	84 (41.6)	48 (23.8)	202 (73.7)
Severe malaria - SM		33 (45.8)	23 (31.9)	16 (22.2)	72 (26.3)
Gender					
Male	32	57 (35.2)	65 (40.1)	40 (24.7)	162 (59.1)
Female	22	46 (41.1)	42 (37.5)	24 (21.4)	112 (40.9)
Age in years (Mean, range)	37.3 (18-58)	33.7 (17-65)	33.5 (16-65)	31.9 (16-65)	33.9 (16-65)
15-25	12	32 (34.4)	35 (37.6)	26 (28.0)	93 (33.9)
26-35	11	32 (41.6)	28 (36.4)	17 (22.1)	77 (28.1)
36-45	17	24 (36.9)	29 (44.6)	12(18.5)	65 (23.7)
>45	14	15 (38.5)	15 (38.5)	9 (23.1)	39 (14.2)
Residence					
Native	42	32 (41.0)	26 (33.3)	20 (25.6)	78 (28.5)
Immigrants	12	71 (36.2)	81 (41.3)	44 (22.4)	196 (71.5)
Education					
No formal education	6	56 (37.1)	63 (41.7)	32 (21.2)	151 (55.1)
Primary	23	32 (43.2)	30 (40.5)	12 (16.2)	74 (27.0)
Secondary	14	12 (34.3)	9 (25.7)	14 (40.0)	35 (12.8)
College level	11	3 (21.4)	5 (35.7)	6 (42.9)	14 (5.1)
Occupation					
Construction worker	5	61 (44.2)	45 (32.6)	32 (23.2)	138 (50.4)
Hotel worker	15	8 (30.8)	12 (46.2)	6 (23.1)	26 (9.5)
Housewives	18	25 (33.3)	32 (42.7)	18 (24.0)	75 (27.4)
Student	9	6 (25.0)	12 (50.0)	6 (25.0)	24 (8.8)
Others	7	3 (27.3)	6 (54.5)	2 (18.2)	11 (4.0)
Knowledge of preventive measures to be taken					
Mosquito nets	31	15 (26.3)	23 (40.4)	19 (33.3)	57 (20.8)
Removal of stagnant water	24	20 (26.0)	32 (41.6)	25 (32.5)	77 (28.1)
Windows netting	48	29 (61.7)	12 (25.5)	6 (12.8)	47 (17.2)
Use of mosquito repellents	43	38 (48.1)	28 (35.4)	13 (16.5)	79 (28.8)
Unaware of preventive measures	5	65 (36.3)	75 (41.9)	39 (21.8)	179 (65.3)

Data represented as number of study participants (percentage- based on total number of infected patients).

Hematological changes during malaria

The hematological parameters of HC and patients withPv, Pfand mixed (Pv and Pf) infections were analyzed and compared. The malarial parasitemia was significantly higher in patients with Pf infections than with Pvand mixed infections (Table 2). Compared to HC, the RBC and Hb levels were significantly lower among malaria patients infected with different parasites ($P \leq 0.05$). Among the infecting species, Hb levels were found to be significantly lower in Pf infections than withPvand mixed infections (Table 2). Compared to HC group, the platelet counts were significantly lower in patients with Pf, Pv and mixed (Pf and Pv) infections ($P \leq 0.001$). The blood sugar levels were also significantly lower in these infected groups compared to HC group ($P \leq 0.001$). The CRP levels were significantly higher in malaria patients than HC group ($P \leq 0.001$), especially during *P. vivax*infection (Table 2).

The levels of TNF-α, in comparison to HC, were significantly increased in all the infecting groups ($P \leq 0.001$). Among the various parasitic groups, Pvinfections resulted in significantly higher TNF-α level. The IL-6 levels were found to be increased in all the parasitic groups, especially during mixed infections. As shown in Table 2, the levels of anti-inflammatory cytokine IL-10, in comparison to HC, were significantly increased in all the groups ($P \leq 0.001$).

Table 2: Changes in hematological and biochemical parameters, oxidative stress biomarkers and inflammatory cytokines across various infecting species.

Parameter	HC	*P. vivax*	*P. falciparum*	Mixed	*P* value (between groups)		
					PvVs Pf	PvVs Mixed	Pf Vs Mixed
% Parasitemia		0.6±0.6	**1.1±1.4**	0.6±0.6	0.0003	0.0506	0.0359
Hemoglobin (g/dl)	12.5±1.0	10.7±3.4	**9.0±3.3**	10.6±3.0	0.0006	0.865	0.0351
RBC ($x10^3/\mu l$)	5.4±0.9	4.4±1.0	4.4±0.9	4.7±1.1	0.9195	0.1448	0.1216
Platelets ($x10^3/\mu l$)	2.0±0.5	1.0±0.6	1.0±0.6	1.1±0.6	0.8389	0.5141	0.3663
Blood Sugar (mg/dl)	106.5±13.1	76.5±20.6	80.0±29.8	81.8±34.4	0.6236	0.644	0.9994
CRP (mg/l)	4.2±1.6	**55.7±14.7**	49.4±20.6	48.0±17.6	0.0027	0.0007	0.5585
Urea (mg/dl)	20.7±5.7	29.6±22.2	30.1±17.8	33.8±20.9	0.3349	0.0108	0.1539
TNF-α (pg/ml)	76.2±35.7	**317.4±212.3**	291.7±263.1	241.0±197.2	0.0474	0.004	0.4881
IL-6 (pg/ml)	102.4±62.0	214.1±136.7	297.7±196.5	**302.8±198.5**	0.002	0.0033	0.0246
IL-10 (pg/ml)	137.8±77.9	698.3±500.5	656.2±472.7	652.8±412.9	0.6155	0.9633	0.7312
MDA (IU/L)	0.8±0.4	2.4±1.6	**3.3±1.7**	2.3±1.6	< 0.0001	0.7125	< 0.0001
UA (mg/dl)	2.2±1.1	3.5±2.0	3.3±1.8	3.5±2.1	0.6053	0.9352	0.7333
SOD (U/ml)	43.8±11.2	46.7±10.3	41.0±11.6	44.2±24.8	0.0005	0.2089	0.0276

Data represented as mean(standard deviation); Comparison of three groups by Kruskal Wallis test; comparison of two groups by Mann-Whitney U test; Significant data are highlighted in bold (i.e., $P \leq 0.05$).

Levels of oxidative stress biomarkers

MDA: The MDA levels, in comparison to HC, were significantly increased ($P \leq 0.001$) across all infecting parasite species. Among the various infecting groups, Pf infections resulted in significantly higher circulating MDA levels (Table 2). Upon comparison between MM and SM patients, the MDA

levels were found to be significantly increased in SM cases across all infecting species, especially during Pf infections (Figure 1, Table 3).A significant positive correlation was observed between the MDA levels and increase in parasitic density duringPf (r=0.2765, *P*=0.0083) infection. The Hb levels were significantly decreased with an increase in MDA levels during Pf (r=-0.3926, *P*<0.0001) and mixed (r=-0.2806, *P*=0.0247) infections. The oxidative stress is known to play a potential role in malarial thrombocytopenia[17]. We observed a significant negative correlation between increased MDA level and decreased platelets levels during Pf(r=-0.1932, *P*=0.0462) infection. The MDA levels also had a positive correlation with increased CRP levels during Pf (r=0.2973, *P*=0.0019) and mixed (r=0.3573, *P*=0.0037) infections.The TNF-α levels were positively correlated with increasing MDA levels during Pf (r=0.3648, *P*=0.0001) and mixed (r=0.3233, *P*=0.009) infections. A significant positive correlation was observed between the MDA and UA levels during Pv(r=0.3939, *P*=0.007) Pf(r=0.1612, *P*=0.0272) and mixed (r=0.2799, *P*=0.0276) infections, whereas a significant negative correlation was observed between MDA and SOD levels in Pf (r=-0.2612, *P*= 0.0066) infections.

UA: The UA levels, in comparison with HC were also found to be significantly increased (*P*≤0.05) regardless of infecting parasite species (Table 2). In comparison to MM patients, the UA levels were significantly higher during severe Pf, Pv and mixed infections(Figure 1, Table 3). A significant negative correlation was observed between the Hb and UA levels during mixed (r=-0.2689, *P*=0.0346) infections. The increased UA levels had a significant negative correlation with decreased platelet levels duringPv(r=-0.3844, *P*<0.0010), Pf (r=-0.2237, *P*=0.0206) and mixed (r=-0.3027, *P*=0.0168) infections. A significant increase in CRP levels was observed with increased UA levels in mixed (r=0.3565, *P*=0.0045) infection cases. TNF-α levels also was also positively correlated with increased UA levels in Pv(r=0.3933, *P*=0.008), Pf (r=0.417, *P*<0.0001) and mixed (r=0.5954, *P*<0.0001) infections.

SOD: The levels of SOD, in comparison to HC were found to be significantly increased (*P*=0.0062) only in patients during Pvinfections (Table 2). The SOD levels, in comparison to MM patients, were significantly decreased (*P*≤0.005) in patients with severe Pv, Pf and mixed infections. Upon comparison among SM patients across various infecting groups, the SOD levels were significantly lower in patients with severe Pf infections (Figure1, Table 3). The SOD levels were also decreased with an increase in parasitic burden in Pf (r=-0.3186, *P*=0.0022) infection cases. There was an inverse correlation between increased CRP levels (inflammatory marker) and SOD levels. However, the correlation was significant only in Pf infections (r=-0.2804, *P*=0.0034) in comparison to Pv(r=-0.1874, *P*=0.058) and mixed infections (r=-0.1566, *P*=0.2166).A significant positive correlation was found between the RBC and SOD levels during Pf (r=0.2307, *P*=0.0168) infections.

Table 3: Levels of MDA, UA and SOD in healthy controls and in patients with mild and severe malaria patients infected with *P. vivax*, *P. falciparum* and mixed infections.

Parameter	HC	*P. vivax*		*P. falciparum*		Mixed		P value (between SM groups)		
		MM	SM	MM	SM	MM	SM	PvVs Pf	PvVs Mixed	Pf Vs Mixed
MDA (IU/L)	0.8±0.4	1.8±1.3	3.6±1.6	2.9±1.5	**4.9±1.6**	1.9±1.3	3.5±1.7	0.005	0.796	0.009
UA (mg/dL)	2.2±1.1	2.8±1.7	5.1±1.6	3.1±1.8	4.0±1.7	3.3±2.1	4.4±1.8	0.014	0.174	0.013
SOD (U/ml)	43.8±11.2	49.9±9.6	40±8.5	43.9±10.6	**30.3±8.8**	47.6±27.4	34.1±9.4	0.0002	0.046	0.0009

Data represented as median (standard deviation); Comparison of three groups by Kruskal Wallis test; comparison of two groups by Mann-Whitney U test; Significant data are highlighted in bold (i.e., p ≤0.05).

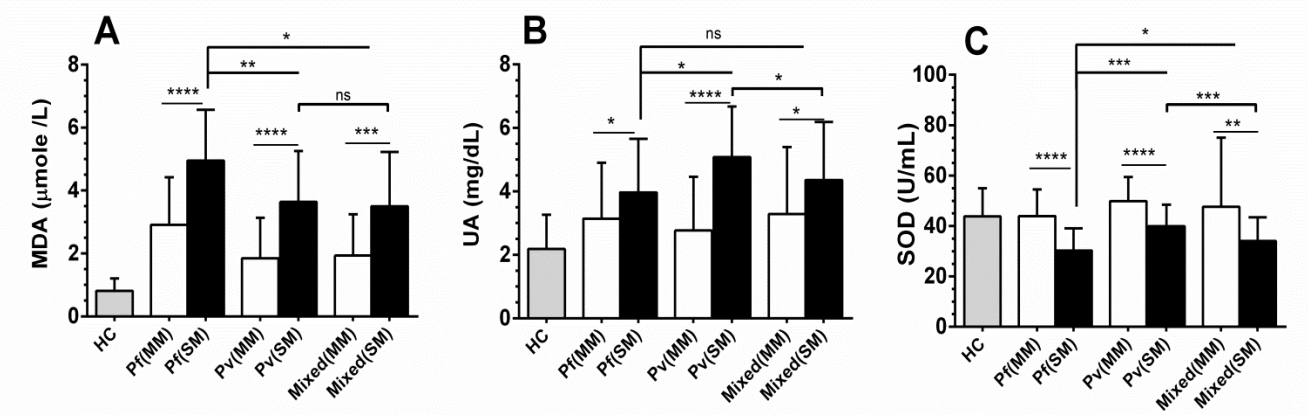


Figure 1.Comparison of (A) Malondialdehyde- MDA, (B) Uric acid–UA and (C) Superoxide dismutase–SOD levels during malarial infection. The levels of MDA, UA and SOD were measured during malarial infection in HC (closed grey boxes) and in patients during mild malaria (MM: open boxes) and severe malaria (SM: closed black boxes) infected with *P. vivax, P. falciparum* and mixed infections. Data represented as mean ±standard deviation were analyzed by one-way non-parametric Kruskal-Wallis test for multiple comparisons, and Mann Whitney U test for comparison between two groups; ** and *** indicate significance at p<0.01 and p<0.001, respectively.

Oxidative stress biomarker levels during malarial anemia and thrombocytopenia

To understand the possible association between morbidity parameters (i.e., malarial anemia and thrombocytopenia) and the levels of the oxidative stress biomarkers, these were analyzed in patients during Pv, Pf and mixed infections.

In this study, all the HC were non-anemic whereas 139 (50.7%) patients had malarial anemia which was contributed byPv(51, 36.7%), Pf (57, 41%), and mixed (31, 22.3%) infections. Based on varying levels of Hb, the participants were further classified into i) Non-anemic (NA) –Hb ≥11g/dl, ii) Mild to moderate anemia (MMdA) – Hb ranged 10.9 -5g/dl and iii) Severe anemia (SA) – Hb<5g/dl. Among the infected, a total of 135 (49.3%) were NA, 113 (41.2%) had MMdA and 26 (9.5%) had SA. The MDA levels, in comparison to NA groups were significantly increased in SA patients during Pv, Pf and mixed infections. The UA levels, in comparison to NA groups were also significantly increased in

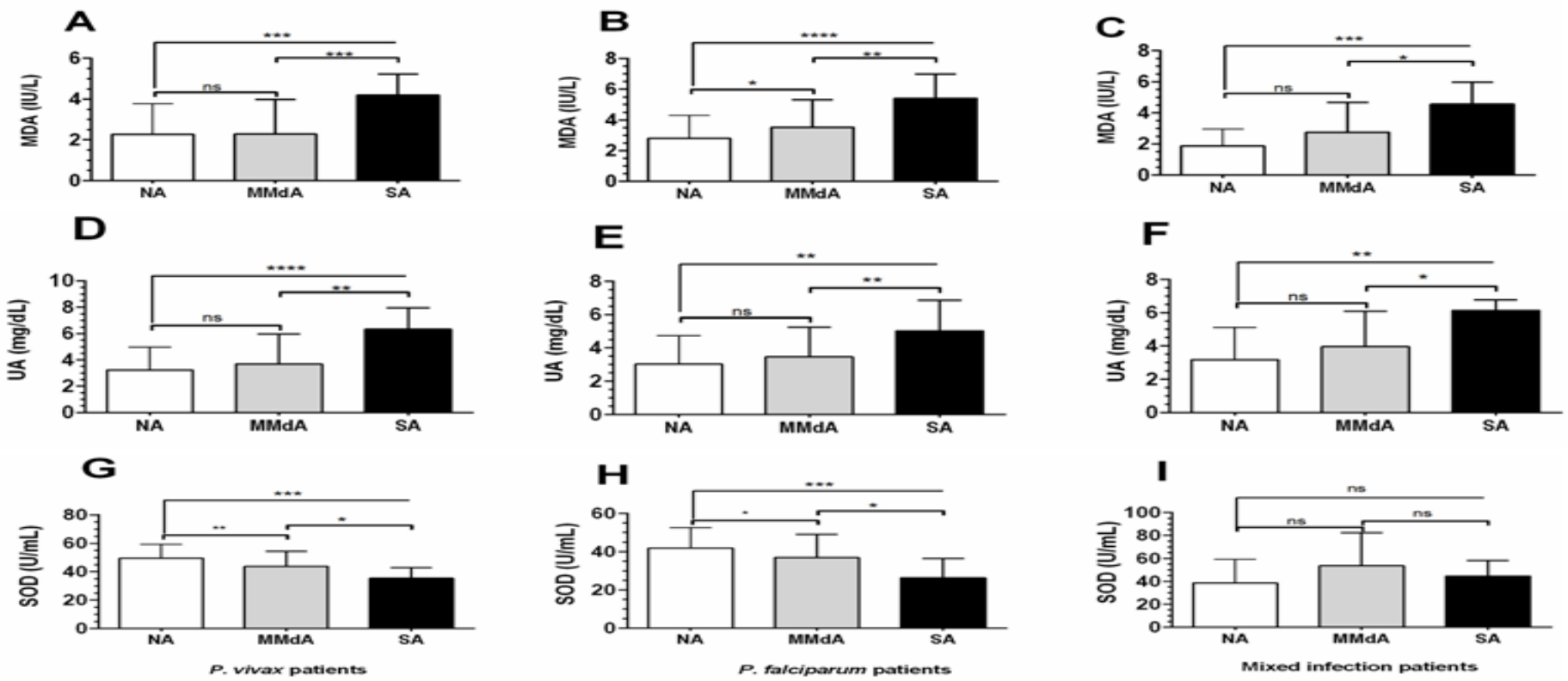
SA group irrespective of infecting species. The SOD levels were significantly decreased in SA patients with Pv and Pf infections (Table 4, Figure 2).

Table 4: Oxidative stress biomarker levels across varying degree of anemia across various infecting species

Parameter	*P. vivax*			*P. falciparum*			*Mixed*			P value (between SA groups)		
	NA	MMdA	SA	NA	MMdA	SA	NA	MMdA	SA	PvVs Pf	PvVs Mixed	Pf Vs Mixed
n (%)	52(50.5)	43(41.7)	8(7.8)	50(46.7)	45(42.1)	12(11.2)	33(51.6)	25(39.1)	6(9.4)	0.121	0.115	0.129
Parasitemia (%)	0.3±0.3	0.8±0.7	1.3±0.8	0.7±0.7	1.4±1.6	1.7±1.6	0.7±0.7	0.8±0.8	1.2±0.8	>0.99	>0.99	0.839
MDA (IU/L)	2.3±1.5	2.3±1.7	4.2±1.0	2.8±1.5	3.5±1.8	5.4±1.6	1.9±1.1	2.8±1.9	4.6±1.4	0.113	0.783	0.347
UA (mg/dl)	3.2±1.7	3.7±2.3	6.3±1.6	3.0±1.7	3.5±1.8	5.0±1.8	3.2±1.9	4.0±2.1	6.1±0.6	0.134	0.913	0.383
SOD (U/ml)	49.6±9.9	43.8±10	35.5±7.4	41.8±10.7	36.9±12.1	**26.4±9**	38.5±20.6	53.7±28.6	44.6±13.5	0.025	0.228	0.010

Data represented as median (standard deviation); Comparison of three groups by Kruskal Wallis test; comparison of two groups by Mann-Whitney U test; Significant data are highlighted in bold (i.e., $P=\leq 0.05$).

Figure 2: Levels of oxidative stress biomarkers in patients during varying intensity of malarial anemia: The levels of Malondialdehyde- MDA(A-C), Uric acid-



UA (D-F) and Superoxide dismutase-SOD (G-I) in Non-anemic (NA- open boxes) and mild to moderate anemia (MMdA –closed grey boxes) and severe anemia (SA- closed black boxes) during *P. vivax*(A,D,G) and *P. falciparum* (B,E,H) and mixed (C,F,I) infections. Data represented as mean ± standard deviation and were analyzed by one-way nonparametric Kruskal-Wallis test for multiple comparisons, and Mann Whitney U test for comparison between two groups; ** and *** indicate significance at $p<0.01$ and $p<0.001$, respectively.

Thrombocytopenia is a common complication during malaria[17].In this study, malarial thrombocytopenia was found in 210 (76.6%) patients with Pv(80, 38.1%), Pf (85, 40.5%), and mixed (45, 21.4%) infections. Based on varying platelet levels, the study participants were classified into i) Non-thrombocytopenic (NT) – platelet levels >150x10^3/µl, ii) Mild to moderate thrombocytopenia(MMdT)–platelet levels ranged 150-50x10^3/µl, and iii) Severe thrombocytopenia (ST)–platelet levels <50x10^3/µl. In this study, 5(9.3%) HC had mild thrombocytopenia. A total of 64 (23.4%) were NT, 139 (50.7%) had MMdT and 71 (25.9%) had ST. In comparison to NT groups, the levels of MDA were found to be significantly increased duringST in patients with Pv and Pf infections. Upon comparison between ST groups across various infecting species, patients with Pf infections had significantly increased MDA levels. The levels of UA, in comparison to NT patients were also found to be significantly increased ($P \leq 0.05$) in ST cases irrespective of infecting species. The SOD levels did not show any significant change across the varying levels of thrombocytopenia irrespective of infecting species (Table 5, Figure. 3).

Table 5: Oxidative stress biomarker levels across varying degree of thrombocytopenia across various infecting species

Parameter	*P. vivax*			*P. falciparum*			Mixed			P value (between ST groups)		
	NT	MMdT	ST	NT	MMdT	ST	NT	MMdT	ST	PvVs Pf	PvVs Mixed	Pf Vs Mixed
n (%)	23(22.3)	51(49.5)	29(28.2)	22(20.6)	59(55.1)	26(24.3)	19(29.7)	29(45.3)	16(25.0)			
Parasitemia	0.5±0.5	0.4±0.6	0.9±0.6	0.9±1.1	1.0±1.3	1.5±1.6	0.8±0.9	0.6±0.6	0.9±0.8	0.156	0.985	0.270
MDA (IU/L)	2.1±1.6	2.2±1.6	3.0±1.7	3.1±1.5	3.1±1.6	**4.1±2.0**	1.9±1.1	2.7±1.9	2.1±1.2	0.032	0.132	0.0009
UA (mg/dl)	2.6±1.5	3.2±1.9	4.7±1.9	2.8±1.4	3.0±1.6	4.5±1.9	2.7±2.0	3.8±2.1	4.0±2.0	0.630	0.256	0.453
SOD (U/ml)	46.9±10	47.8±10.6	44.5±10.1	43±13	42±11	38±12	43.6±24.3	48.5±26.2	37.2±22.6	0.032	0.309	0.995

Data represented as median (standard deviation); Comparison of three groups by Kruskal Wallis test; comparison of two groups by Mann-Whitney U test; Significant data are highlighted in bold (i.e., $P=\leq 0.05$).

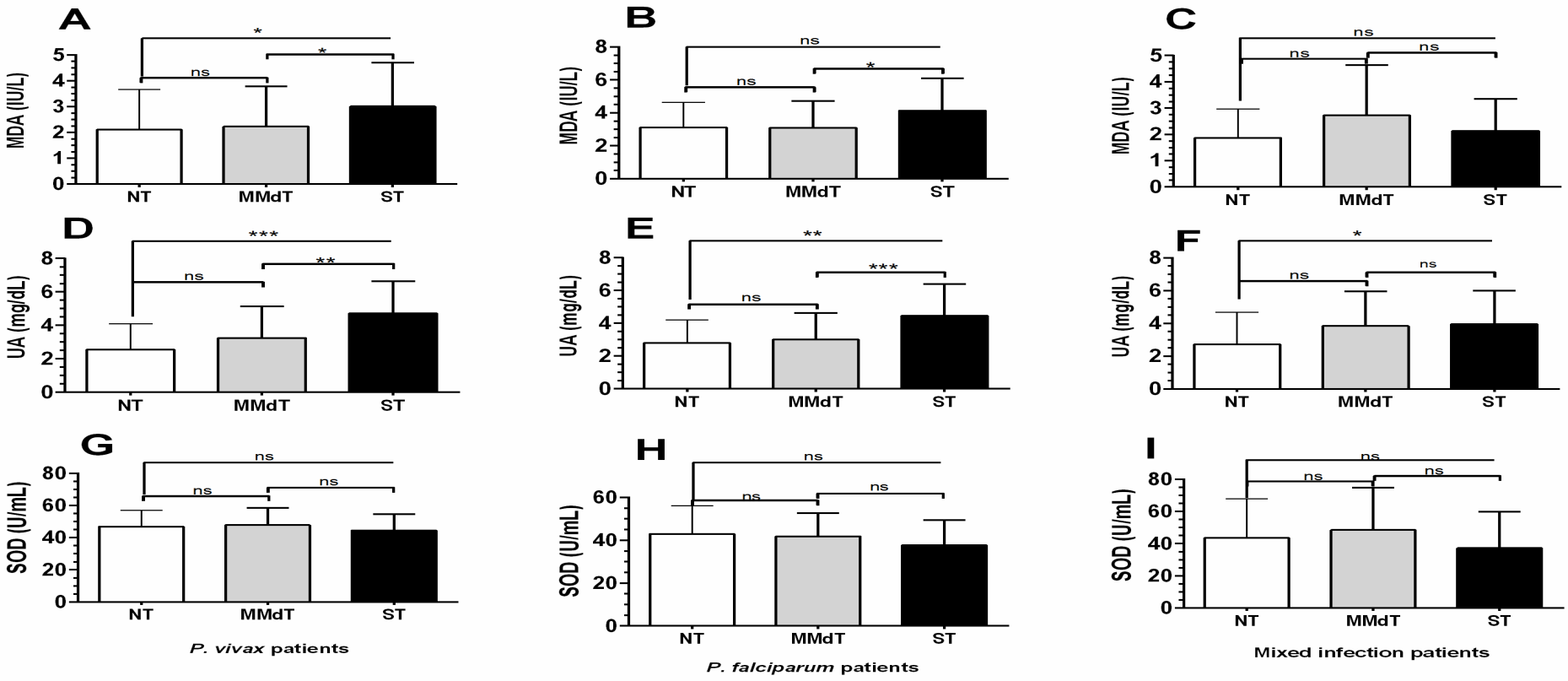


Figure 3: Levels of oxidative stress biomarkers in patients with varying intensity of thrombocytopenia during malarial infections: The levels of Malondialdehyde-MDA (A-C), Uric acid-UA (D-F) and Superoxide dismutase-SOD (G-I) in Non-thrombocytopenic (NT- open boxes) and mild to moderate thrombocytopenia (MMdT – closed grey boxes) and severe thrombocytopenia (ST- closed black boxes during *P. vivax*(A,D,G) and *P. falciparum* (B,E,H) and mixed (C,F,I) infections. Data is represented as mean ± standard deviation andwere analyzed by one-way nonparametric Kruskal-Wallis test for multiple comparisons, and Mann Whitney U test for comparison between two groups; ** and *** indicate significance at $p<0.01$ and $p<0.001$, respectively

DISCUSSION:

The results of present study confirm thatoxidative stress levels are increased in patients during mild and severe malarial infections. In malaria, the oxidative stress is initiated during erythrocyticschizogony mainly due to destruction of infected red blood cells (iRBCs). The higher levels of free radicals generated and theinsufficient antioxidant levels, leads to an imbalance between the ROS and the antioxidant defense system resulting in oxidative stress[1].Our findings of increased MDA levels (a lipid peroxidation marker) during malarial infections corroborate with the previously published reports, especially in patients suffering with Pf infections[18,19]. Increased ROS production induces increased tissue damage which results in disease severity[20]. In our studyalso the MDA levels, in comparison with MM, were increased in patients with SM as reported earlier[20-25].

Uric acid levels are known to be increased during malaria infections due to increased hemolysis and oxidation of nucleic acids by ROS. Various worldwide studies have shown that UA crystals are able to induce inflammatory responses via NALP3 in flamma some pathway[26,27].However, the exact mechanism by which the UA induces the activation of inflammatory responses during malarial infections needs to be explored. Our study also supports the hypothesis that UA plays a major role in malarial pathogenesis[26]. We also observed a significant increase in UA levels during Pf, Pv and mixed infections[28]. Though we did not observe any significant correlation between increase in parasitic density and UA levels, as reported in earlier we also observed a significant increase in UA levels during SM cases[26.] The UA is known to induce inflammatory responses and we also found a positive correlation with increasing levels of C-reactive protein (an acute phase protein) and TNF-α levels. This suggeststhat anaggravated inflammatory responsedue to increased UA levelscould be the reason behind progression of milder forms of malaria into SM[10, 29].

The neutralization of large quantities of toxic redox-active by-products resulting from high metabolic rate of rapidly multiplying parasites is essential for the host survival during *Plasmodium* infections. We found that even though there was no significant decrease in SOD levels in patients with MM, SOD levels were found to be significantly decreased in patients with SM in accordance with other studies[19, 30-32].This supports the hypothesis that, during malarial infections, insufficient levels of host antioxidant defense mechanism failed to adequately neutralize the increased ROS generated thusaggravating MM into SM by reduced effectiveness of the host antioxidant defense system.

Malarial thrombocytopenia, though not considered as a criterion for severe malaria by World health organization (WHO), it is one of the most common complications during malarial infections[17].

In our study, a significant proportion (76.6 %) of patients experienced malarial thrombocytopenia, among which 71(25.9%) experienced severe thrombocytopenia. Though mechanism of thrombocytopenia during malarial infections is not clear, generally it seems to occur by coagulation disturbances, splenic sequestration, antibody mediated platelet destruction and oxidative stress[17].The platelet membranes are known to be fragile in comparison to RBC membranes, and are thus expected to get damaged during increased ROS levels[33]. In this study, we found that the MDA and UA levels were increased significantly during thrombocytopenia,especially among ST patients.It was also observed that, there was a significant inverse relationship between increased MDA/UA levels and decreased platelet counts. The results of OS in malarial thrombocytopenia support the hypothesis that among several other speculated mechanisms of thrombocytopenia in malaria, OS might play an important role.

Anemia is most commonly observed during malarial infections[34]. In this study, a significant proportion of patients experienced malarial anemia (50.7%) of which 26(9.5%) suffered from SA. Anemia during malarial infections is multi-factorial. The etiology of malarial anemia is primarily due to lysis of infected and uninfected RBCs, splenic sequestration of RBCs, bone marrow suppression and dyserythropoiesis[34]. It is also known that increased accumulation of free radicals during hemoglobin digestion in the *Plasmodium* infected erythrocytes leads to oxidative stress and damage of the erythrocyte membrane of both the iRBC and uninfected RBC, resulting in the development of anemia[35, 36]. It has been shown that ROS during *Plasmodium* infection can also result from immune activation which can lead to further damage of uninfected erythrocytes, thus aggravating the severity of anemia[37]. In this study, we have shown that,in comparison to NA groups, the MDA and UA levels were significantly increased in SA patients whereas the SOD levels were found to be significantly lower during SA. We also observed a significant negative relationship between the decreased RBC counts and increased MDA levels; decreased Hb levels and increased UA levels. These results suggest that, during malarial anemia, the increased ROS and insufficient levels of the antioxidants results in failure to adequately neutralize the increased ROS generated that eventually result in progression of milder forms of malarial anemia into severe anemia.

CONCLUSION

In the present study, we conclude that the malaria induced oxidative stress contributes to the progression of mild malaria, anemia, and thrombocytopenia into severe forms (i.e. severe malaria,

severe anemia and severe thrombocytopenia). The MDA and UA may have a potential inflammatory role in malarial pathogenesis. Supplementation of diet and/or anti-malarial drugs with antioxidants during malaria treatment could reduce oxidative stress and improve outcomes of infections.

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CONFLICT OF INTEREST

The authors declare that they have no competing interest.

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